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Evaluation of the Antibacterial Effect of Nano Salvadora Persica roots as Root Canal Irrigant Using Confocal Laser Microscopy (An In-Vitro Study)

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Aim: This study aimed to assess the antibacterial efficacy of Salvadora persica nanoparticles and 2.6 % sodium hypochlorite as root canal irrigants against *E. Feacalis* biofilm using confocal laser microscopy. **Methods and Materials:** 48 single canaled mandibular premolar teeth that were recently extracted and randomly split into three groups were used for this study. To guarantee the development of bacterial biofilms, E. fecalis bacteria are incubated in root canals for three weeks. A confocal laser scanning microscope was utilized to evaluate the specimens after they had been stained using the live/dead technique. One Way ANOVA was used to compare the various groups, and the results showed that there was a significant difference ($P<0.05$) in the percentage of dead cells across all groups. Tukey's Post Hoc test was then used to account for multiple comparisons. **Results:** Regarding percentage of dead cells Salvadora persica nanoparticles group (52.17 % ± 2.31) was significantly the highest, while control group (1.47 % ± 0.24) was significantly the lowest. **Conclusion:** Salvadora persica nanoparticles proved to be an effective and all-natural root canal irrigant against the biofilm of *E.faecalis*.

Keywords: *Salvadora persica, nanoparticles, confocal laser microscopy, bacterial biofilm.*

Introduction

A primary objective of root canal therapy is to eliminate as many harmful bacteria as possible from the root canal system while undergoing endodontic treatment. The development of pulpal inflammation and pulpal tissue necrosis, and therefore the formation of periapical lesions, are significantly influenced by microbial germs such the facultative bacteria Enterococcus faecalis[1]. The removal of microorganisms is crucial. However, the anomalies and anatomical differences of the root canal represent a challenge. Furthermore, mechanical instruments are unable to reach every dentinal canal wall, leaving untapped regions. In order to completely eradicate microorganisms from root canal configuration without compromising the healing process, which is essential for the attainment of long-term endodontic root canal success, mechanical enlargment in addition to chemical disinfection is therefore very important [2,3].

Endodontic irrigants have been introduced using a variety of synthetic antibacterial agents for a number of years. Bacteria are growing increasingly resistant to conventional antimicrobial drugs, leading to the development of new, inexpensive, non-toxic alternatives. It has been discovered that natural plant extracts can successfully and effectively replace endodontic irrigants[4].

Sodium hypochlorite, which has strong antibacterial qualities, has long been the preferred irrigant for root canal therapy. If it is forced past the root canal apex, though, negative consequences result. Natural materials derived from medical implants are increasingly being used in the dentistry industry to combat the chemosynthetic character of traditional irrigating solutions. Salvadora persica, often known as meswak, is an herbal substance that has been utilized in the dental sector and has been shown to have a potent antibacterial effect since it contains a variety of active components[5].

The use of nanotechnology has improved the chemical and physical characteristics, as well as the antibacterial activity, of irrigating solutions. By using nanotechnology, this can be accomplished by reducing their solid state to the tiniest nanoparticles. Salvadora persica nanoparticles can maximize the solubility of its active components, enhancing their antibacterial effectiveness[6].

MATERIALS AND METHODS

(A) Sample selection:

In this study, 48 mature single canaled human mandibular premolar teeth were obtained from the oral surgery department at October 6 University's Faculty of Dentistry. The presence of any fractures, cavities, craze lines, curvature, or external resorption in teeth was examined. Radiography was used to rule out any teeth samples that had internal resorption or calcification.

(B) Sample preparation and Sterilization:

An ultrasonic scaler (Suprasson P5 Booster, France) was used to plane the selected samples to remove any hard deposits, calculus, or soft tissue remnants. At the cementoenamel junction, decoronation was carried out using a fine tapered diamond stone set on a high-speed hand piece (PANA AIR, NSK, Japan) with a generous supply of coolant flowing through it. The selected samples' length was adjusted and fixed at lenght 16 mm. Each tooth's root apices were sealed with a little mount of composite resin. This was done to prevent seepage of the bacterial suspension during the incubation period in the autoclave, as well as to aid in retention of the irrigating solution within root canal to ensure their presence among the contact period to get maximum and standardized effect to simulate in vivo apical counter pressure. after which they were sterilized by autoclaving at 121°C for 30 minutes, twice, and then immersed in a 5.25 percent sodium hypochlorite solution for 14 minutes.

(C) Root canal preparation:

All selected samples were prepared as the follow:

Root canal patency and Pulp tissue extirpation from all root canals using # 15 -20 K-St St file (MANI, Inc, Japan). Root canal preparation by the crown down technique using ProTaper Next (PTN, Dentsply Maillefer, Switzerland) rotary system. ProTaper Next files

were mounted on a contra-angle hand piece that was attached to a torque-limiting motor E-connect Pro endomotor rotary device (Changzhou Eighteeth Medical Technology Co., Ltd, China) at torque (0.8 N) and a speed 300 RPM with a sequence X1, X2, X3 to a final size X4 (40/0.06).Irrigation was performed between each rotary file and the next with normal saline.

(D) Preparation of irrigating solutions:

2.6 % Sodium hypochlorite: 5.25% NaOCl solution was diluted to 2.6 % as half to half NaOCl and distilled water.

Salvadora perisca 60 % ethanolic extract preparation:

Eight hundred grams of Salvadora Persica chewing sticks were sun-dried for ten days at Botany Garden in Mecca, Saudi Arabia. Next, chop with a sharp knife, and finally blend into a powder with a food processor that is sold commercially. In a sterile, well-capped flask, fourty grams of powder were mixed with 120 milliliters of 60% ethanol, allowed to stand at room temperature for three days, and then filtered through number one filter paper. After that Miswak extract was kept in sterile screw-capped vials in the refrigerator after being kept at 37°C until it was dried out. In order to achieve 100% concentration, 1 gram of miswak alcoholic extract was dissolved in 2.5 ml of sterilized Ringer's lactate to create serial dilutions of the extract.

Synthesis of polymeric PEG-PVP-linker biomaterial

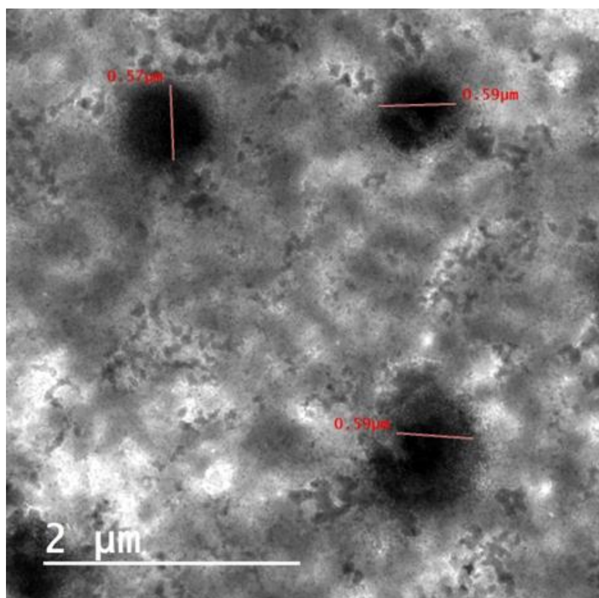
PEG-PVP linker biomaterial was prepared by adding 2:1(w/w)-PEG-PVP dissolved in 25 ml of isopropanol dropwised with continues starring.

Synthesis of miswak encapsulation PEG-PVP biopolymer

The polymer mixture, known as PEG-PVP linker biopolymer, was combined dropwise with 1% of 25 ml of alkalized miswak while being continuously stirred at room temperature for a full day., then filtration the mixture and 2.5 ml hydrochloric acid (HCL) was then added to neutralize the pH of the solution. The product was precipitated from the supernatant liquid by adding 2:1 (v/v) mixture of acetone and ethanol. The solution was then heated under reflux for 24 h. After the reaction, the solvents were evaporated under high vacuum then the miswak-PVP-PEGs were obtained encapsulated and sparingly soluble in 75 ml phosphate buffered (PBS) were prepared in 100 ml glass bottles. The mixture was stirred and vortexed several times for complete dissolution and encapsulation and stored at 4 °C.

Characterization of Salvadora persica nanoparticles:

Using a transmission electron microscope (TEM), additional proof of their size, shape, and abundance was identified as depicted in (Figure1). With an average size of 10.6 nm and a spherical shape, the nanomiswak particles were displayed in extremely small size ranges from 6.49 to 21.0 nm.Material absorption: 0.100, viscosity (cP): 0.8872, dispersant RI: 1.330, and dispersant name: water



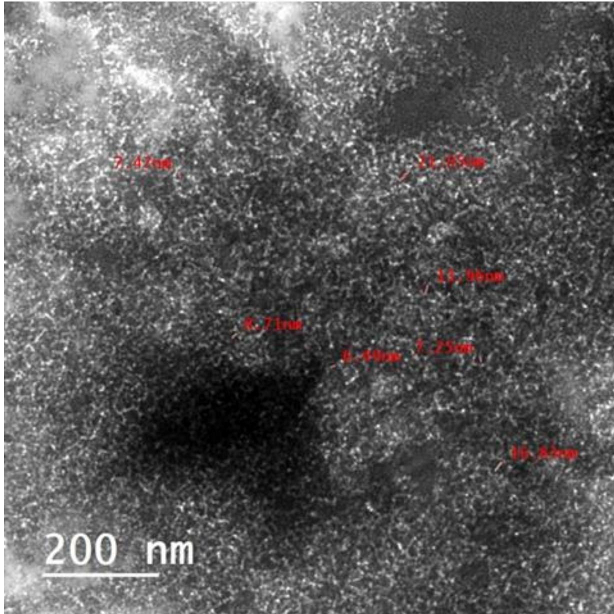


Figure (1): Salvadora persica nanoparticles characterization

Evaluation of antimicrobial effect:

(A) Sample sterilization:

Sterilization of samples after mechanical preparation by autoclaving at temperature 121 ◦C for 30 minutes for two sterilization cycles was done. Each tooth sample was stored in separate autoclavable eppendorf until time of inoculation.

(B) Microorganisms biofilm preparation:

Step one: Proper growth media preparation brain heart infusion (BHI) followed by pure bacterial culture of gram+ *cocci* (#29212, ATCC Manassas, VI)

Step two: *Enterococcus faecalis* was introduced in 7 ml of brain heart infusion**,** kept at temperature 37 ◦C for 24 hours. After that suspension was prepared on the BHI plates. Bacterial cells after that were resuspended in saline and adjusted to #1 mcfarland turbidity standard ($3x10^8$ cells/ ml).

Step three: 5 ml of sterilized brain heart infusion was mixed with five ml of bacterial inoculum. The samples were inoculated with *Enterococcus faecalis* by using micropettes in order to fill each root canal for two weeks to allow bacterial biofilm formation. Every 72 hours interval this process was repeated.

Step four: Samples were kept under suitable atmospheric condition and humidity at temperature 37 ◦C for three weeks at the incubator to ensure complete biofilm formation.

Step five: Centrifuging the samples twice during incubation period 'time interval' was done.

(C) Biofilm confirmatory group:

16 Samples were used as control group to ensure complete bacterial biofilm formation before irrigating root canals that was scanned using confocal laser microscopy.

(D) Samples classification and irrigation:

32 Samples were classified according to final irrigation solution into two experimental groups.

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

Group (B): 16 samples were irrigated with 5 ml SP nanoparticles 5 mg/ml using plastic syringe with 23 gauge needle for two minutes.

Using paper points all root canals were dried after ten minutes and then irrigated with sodium thiosulfate to counteract effect of sodium hypochlorite. Samples were kept at low temperature -5 ◦C to prevent growth of microorganisms.

Method of evaluation:

1. Antibacterial effect using confocal laser scanning microscopy:

Sample cutting:

. The extracted teeth were cut using a 0.3mm Isomet saw and kept under coolant through sterile distilled water so they could be imaged. Every root was divided into two sections of one millimeter, which represent the middle and apical thirds of each root. Since the dentin segments from the in situ experiment were flat prior to infection, sectioning them for confocal microscopy analysis was not necessary, with a sample size of 5 x 5 x 1 millimeters was not necessary. All of the samples required for the fixation of microorganism growth were stored in an ice box at a temperature of -4 °C.

Sample preparation for imaging by confocal laser microscopy:

- Dentin segments were washed carefully by 100 µl sterilized distilled water for one minute and then dried samples smoothly.

- Dentin segments were then placed at the bottom of the Eppender of tube.

- 10 µL of propidium iodide (PI) red fluorescence and 100 µL of each 0.01 % acidrine orange (AO) green fluorescence were added to the selected specimens to be stained in a dark room, then kept for 15 minutes after centrifuging for ten seconds.

- Specimens were removed from the tube and washed twice with 100 µl sterile distilled water to remove excess dye. After that specimens were transferred to glass covered slips, covered with immersion oil before performing any image acquisition.

Laser and stain specifications

Confocal illumination was performed by using argon laser microscope that was adjusted 460 nm emission for (PI) and to 500 nm for (AO). Fluorescence from the stained specimen cells was viewed through using cone focal laser scanning microscopy. Dye material has the ability to bind bacterial DNA emitting green fluorescence while bacterial RNA emitting red fluorescence, after staining, corresponding specimens were immediately analyzed by CLSM technique. PI enters only the dead cell membrane exhibiting

red fluorescence, while AO stains the living cells exhibiting green fluorescence. For the acridine orange stain the excitation and emission for DNA was 500 and 526 nm respectively, while for RNA the excitation and emission was 460 and 650 nm. Sequential frame scan mode was used to prevent crosstalk.

Magnification of specimens:

Observing mounted specimens through using 40X magnification oil immersion objective with a numeric aperature of 1.4, confocal pinhole was set to diameter 164 Mm for channel two, and 88 Mm for channel one

Image analysis:

Images of confocal laser microscopy were analyzed using Zeiss laser scanning microscope software version ZEN 3.3 (2012). 5-10 µm in dentin structure as deep scans obtained from each specimen (twenty –forty sections of 2 µm step size in format of 1024 X1024 pixels).

Statistical analysis was performed with SPSS 20, Graph Pad Prismand Microsoft Excel 2016. All quantitative data were presented as minimum, maximum, mean and standard deviation. One Way ANOVA test was used to compare between different groups followed by Tukey`s Post Hoc test for multiple comparisons.

Results:

Regarding antibacterial effect Comparison between different groups was performed by using One Way ANOVA test which revealed significant difference between all groups regarding percentage of dead cells as $P<0.05$, followed by Tukey`s Post Hoc test for multiple comparisons which revealed that % of dead cells of Salvadora persica nanoparticles group (52.17 % ± 2.31) was significantly the highest ***figure (2)*** , followed by 2.6% NaOCl group (50.36 % ± 1.42) while control group (1.47 % ± 0.24) was significantly the lowest ***table(1) , figure (3)***.

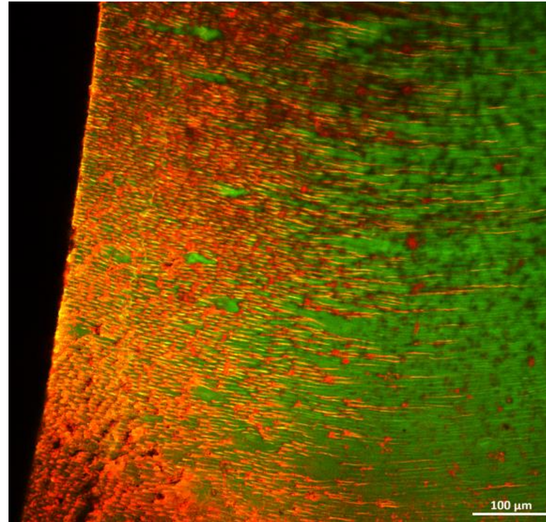


Figure (2): Live and dead cells shown by CLSM in Salvadora persica nanoparticles samples.

Table (1): **Mean and standard deviation of dead cells percentage in all groups and comparison between them using One Way ANOVA test followed by Tukey`s Post Hoc test:**

Bacterial count reduction and Biofilm	% of dead cells	
	Mean	Std. Deviation
Salvadora persica Nanoparticles	52.17 [a]	2.31
Sodium hypochlorite solution (2.6%).	50.36 [b]	1.42
Control group	1.47 [c]	0.24
P value	<0.0001*	

**Significant difference as $P<0.05$.*

Means with the same superscript letters were insignificantly different a $P>0.05$.

Means with different superscript letters were significantly different a $P<0.05$.

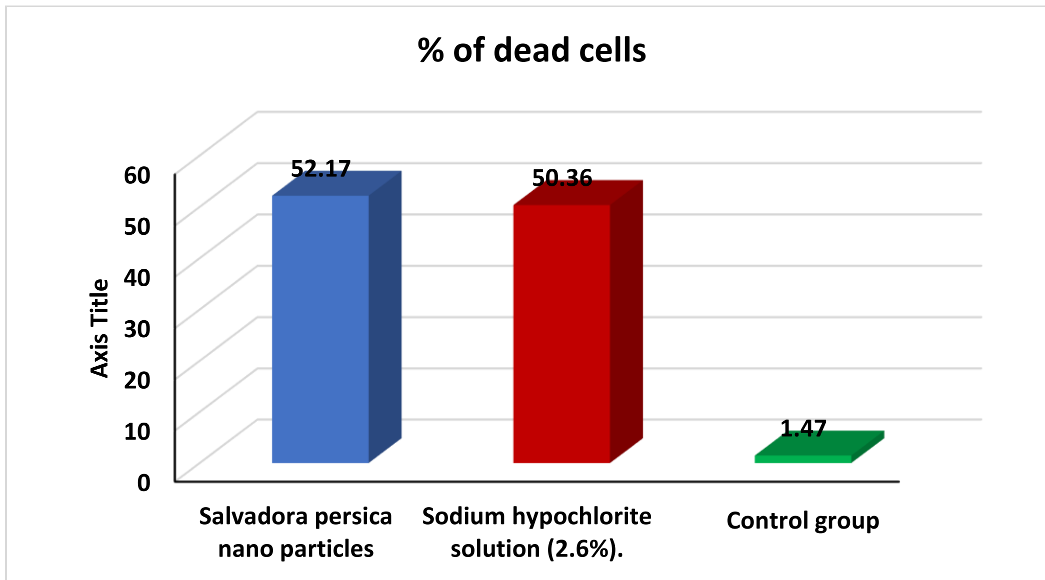


Figure (3): Bar chart showing percentage of dead cells in all groups

Discussion:

During root canal treatment, the conventional mechanical method of cleaning and shaping can't provide the desired removal of microorganisms penetrating dentinal tubules. Furthermore, after mechanical instrumentation 9.6 % - 47.6 % of root canal system is left untouched, this is covered with dentin chips, bacteria and pulp tissues that act as nidus for reinfection[7].

The number of facultative anaerobes in the root canal system increases with the duration of the infection. One of the most prevalent pathogens that can be grown from retreatment-treated failing root canals is Enterococcus faecalis. By making the bacteria a thousand times more resistant to antibodies, phagocytosis, and antimicrobial chemicals, the biofilm that surrounds them helps the germs survive the destruction. This is because the extracellular matrix acts as a protective barrier. Additionally, through horizontal gene transfer, biofilms facilitate the transmission of antibiotic-resistant genes among several clinically relevant species, giving microorganisms the resistance to chemo mechanical preparation. For this reason, the best intracanal irrigant solutions are those with strong antibacterial properties to enhance the results of the instrumentation processes[8].

Previous studies have reported that *S. persica* roots have antiseptic, antimicrobial, anticariogenic, and analgesic activities [9]. Trimethylamine, salvadorine, chloride, fluoride, and trace amounts of tannins, saponins, flavonoids, and sterol have all been shown to be present in Salvadora persica. It has been demonstrated that certain elements have strong antibacterial properties [10].

Nanotechnology in the field of endodontics has become widely used in an effort to improve the antimicrobial efficiency of conventional antibacterial agents and to obtain promising results in endodontic therapy. Numerous benefits, such as their ultra-small size, high surface-area-to-volume ratio, and superior physical and chemical properties, can be obtained from antimicrobial nanoparticles. A recent development in the management of dental infections is the use of nanoparticles (NPs). Increased antibacterial impact is produced by NPs' ability to react with negatively charged bacterial cells due to their larger surface area and positive charge [11]. Additionally, Salvadora persica root nanoparticle powder increases the bactericidal activity of the product by maximizing the solubility of its active components [12]. Nevertheless, there aren't many studies comparing the antibacterial properties of Salvadora persica roots to sodium hypochlorite as an irrigating solution [6].

The process of creating nanoparticles was done top-down. This method involves employing physical (lithographic) instruments or chemical processes to deconstruct the bulk material or solid until the desired tiny structures are formed, ultimately achieving the desired size and shape[13].

Nanoparticles have the ability to infiltrate biofilms and engage in electrostatic interactions with bacterial cell walls, resulting in damage to cell membranes, heightened permeability, production of reactive oxygen species, disruption of cellular processes, degradation of proteins, damage to DNA, and ultimately, cell death.[14].

Human mandibular single-canaled teeth (premolars) were the study's sample of choice because they allowed us to assess the effectiveness of the tested irrigating solutions in the root canal system and replicate a clinical setting. In order to rule out anatomical variances and complexity concerns, the samples that were chosen were single-canaled teeth

[15, 16]. Every test sample was autoclaved to ensure secure handling. These samples were then kept in an aqueous solution with a disinfectant to prevent dehydration and the formation of more bacteria [17]. To standardize the specimens, teeth were decoronated to a length of 16 mm. The Protaper Next set of nickel titanium rotary tools[18], [19]was used for root canal preparation. Each tooth's root apex was capped with a little amount of composite resin to stop the bacterial suspension from seeping through while the tooth was in the autoclave for incubation. In order to replicate in vivo apical counter pressure, it will also help retain the irrigating fluid inside the root canal and guarantee its presence throughout the contact period [20].

The advancement of direct viable staining techniques and the confocal microscope (CLSM) have sparked interest in the study of bacterial viability in recent years, particularly in the field of biofilms. Some early researches have reported using CLSM to analyze dentin infection and dental biofilms. The use of vital staining techniques to observe live bacteria in dentinal tubules and root canal walls, in situ hybridization to identify labeled bacteria, and three-dimensional visualization of structural organization in biofilms are some potential benefits of confocal microscopy for the study of dentin infection[17].

As a non-destructive method of studying these ecosystems and the hydrated spatial arrangement at the cellular level, confocal laser scanning microscopy has shown to be an extremely useful tool in the recent years for studying the structure of biofilms. Targeting certain extracellular matrix elements and cell types is made possible by the use of fluorescent markers. It is possible to distinguish between live and dead bacteria using particular stains, as shown by red or green fluorescence. By employing a three-dimensional reconstruction of the biomass to examine the architecture of the biofilm, this technique enables us to have a comprehensive understanding of the efficacy of irrigating solutions and procedures in disturbing the biofilm structure [21].

This study aimed to evaluate the antibacterial effect of Salvadora persica roots nanoparticles versus sodium hypochlorite. Using statistical results obtained by cone focal laser microscopy we calculated the mean value of percentage of dead cells. Both Salvadora persica nanoparticles and NaOCl showed antibacterial effect against *Enterococcus faecalis*

biofilm. Salvadora perisca nanoparticles showed the highest dead cells percentage (52.17 %) that was significantly higher than NaOCl group (50.36%) as ($P<0.05$), while control group showed the least dead cells percentage (1.47 %).

Actually, the wide range of chemical components in S. persica is responsible for its antimicrobial activity. Gram-negative bacteria are effectively inhibited by benzoyl isothiocyanate (BITC), a significant component found in S. persica roots. Because of its lipophilic and electrophilic qualities, it has been suggested that BITC may penetrate bacterial membranes and inhibit their redox processes, which would disrupt the potential of the bacteria's membrane[22].

Efficiency of NaOCl is attributed to its effectiveness in eliminating intracanal microorganisms. Hypochlorite acid which is powerful oxidizing agent that produces an antibacterial effect by irreversible oxidation of hydrosulphuric groups of bacterial enzymes. As essential enzymes are inhibited, disturbance of the metabolic functions of the bacterial cell occurred which lead to death of bacterial cells. Highly toxic N-chloro components that have antimicrobial effect can be formed by Chlorine adherence to bacterial cytoplasm [23, 24].

Our study's results align with previous research demonstrating the antimicrobial efficacy of Salvadora persica extracts at varying concentrations against bacterial biofilms [6, 25, 26, 27.] The antibacterial effect of miswak extract against Streptococcus faecalis, Pseudomonas aeruginosa, and Staphylococcus aureus was also reported by ***Darout et al.*** [28]. This activity may have been attributed to the extract's nitrate content.

On the other hand, other studies have shown a significant antibacterial effect of sodium hypochlorite against common oral pathogens than Salvadora persica nanoparticles [29, 30, 31].

Conclusion:

Salvadora persica nanoparticles represent an efficient, natural, promising root canal irrigant regarding reducing *E.faecalis* biofilm.

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