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Evaluation of cytotoxicity and the antibacterial effect of *Salvia officinalis* as an intra-canal medicament using Confocal microscope

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Aim: The principal objective of root canal therapy is to control the infection in the root canal system after mechanical preparation and irrigation by eliminating the current infection and preventing any future recurrence. The present study investigated the antibacterial efficacy and cytotoxicity of *Salvia officinalis* (SOL) extract and its nanoform (SOL-NPs) as intracanal medicaments against *Enterococcus faecalis*, compared to calcium hydroxide.

Materials and methods: Cytotoxicity of SOL extract and SOL-NPs against the normal human epithelial cell line 1-BJ1 (Normal skin fibroblast) using MTT assay. 70 teeth were collected and evenly split into three experimental groups (n=20): A, B, and C, and one negative control group (n=10). Group A teeth were injected with ready-to-use Metapaste calcium hydroxide. Group B received *S. officinalis* extract paste injections. roup C teeth received nanoform *S. officinalis* paste injections. The negative control samples were infected with root canal-isolated *Enterococcus faecalis*.

Results: The IC_{50} values were 594 µg/ml for SOL extract, 1468.8 µg/ml for SOL-NPs, and 1219 µg/ml for Metapaste, highlighting the SOL extract as the most potent. Both the SOL extract and SOL-NPs showed significant antibacterial activity against *E. faecalis*, with SOL-NPs proving more effective than CH in reducing bacterial counts.

Conclusion: SOL extract and SOL-NPs effectively reduced bacterial cell counts and dead cells, with SOL-NPs showing superior antibacterial properties, potentially treating *Enterococcus faecalis* in intracanal settings. The findings underscore the potential of plant-based solutions in managing dental infections while minimizing cytotoxic risks.

Keywords: Intracanal medicaments; *Salvia officinalis*; Nanoparticles; Cytotoxicity; Antibacterial inhibition

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Introduction

The primary objective of root canal medicaments is to eliminate bacterial infection from the root canal system.[1] Chemo-mechanical preparation of the root canal system alone is insufficient, as microorganisms persist in the root canal and dentinal tubules following instrumentation. Consequently, the application of antimicrobial dressings following canal preparation is typically advised. These medicaments must exhibit maximal antimicrobial efficacy against the diverse bacterial species present in the infected root canal while avoiding irritation to the periapical tissue. [2] Throughout the years, calcium hydroxide (CH) has been regarded as a gold standard intracanal medicament. [3] It is the primary intracanal medicament used in endodontics, and it is commonly employed to eliminate any remaining microorganisms after chemomechanical preparation. [4] It induces specific effects, including ionic diffusion and pH modification upon contact with tissues, [5] leading to the inhibition of bacterial toxins associated with pathogenicity and the activation of alkaline phosphatase, which boosts the formation of mineralized bone tissue and assists in bone repair. [6] Many microorganisms in root canals develop resistance to CH and other medicaments, highlighting the need for alternatives that demonstrate high antibacterial activity and low cytotoxicity. [1,7] Through the years several alternatives were employed including medicinal plants which serve as a viable alternative for controlling microorganisms that cause human infections. [8] Phytotherapy is receiving growing interest in dentistry, especially in specialized fields like endodontics, due to its antimicrobial properties and biocompatibility. [9] The utilization of botanical solutions in dental applications indicates a broader trend, leveraging their established antimicrobial properties and compatibility with oral health treatments. [10]

Salvia officinalis Linnaeus (sage) is a member of the Lamiaceae family, originating from the Mediterranean region, yet it is found across various continents. This aromatic plant serves culinary, medicinal, and commercial functions. [11] Furthermore, various biological effects have been associated with S. officinalis products, including antibacterial, antifungal, antileishmanial, anti-inflammatory, antitumor, antioxidant, antinociceptive, mnemonic, and antiangiogenic activities, as observed in extracts, essential oils, and bioactive molecules. [12] Numerous studies over the years have demonstrated the antibacterial efficacy of S. officinalis against various intracanal bacteria, including those responsible for pharyngitis, cariogenic bacteria, and infected root canals.[13] Furthermore, biocompatibility and stability are essential characteristics of intra-canal medicaments. Evaluating the cytotoxic activity of these medicaments is crucial, as it affects the biological and physiological behavior of cells. [10]

To our knowledge, there is a scientific lack of evidence supporting the antibacterial efficiency and cytotoxicity effect of the S. officinalis extract and its nanoform against the anaerobic bacterium, Enterococcus faecalis, and its cytotoxicity against dental tissues. Therefore, the present study aims to assess the cytotoxicity and antibacterial effect of S. officinalis extract (SOL) and its nanoform (SOL-NPs) paste as intracanal medicaments for infected root canals with E. faecalis using a confocal microscope.

Materials and methods

Study design, ethical approval, and sample selection

The study was conducted as an in-vitro study parallel-group design, Three-arm study, with an allocation ratio of 1:1:1 and a negative control. The Research Ethics Committee of the Faculty of Dentistry at October 6 University reviewed and approved the current study on May 8, 2023, under ethical approval (RECO6U/20-2023). The samples comprised 70 recently extracted mature single, straight-canaled human permanent teeth, devoid of root resorption or fracture, obtained from the outpatient clinic of the oral surgery department, October 6 University. Teeth exhibiting highly curved canals, fractures, internal resorption, or dilacerated roots were excluded from the study samples. Teeth were radiographically examined to eliminate any samples exhibiting internal resorption, calcification, or possessing multiple canals. All teeth underwent mechanical scaling utilizing an ultrasonic scaler (Suprasson P5 Booster, Satelec, France) to eliminate residual bone, calculus, or soft tissue.

Sample size calculations

The sample size was determined for cytotoxicity evaluation based on De Oliveira et al. [14], who indicated that the responses within each subject group followed a normal distribution, with a standard deviation of 0.279. If the actual difference between the experimental and control means was 0.33, a minimum of 12 subjects in each group was required to reject the null hypothesis that the population means of the experimental and control groups are equal, with a power of 0.8. The probability of a Type I error for this null hypothesis test was 0.05. The total sample size was increased to 15 specimens for each group to compensate for 20% dropout.

The sample size was determined for the antibacterial effect according to De Oliveira et al. [14], who indicated that the responses within each subject group followed a normal distribution, with a standard deviation of 0.71. If the actual difference between the experimental and control means was 0.8, a minimum of 13 subjects in each group was required to reject the null hypothesis that the population means of the experimental and control groups are equal, with a power of 0.8. The probability of a Type I error for this null hypothesis test was 0.05. The total sample size was increased to 20 specimens for each group to compensate for a 20% dropout.

Intra-canal medicaments

Salvia officinalis extract preparation Leaves of Salvia officinalis were collected from the Botany Garden in the North Sinai desert, Egypt. The extract of S. officinalis leaves (SOL) was prepared using the Soxhlet extraction method described by Ozel and Kaymaz.[15]

Salvia officinalis extract nanoform preparation and characterization

A 10 mg of aqueous extract was solubilized in a chloroform-ethanol mixture at a ratio of 1:1. A rotary evaporator was utilized at 100 rpm to aid in the formation of a thin film, which was subsequently hydrated with 10 ml of PBS at pH 7.4. Bath sonication for five minutes was utilized to reduce the particle size of the produced vesicles.

The resulting SOL-NPs were characterized using transmission electron microscopy (TEM) (Tecnai 20 G2 S TWIN at IIT Roorkee, operating at 200 kV). The average size, polydispersity index, and zeta potential of SOL-NPs were assessed using

dynamic laser scattering (Malvern Zeta sizer (Malvern Instruments, UK)). The UV-Vis absorption spectra of SOL-NPs and free-SOL were recorded at 25 °C within the 200-400 nm range at 1.0 nm intervals utilizing a spectrophotometer (Jasco, Japan). All records were computed in triplicate. FTIR spectra of SOL-NPs and free-SOL were obtained using a Bruker instrument (Karlsruhe, Germany). The spectra were obtained at a resolution of 4 cm^{-1} over the range of 400 to 4000 cm^{-1} at a temperature of 25 °C. [2,16]

Metapaste calcium hydroxide

A commercial ready-to-use Metapaste calcium hydroxide (MetaPaste, Meta Biomed Co., Cheongju, Korea).

Sample preparation

Teeth crowns were excised utilizing a fine-tapered diamond stone attached to a high-speed handpiece (PANA AIR, NSK, Japan) at the cemento-dentinal junction, accompanied by a substantial flow of coolant. The length was standardized to 16mm, and the root apices were sealed using a small quantity of composite resin (Esthet-X, Dentsply, York, PA, USA). Samples were immersed for 15 minutes in a 5.25% sodium hypochlorite solution and subsequently sterilized by autoclaving (Class B Autoclave IcanClave STE-18-D, Valencia, Spain) for 30 minutes at 121°C for two cycles. The patency of root canals was assessed using a size #15 K-ST File (Mani, Inc. Japan). Each root canal was irrigated with 5 mL of 0.9% saline solution after the use of each file. The canal was thoroughly irrigated with 3 ml of 17% EDTA for one minute after mechanical instrumentation. Then a final flush with water was done.

Estimation of cytotoxicity of tested intracanal medicaments

The cytotoxicity of SOL extract (Powder), SOL-NPs (emulsion), and $Ca(OH)_2$ (Metapaste) was conducted and determined by the Bioassay-Cell Culture Laboratory, National Research Center, El-Tahrir St., Dokki, Giza, Egypt. Cytotoxicity of SOL extract and SOL-NPs against the normal human epithelial cell line 1-BJ1 (Normal skin fibroblast) using MTT assay. A range of concentrations (5, 10, 20, 40, 78, 156, 312, 625, 1250, 2500, 5000, and 10000 µg/ml) of each intracanal medicament was employed to determine the IC_{50} 48 hours post-treatment using SPSS 20.0 (IBM, USA, Version 20.0.0).

Assessment of the antibacterial effect

A 7mL aliquot of Brain Heart Infusion (BHI) artificial medium was inoculated with Enterococcus faecalis (#29212, ATCC Manassas, VI) sourced from the American Type Culture Collection and subsequently kept at 37°C for 24 hours. Following this, suspensions were prepared on the surface of BHI plates in saline under the same incubation conditions and adjusted to the #1 McFarland turbidity standard (3×10^8 cells/mL) using sterile loops (Difco Laboratories, Detroit, MI, USA). Five milliliters of sterilized BHI were mixed with 5 ml of bacterial inoculum, and E. faecalis was introduced to fill each root canal for four weeks for biofilm formation. The procedure was conducted at 72-hour intervals, employing pure cultures that were prepared and calibrated to the #1 McFarland standard. To verify the establishment of biofilm before the application of any treatments to the roots, a negative control group was employed and examined using a Confocal Laser Scanning Microscope (CLSM).

Sample evaluation

Samples were bisected with a 0.3 mm Isomet (IsoMet 4000 Precision Saw, Secunderabad, Telangana, India) saw while maintaining constant cooling with sterile distilled water. The specimens were placed in the bottom of an Eppendorf tube, to which 100 µl of 0.01% acridine orange (Acridine orange, Shanghai Yueteng Biotechnology Co., Ltd., China) (AO) (green fluorescence) and 10 µL of propidium iodide (Propidium Iodide, Shanghai Yueteng Biotechnology Co., Ltd., China) (PI) (red fluorescence) were added. The samples were incubated in a dark room for 15 minutes. The specimens were subsequently placed on glass coverslips and coated with immersion oil prior to image acquisition. Confocal illumination utilized an argon laser microscope set to a 500 nm wavelength for AO and a 460 nm emission for PI. The fluorescence emitted by the stained cell was observed using a confocal laser scanning microscope (Carl Zeiss, ZEISS, Jena, Germany). The mounted specimens were examined using a 40X magnification oil immersion objective with a numeric aperture of 1.4. The confocal pinhole was adjusted to a diameter of 88 µm for channel one and 164 µm for channel two. Confocal images were acquired and analyzed with the Zeiss laser scanning microscope software, version 2012. Deep scans were obtained from each specimen within the dentin structure at depths of 5-10µm, comprising 20-40 sections with a step size of 2 µm in a resolution of 1024 × 1024 pixels. Bacterial survival was quantified as the percentage of green voxels relative to total fluorescence. The percentage of biofilm cell death, as determined by the LIVE/DEAD technique, was calculated by assessing the biovolume of the red subpopulations relative to the total biovolume of the biofilm. [17]

Statistical analysis

The data is presented as the mean and standard deviation (SD). The Kolmogorov-Smirnov test is used to determine normality. To analyze irrigation materials for each activation approach, one-way ANOVA was employed, followed by pairwise comparisons using Tukey's HSD test. Statistical analysis was performed using SPSS Version 20.0.

Results

Further evidence for the size, shape, and abundance of produced SOL-NPs was recognized by the transmission electron microscope (TEM) as shown in Figure 1. The SOL-NPs were observed in tiny sizes ranging from 6.4 to 51.7 nm with an average size of 22.6 nm and a uniform round shape.

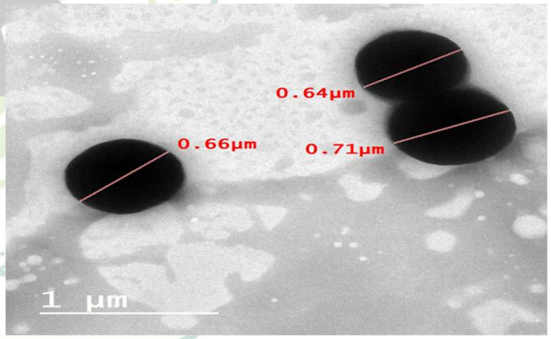

Figure 1: Transmission electron microscope photograph showing the particle size, shape, and abundance of SOL-NPs

Cytotoxicity of SOL extract, SOL-NPs, and $Ca(OH)_2$ against the cell line 1-BJ1

The cytotoxicity activity of the three medicaments is shown in Figure 2.

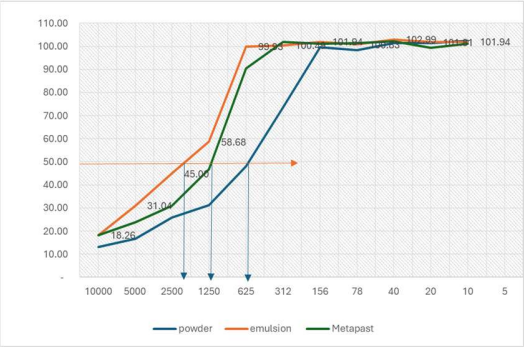

Figure 2: Cytotoxicity effect of SOL extract (Powder), SOL-NPs (Emulsion), and $Ca(OH)_2$ (Metapaste) against the cell line 1-BJ1

The results indicated that the three compounds (SOL extract, SOL-NPs, and $Ca(OH)_2$) exhibited concentration-dependent effects concerning their cytotoxicity against the normal skin fibroblast cell line 1-BJ1, with concentrations ranging from 10,000 µg/ml to 5 µg/ml. The results show that all three compounds exhibit a dose-dependent response, with higher concentrations generally leading to high inhibitory effect. The SOL extract showed a significant increase in response at higher concentrations. The SOL-NPs also revealed a high cytotoxic effect at higher concentrations. The Metapaste consistently increased in effect with higher concentrations. Control data confirmed the reliability of the experimental setup, showing consistent responses. The IC_{50} values, which indicated the concentration at which 50% of the maximum cytotoxicity was observed, were 594 µg/ml for SOL extract, 1468.8 µg/ml for SOL-NPs, and 1219 µg/ml for Metapaste, highlighting the SOL extract as the most potent. Overall, these results suggested that all three compounds exhibited inhibitory effect in the MTT assay, with varying degrees of efficacy.

The antibacterial effect of tested intercanal medicaments

The antibacterial effect of S. officinalis leaves extract and SOL-NPs against the anaerobic bacterium Enterococcus faecalis was evaluated on two depths: 50 and 100 µm (Tables 1 and 2). Data in Table 1 shows the inhibitory effect of SOL extract and SOL-NPs compared to Metapaste against the intra-canal existence bacterium, Enterococcus faecalis, at 50 µm. There was a significant reduction in bacterial cell counts after the application of Metapaste, SOL extract, and SOL-NPs compared to the untreated canals.

Table 1: The antibacterial effect of *Salvia officinalis* leaves extract (SOL) and its nanoform (SOL-NPs) compared to Metapaste (Calcium hydroxide paste) at a depth of 50 µm

Treatment	Bacterial count		
	Alive (Mean ± S. D.) (CFU/ml)	Dead (Mean ± S. D.)	Reduction (%)
Metapaste	29106.4±10310.11^{ab}	21819.8±7586.92^{a}	43.08±6.64^{a}
SOL extract	22559.7±12761.50^{a}	25021.3±12655.64^{a}	53.10±4.13^{a}
SOL-NPs	12959.2±6018.50^{c}	35747.2±13630.96^{a}	73.55±3.73^{b}
Negative control	31066.3±4338.59^{b}	1429.0±445.89^{b}	4.45±1.42^{c}
p-value	0.0001*	0.0001*	0.0001*

*significant difference as P<0.05.
Means with different superscript letters per row were significantly different as P<0.05.
Means with the same superscript letters per row were insignificantly different as P>0.05.

The live bacterial cell counts were 29106.4±10310.11, 22559.7±12761.50, and 12959.2±6018.50 CFU/ml after the application of Metapaste, SOL extract, and SOL-NPs, respectively. A statistically significant difference was observed when SOL extract and SOL-NPs compared to Metapaste regarding alive cells ($P < 0.05$). In addition, there was a significant difference between SOL extract and SOL-NPs regarding the mean alive cells. The dead cells for each treatment were 21819.8±7586.92, 25021.3±12655.64, and 35747.2±13630.96, respectively, and there was a significant difference (P<0.05). When comparing SOL extract and its nanoform with Metapaste no significant difference was observed

regarding the dead cells. The application of intracanal medicaments caused a significant reduction of 43.08±6.64, 53.10±4.13, and 73.55±3.73%, respectively (P<0.05), but no significant difference when comparing SOL extract with Metapaste.

Table 2: The antibacterial effect of *Salvia officinalis* leaves extract (SOL) and its nanoform (SOL-NPs) compared to Metapaste (Calcium hydroxide paste) at a depth of 100 µm

	Bacterial count		
	Alive (Mean ± S. D.) (CFU/ml)	Dead (Mean ± S. D.)	Reduction (%)
Metapaste	24157.3±7128.02^{a}	16618.9±4036.12^{a}	41.23±6.95^{a}
SOL extract	17625.4±11312.10^{b}	25883.4±11289.39^{ab}	61.01±7.72^{b}
SOL-NPs	10562.5±5285.54^{b}	28767.2±12847.73^{b}	72.95±4.24^{b}
Negative control	49349.2±72579.85^{a}	1039.4±331.89^{c}	3.74±1.85^{c}
p-value	0.0001*	0.0001*	0.0001*

*significant difference as P<0.05.
Means with different superscript letters per row were significantly different as P<0.05.
Means with the same superscript letters per row were insignificantly different as P>0.05.

Data in Table 2 shows the inhibitory effect of SOL extract and SOL-NPs compared to Metapaste against the intra-canal existence bacterium, Enterococcus faecalis, at 100 µm. There was a significant reduction in bacterial cell counts after the application of Metapaste, SOL extract, and SOL-NPs compared to the untreated canals. The live bacterial cell counts were 24157.3±7128.02, 17625.4±11312.10, and 10562.5±5285.54 CFU/ml after the application of Metapaste, SOL extract, and SOL-NPs, respectively. A statistically significant difference was observed when comparing the effect of SOL extract and SOL-NPs with Metapaste (P= 0.0001), but there was no significant difference between SOL extract and its nanoform, concerning the mean alive cells. The dead cells for each treatment were 16618.9±4036.12, 25883.4±11289.39, and 28767.2±12847.73, respectively, and they were statistically significantly different (P= 0.0001) but there was no significant difference between SOL extract and SOL-NPs. The application of intracanal medicaments caused a significant reduction of 41.23±6.95, 61.01±7.72, and 72.95±4.24%, respectively, and a significant difference was observed when comparing SOL extract and SOL-NPs with Metapaste (P= 0.0001).

Discussion

The fundamental principle of root canal therapy is to control the infection in the root canal system after mechanical preparation and irrigation by eliminating the current infection and preventing any future recurrence. [18] The introduction of antibacterial and disinfecting chemical agents into the root canal may directly inhibit or eliminate microorganisms, neutralize toxins, adjust ambient pH, and provide conducive biological circumstances for the repair and regeneration of periapical tissue.[19] Moreover, the intracanal drugs must have poor cytotoxicity and potent biocompatibility. Optimal intracanal medicaments must possess strong antibacterial properties, neutralize contaminants, and provide prolonged disinfection efficacy. It should also possess permeability and flowability. [20] It must possess the capability to establish a physical-chemical barrier inside the root canal, demonstrate superior biocompatibility, and mitigate inflammation in periapical tissues without inducing more irritation to the apical tissue. Conversely, it should not impede the repair, induction of healing, or creation of hard tissue in periapical tissues. [21] The medications now available in clinical practice have not fulfilled all these criteria. [22] Therefore, this study aimed to investigate the effectiveness of Salvia officinalis (sage) extract (SOL) and its nanoform (SOL-NPs) as potential treatments for infected root canals caused by E. faecalis compared to calcium hydroxide. E. faecalis is one of the most prevalent species identified in the canals of teeth with post-treatment apical periodontitis. However, current evidence

indicates that post-treatment infections are typically mixed and result from various bacterial combinations.[23] In addition, available medicaments possess several disadvantages including alterations to the oral microbiome, tooth discoloration, limited cost-effectiveness, and the promotion of pathogenic bacterial resistance. Consequently, it is essential to identify alternate therapeutic and preventative strategies to sustain oral hygiene. [24] Herbal extracts and their derivatives provide a cost-effective, safe, and biocompatible alternative to manufactured pharmaceuticals for addressing oral issues.[25] This aligns with the global trend the World Health Organization reported, indicating that over 75% of people worldwide depend on herbal plants for their primary healthcare needs owing to their remarkable physicochemical and pharmacological attributes. Herbal therapy may be used to manage oral problems as an antibacterial, anti-inflammatory, astringent, anesthetic, and anti-cariogenic agent.[26] In this study, the cytotoxicity and antibacterial effectiveness of SOL extract and its nanoform were compared to calcium hydroxide paste. Calcium hydroxide is an established and well-studied intracanal medication with antibacterial, pulp-dissolving, and dentin-repairing capabilities.[4] The study was meticulously crafted as an in vitro, parallel-group, single-centered, three-arm study with an equal allocation ratio of 1:1:1. This design ensures rigorous comparison and control among the three groups tested.[27] In this study recently extracted mature single, straight-canaled human permanent teeth devoid of root resorption or fracture were included. Using teeth with these characteristics ensured the reliability and consistency of the obtained results. [28] On the other hand, teeth exhibiting highly curved canals, fractures, resorption, or dilacerated roots were excluded from consideration. These exclusion criteria were employed to ensure a controlled experimental environment and to minimize potential confounding variables that could influence the results.[29] The SOL extract was prepared by water using the Soxhlet extraction method. This extraction method exhibited superior effectiveness in recovering hydrophilic chemicals from S. officinalis, as the combination of water and prolonged heating facilitated more effective cell disintegration, hence enhancing extraction capacity. [30] The primary outcome of this study examined the cytotoxic potential of SOL extract and SOL-NPs compared to calcium hydroxide on the normal human epithelial cell line 1-BJ1 using the MTT test, which assesses cell metabolic activity as a measure of cell viability, proliferation, and cytotoxicity.[31] The cytotoxicity effect of SOL extract, SOL-NPs, and $Ca(OH)_2$ against the normal skin fibroblast cell line 1-BJ1 was determined. The secondary outcome of this study determined the antibacterial activity of the SOL extract and SOL-NPs at two depths: 50 and 100 µm. A confocal scanning microscope was utilized to visualize the biofilm structure and quantify bacterial growth.[10,32]

The results showed that all three compounds exhibited concentration-dependent effects concerning their cytotoxicity, with higher concentrations generally leading to a high inhibitory effect. The SOL extract showed a significant increase in response at higher concentrations, while the SOL-NPs revealed a high cytotoxic effect at higher concentrations. The Metapaste consistently increased in effect with higher concentrations. The IC_{50} values were 594 µg/ml for SOL extract, 1468.8 µg/ml for SOL-NPs, and 1219 µg/ml for Metapaste, highlighting the SOL extract as the most potent.

The results also demonstrated that SOL extract and its nanoform exhibit substantial antibacterial activity against E.

faecalis in human root canals, with SOL-NPs displaying considerably more effectiveness than calcium hydroxide against E. faecalis. The findings indicate that SOL-NP is a viable choice for continued development as an antibacterial intracanal drug, especially for chronic E. faecalis infections. Potent antibacterial intracanal agents may mitigate the shortcomings of instrumentation to diminish germs in the intricate architecture of root canals. It was reported that S. officinalis extract has superior efficiency compared to antibiotics and has also less chance of drug resistance when used in high concentrations.[33].The results were also consistent with Shahriari et al.[34] who found potent antibacterial activity which was increased with the increased dose. They reported that this inhibitory activity may be due to the interactions of the extract with the oral fluids. Many studies also reported the inhibitory effect of the extract on many oral bacteria and fungi responsible for carious and periodontal infections. [32,35]

Calcium hydroxide ($Ca(OH)_2$) has been used as a standard intracanal treatment; nevertheless, it proved ineffective against E. faecalis. [36] The antibacterial effectiveness of $Ca(OH)_2$ was well demonstrated in endodontics. Nevertheless, fewer studies indicated its diminished efficiency against E. faecalis, even after prolonged application. [37–40]

Conclusion

SOL extract and SOL-NPs effectively reduced bacterial cell counts and dead cells, with SOL-NPs showing superior antibacterial properties, potentially treating Enterococcus faecalis in intracanal settings. The findings underscore the potential of plant-based solutions in managing dental infections while minimizing cytotoxic risks.

Ethics approval

The Research Ethics Committee of the Faculty of Dentistry at October 6 University reviewed and approved the current study on May 8, 2023, under ethical approval (RECO6U/20-2023).

Competing interest

The authors declare that they have no competing interests.

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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