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ANTIBACTERIAL ACTIVITY OF NANO HERBAL MEDICATIONS AGAINST *ENTEROCOCCUS FAECALIS* BIOFILM

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ABSTRACT

Aim: This study aimed to compare the antibacterial efficacy of nano-pomegranate loaded chitosan nanoparticles paste, curcumin nanoparticle gel, and 2% CHX gel against *E. faecalis* biofilm.

Methods: the three tested groups were compared as: group 1: Nano-pomegranate loaded chitosan nanoparticles (PN), group 2: curcumin nanoparticle gel (CN), group 3: 2% chlorhexidine (CHX) gel. Size distribution and average size of nano materials were confirmed by Transmission Electron Microscope (TEM). The antibiofilm activity against *E. faecalis* biofilm was tested; The minimum biofilm inhibitory concentration (MBIC), minimal biofilm-eradicating concentration (MBEC), *E. faecalis* cell suspension in the absence or presence of the best promising nano-material (PN) was examined under scanning electron microscope (SEM), and a preformed *E. faecalis* biofilm was established on teeth sections and the best promising nano-material (PN) was added and examined by a confocal laser scanning fluorescence microscope (CLSM). In addition, cytotoxicity evaluation of tested materials was done against human gingival fibroblast cells. Data were analyzed by ANOVA and Tukey's test ($p \leq 0.05$).

Results: The three tested materials recorded MBIC of 7.8 μg/ml**.** However, PN presented the lowest MBEC (15.6 μg/ml) compared to (CHN; 31.25 μg/ml), and CN recorded the highest MBEC (62.5 μg/ml) with statistically significant differences among them. Cytotoxicity studies revealed that CHN was the safest (446.99 μg/ml), followed by PN (IC_{50}= 30.74 μg/ml), then CN (14.47 μg/ml). SEM images of *E. faecalis* biofilm treated with PN demonstrated reduced count of adhering bacteria and distorted cells with surface irregularities and roughness. CLSM showed increase in red fluorescence intensity after biofilm treatment with MBIC (7.8 μg/ml) and MBEC (15.6 μg/ml) due to increased count of dead cells. A dramatic reduction in the biofilm thickness (83.3%) after treatment with MBECof PN (15.6μg/ml) with Statistical significance compared to the untreated

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control biofilm ($P < 0.05$). Moreover, subjecting *E. fecalis* cells to 7.8μg/ml resulted in a down regulation (45 to 65%) in expression levels of *ASA, CYT1, Esp,* and *ragg* genes involved in biofilm of *E. fecalis*.

Conclusion: Regarding cytotoxicity, CHN was the safest, followed by the PN and CN. Regarding antibacterial effect; the three tested materials were equally effective in the inhibition of biofilm formation, while PN loaded in chitosan was the most effective one in eradication of an already established biofilm.

KEYWORDS: Nanoparticles, Pomegranate peel extract, Biofilm, Persistent, Endodontic, *Enterococcus faecalis*

INTRODUCTION

Bacterial infections in root canals can be either free-floating planktonic or in the form of biofilms, which are colonies of microorganisms linked by an exopolysaccharide matrix [1]. *Enterococcus faecalis* (secondary and persistent endodontic infections) and Gram-positive bacteria with black pigmentation (primary endodontic infections) are the most frequent bacteria affecting a root canal[2]. Some highly resistant bacteria, such as *E. faecalis*, can build complex biofilms, which enhance the bacteria's resistance to different antibacterial medications [3, 4]. Due to its strong resistance, *E. faecalis* is the most often detected bacteria in endodontic treatment failure cases (24–77%) [5].

The removal of intracanal bacterial biofilms is the primary factor determining the efficacy of root canal therapy [6, 7]. To get rid of root canal germs, mechanical root canal preparation is crucial. It does not, however, totally eradicate root canal bacteria because it leaves more than 35% of the root canal's surface untreated. Therefore, it's essential to utilize medication, irrigation, and antibacterial agents to eliminate any leftover pathogens in the root canal[8,9].

E. faecalis can be effectively killed by the broad-spectrum antibacterial agent chlorhexidine gluconate [10,11]. Despite having strong antibacterial properties, Chlorhexidine 2% is highly toxic to periapical tissue, slows down the healing process, and causes an inflammatory reaction when it encounters healthy tissue[12,13]. Due to these shortcomings, other antibacterial agents with good biocompatibility were sought after.

Biologically active plant compounds have garnered considerable interest as viable substitutes for synthetic hazardous products in recent times. This is because many microorganisms have become resistant to these compounds, and plant extracts are thought to be safer than synthetic compounds [14].

The bioactive curcuminoid polyphenol curcumin has been isolated from the rhizomes of Curcuma longa and is well-known for its antibacterial properties. Curcumin's main components that demonstrate antioxidant properties are called curcuminoids. Its antibacterial properties are extensive and effective against a variety of microorganisms, including antibiotic-resistant ones. Additionally, curcumin has the potential to be antiviral and antifungal [15]. Furthermore, by inhibiting the pathogen's virulence factors and enhancing host-mediated immunity, it functions as an immunomodulator to combat bacterial infections[16]. Since curcumin's main drawbacks are poor water solubility, low bioavailability, and fast degradation, the use of nanotechnologies for the delivery of curcumin could increase the prospects for its clinical application [15].

Growing mostly in Spain, pomegranate (*Punica granatum*, Linneo) fruit is a crop grown in Europe. Pomegranates have been shown in numerous studies to exhibit insecticidal, anthelmintic, antifungal,

antibacterial, and antiviral properties against plant and human infections. A methanolic pomegranate peel extract demonstrated antibacterial activity against *S. aureus, Lactobacillus acidophilus, Streptococcus mutans, and Streptococcus salivarius*, in accordance with research by Abdollahzadeh et al. [18]. *P. granatum* has also been demonstrated to inhibit oral pathogenic bacteria [17]. Pomegranate fruit, especially the peel, may therefore have antibacterial properties that make it safe to use in place of synthetic antimicrobial agents [17].

The use of nanoparticles in endodontic treatment has created new and interesting opportunities for disinfection strategies and treatment efficacy. To ascertain their individual and combined benefits as safe antibacterial agents in endodontics, this study analyzed the cytotoxicity and antibacterial efficacy of nano-curcumin (NC), nano-pomegranate (NP) loaded in chitosan in comparison to Chlorhexidine against *E. Faecalis* bacteria in biofilm to determine their benefits as safe antibacterial agents in the endodontics. The zero hypothesis was that there is no difference between the experimental medications in terms of their antimicrobial activity against *E. faecalis*.

MATERIALS AND METHODS

Chemicals used:

Dimethyl sulfoxide (DMSO), MTT, and trypan blue dye were purchased from Sigma (St. Louis, Mo., USA). Fetal Bovine serum, RPMI-1640, HEPES buffer solution, L-glutamine, gentamycin, and 0.25% Trypsin-EDTA were purchased from Lonza (Belgium). Chitosan medium molecular weight with DD>85%, and glacial acetic acid were purchased from Loba-Chemie, India. Penta sodium tripolyphosphate (TPP) and Methanol HPLC were supplied from Millipore Merck, Germany. Bile esculin agar was purchased from OXOID (UK). Sodium chloride was purchased from El Nasr Pharmaceutical Chemicals Co., Cairo, Egypt.

Bacteria and cell culture

Test Microorganisms

E. faecalis OG1RF, was used in the present study as a reference strain. Ten *E. faecalis* isolates and biofilm-forming were also used. All of them were obtained from the Department of Microbiology and Immunology, Faculty of Pharmacy, Tanta University, Egypt. A freeze-dried cryotubes were recultivated using bile esculin agar as a selective medium.

Cell Line

Mammalian cell lines: Human Gingival Fibroblast cells (ATCC® PCS-201-012™) were purchased from the laboratory of the Tissue Culture Department of the Holding Company for Biological Products and Vaccines (VACSERA), Cairo, Egypt.

Teeth used for *ex vivo* model

Six single-rooted permanent mandibular premolars extracted for periodontal reasons were authorized by the research ethics committee of the Faculty of Dentistry, Tanta University. A dental operating microscope (Zumax, Suzhou New District, China) set at a magnification level of × 8 was used in teeth examination, and radiographs were captured to exclude teeth with fractures, resorption, or calcification, as well as teeth with multiple root canals.

Teeth cleaning was done using an ultrasonic scaler (Satelec, Cedex, France) and decoronation was done by a diamond stone attached to a handpiece operating at a high speed under sufficient water. The roots were immersed in 5.25% sodium hypochlorite (NaOCl) solution for 30 minutes. Subsequently, they were placed in a saline solution

The samples were further sectioned using a 0.3-mm IsoMet saw (IsoMet 4000 Precision Saw, Secunderabad, Telangana, India) maintaining a consistent cooling process with sterile distilled water. Two pieces of 1 mm thickness were extracted

from each root, specifically from middle third. The root portions were rinsed with sterile distilled water for a duration of 1 minute and subsequently dried uniformly.

Ethical consideration:

According to the research guidelines adopted by the Research Ethics Committee, Faculty of Dentistry, Tanta University, the purpose of the present study was explained to the patients and informed consents were obtained to use their teeth in the research and the design and all procedures of the present study were accomplished.

Preparation Method of nanomaterials

Both nano herbal medications and Chlorhexidine gel were prepared by Nano Gate Company (Cairo, Egypt) as follows:

Herbal nanoparticles preparation:

Punica granatum loaded chitosan nanoparticles were obtained upon the addition of a tripolyphosphate (TPP) aqueous solution to a Chitosan solution with little modifications. Briefly, chitosan (0.2 g) was added to 40 mL acetic acid (1% v/v) to achieve a 0.5% w/v concentration, and it was then kept overnight under magnetic stirring at ambient temperature to get a clear solution, and its pH was adjusted to 4.6 using a 1 M NaOH. Chitosan solution was then filtered through a 0.45 μm syringe filter. PPE was added to the CS solution 1.0 ml (22.50 mg).

About 0.3 gm of *Punica granatum* nanopowder were added to 15 ml distilled water with sonication and stirring for 1h to get a concentration 2 %w/v then, 0.6 gm of Hydroxy propyl Methyl Cellulose (Loba Chemie, India) was sprinkled gently and gradually over the solution under mild temperature with vigorous stirring to get homogenous paste.

Curcumin powder (Loba Chemie, India) was milled by using ball mill machine (planetary-ball-mill-pm-400) for 10h, speed 350rpm and 3min intervals. After that, suspend 0.3 gm of nano Curcumin powder in DH2O with sonication and stirring for 1h to get a concentration 2 %w/v then, 0.6 gm of Hydroxy propyl Methyl Cellulose (Loba Chemie, India) was sprinkled gently and gradually over the solution under mild temperature with vigorous stirring to get the homogenous gel.

Formulation of 2% chlorhexidine gel

Chlorhexidine gel was prepared by dispersing sodium carboxymethyl cellulose (3%) as a gelling agent in chlorhexidine gel (2%) for a specified period of time dissolved. Glycerin and preservative propyl paraben were added to the above dispersion and were stirred continuously till it formed a homogeneous product.

Characterizations:

Nanomaterials size and shape:TEM studies were conducted on JEOL JEM-2100 high-resolution transmission electron microscope at an accelerating voltage of 200 kV. TEM samples were prepared by adding a droplet of colloid suspension in respective solvent on a Formvar carbon-coated, 300-mesh copper grid (Ted Pella) and permitting them to evaporate in air at ambient conditions. An image analysis software package was used to determine size distribution and average size.

Assessment of Cytotoxic Effects of the test nano-materials

Cell line Propagation:

The cells were grown on RPMI-1640 medium supplemented with 10% inactivated fetal calf serum and 50μg/ml gentamycin. The cells were preserved at 37ºC in a humidified atmosphere with 5% CO_2 and were subcultured two to three times a week.

Cytotoxicity assessment using viability assay:

The cell line was suspended in a medium at a concentration of $5x10^4$ cell/well in Corning® 96-well tissue culture plates, then incubated for

24 h. The tested nanomaterials were then added into 96-well plates (three replicates) to achieve eight concentrations for each compound. Six vehicle controls with media or 0.5% DMSO were run for each 96-well plate as a control. After incubating for 48 h, the numbers of viable cells were determined by the MTT test. Briefly, the media was removed from the 96 well plate and replaced with 100 μl of fresh culture RPMI 1640 medium without phenol red then 10 μl of the 12 mM MTT stock solution (5 mg of MTT in 1 mL of PBS) to each well including the untreated controls. The 96 well plates were then incubated at 37°C and 5% CO_2 for 4 h. An 85 μl aliquot of the media was removed from the wells, and 50 μl of DMSO was added to each well and mixed thoroughly with the pipette and incubated at 37°C for 10 min. Then, the optical density was measured at 590 nm with the microplate reader (SunRise, TECAN, Inc, USA) to determine the number of viable cells and the percentage of viability was calculated as [(ODt/ODc)]x100% where ODt is the mean optical density of wells treated with the tested sample and ODc is the mean optical density of untreated cells. The survival curve of the cell line after treatment with the specified compounds was made by plotting the relation between surviving cells and drug concentration. The 50% inhibitory concentration (IC_{50}), the concentration required to cause toxic effects in 50% of intact cells, was estimated from graphic plots of the dose-response curve for each concentration using Graphpad Prism software (SanDiego,CA.,USA) (https://www.sciencedirect.com/science/article/abs/pii/S1773224723009437).

Antibiofilm assay

Inhibitory effect on *E. faecalis* biofilm formation and pre-established biofilm

The biofilm assay was performed in polystyrene flat-well microtiter plates using Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 mg/mL sucrose. Mid-logarithmic phase cultures of *E. faecalis* isolates were diluted to achieve a cell density of 5×10^5 CFU/mL. A volume of 100 μL of each bacterial suspension was then aseptically transferred to the wells, followed by static incubation for 24 h. This assay was conducted in the absence and presence of the test nanomaterials, which were added at concentrations ranging from 3.9 to 500μg/mL. Following incubation, the culture medium was carefully removed by aspiration to eliminate planktonic cells, and the wells were rinsed three times with phosphate-buffered saline (PBS, pH 7.4) to further remove non-adherent bacteria. Adherent cells were subsequently fixed with 99% (v/v) methanol. After a 15-minute fixation period, the methanol was removed, and the wells were allowed to air dry for 30 min. The wells were then stained with 0.1% (w/v) crystal violet for 15 minutes at room temperature. After staining, the wells were gently washed three times with PBS to remove excess dye, followed by another 30-minute air-drying period. Finally, the crystal violet stain was solubilized by adding 100 μL of 95% methanol to each well, and biofilm formation was quantitatively assessed by measuring absorbance at 595 nm using a microplate ELISA reader. The minimum biofilm inhibitory concentration (MBIC) was defined as the lowest concentration of the antimicrobial agent required to inhibit biofilm formation, as described by Elshinawy el al.[19].

The challenge of eradicating the biofilm of *E. faecalis* using test nanomaterials Through an *ex-vivo* Teeth Model

After being incubated for 24 h at 37°C, a cell suspension of E. *faecalis* was added to the microtiter plate to create a biofilm on thin sections of premolar teeth in order to study the impact of nanomaterials on the developed biofilm. After incubation, non-adherent cells were eliminated from the wells by carefully aspirating the medium and thoroughly washing them with sterile PBS. The

corresponding wells were filled with a fresh medium DMEM containing the test substance at the proper concentration, followed by incubation for 24 h at 37°C. After that, the well was stained as previously mentioned and the medium was aspirated. We conducted three replications of each experiment. The nanomaterials' minimal biofilm-eradicating concentration (MBEC) was defined as MBEC80 where the drug concentration eradicated 80% of *E. faecalis* biofilm compared to the untreated control[19].

Scanning electron microscopy (SEM)

A suspension of *E. faecalis* cells in DMEM was introduced into a 6-well microtiter plate containing sterile coverslips, enabling cell adhesion. The experiment was conducted with or without the addition of the most promising nanomaterial (PN), applied at its MBIC (7.8 µg/ml) or MBEC (15.6 µg/ml) concentrations. Plates were then incubated under anaerobic conditions at 37°C for 24 h. Following incubation, the medium was carefully aspirated, and the wells were gently washed with PBS to remove any non-adherent cells [22]. The coverslips were then transferred to a fixation solution containing 2.5% glutaraldehyde for 4 h at room temperature before being transported to the Scanning Electron Microscopy Unit at the Nanotechnology Institute, Kafr-Elsheikh University, for processing and imaging.

Confocal laser scanning microscopy (CLSM)

A pre-established biofilm of *E. faecalis* was cultivated on tooth sections within a microtiter plate for 24h, as mentioned previously. The selected nanomaterial, identified as the most promising candidate (PN), was introduced at ½ MBEC (7.8µg/ml) or the MBEC (15.6 µg/ml) along with fresh medium into the test wells and incubated anaerobically at 37°C for 24 h[22]. After the incubation period, the medium was carefully removed, and the tooth sections were gently rinsed three times with PBS. Subsequently, the samples were stained with the Live/Dead™ staining kit for approximately 15 min at room temperature. In this assay, live bacterial cells within the biofilm fluoresced green due to acridine orange uptake, while dead cells appeared red due to staining with propidium iodide stain. The biofilms were then visualized using a confocal laser scanning fluorescence microscope (CLSM) following the manufacturer's protocol, and Z-stack images were generated. Additionally, measurements of biofilm thickness were recorded.

qRT-PCR

To quantify the expression levels of *E. faecalis* biofilm-associated genes, the qRT-PCR was performed. Bacterial RNA was extracted from mature biofilms using RNA extraction kit. Complementary DNA (cDNA) synthesis was carried out using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific), as per the manufacturer's protocol. Quantitative PCR was then performed using specific primers (Table 1) for the biofilm genes of interest (*ASA*, *CYT1*, *Esp*, and *ragg*), with *recA* employed as the housekeeping gene for normalization. Amplifications were conducted in a 96-well plate using SYBR Green Master Mix on a StepOnePlus™ Real-Time PCR System (Applied Biosystems). The reaction conditions included an initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Relative expression levels of the target genes were calculated using the ΔΔCt method, with *recA* as the internal control to normalize gene expression.

The transcriptional level of virulence and biofilm-related genes of *E. feacalis* was evaluated in the absence and presence of 7.8 µg/ml of the PN, and was analyzed to further investigate the potential mode of action of PN as antibiofilm agent.

TABLE (1) Sequences of primers for test genes of *E. faecalis* and their role in biofilm formation.

Gene name	Function	Oligonucleotide sequence
ASA	Aggregation substance; The asa1 gene encodes aggregation substance, a surface-associated protein that facilitates the adhesion of E. faecalis to host tissues and other bacterial cells, a critical first step in biofilm development.	GCACGCTATTACGAACTATGA
		TAAGAAAGAACATCACCACGA
CYT1	Cytolysin; codes cytolysin, a potent virulence factor that has been implicated in biofilm-associated infections due to its cytotoxic effects on host cells and immune evasion properties. Cytolysin not only assists in biofilm stability by lysing competing bacteria and immune cells, but it also promotes the dissemination of the biofilm in host tissues by damaging epithelial barriers. This protein plays a critical role in maintaining biofilm integrity under environmental stresses, allowing *E. faecalis* to persist and thrive within hostile host environments	ACTCGGGGATTGATAGGC
		GCTGCTAAAGCTGCGCTT
ESP	This gene encodes for the enterococcal surface protein, a well-characterized factor essential for biofilm formation on abiotic surfaces, such as catheters and implants. *Esp* facilitates initial adherence to surfaces and enhances the stability and robustness of the biofilm matrix by anchoring cells to extracellular structures.	AGATTTCATCTTTGATTCTTGG
		AATTGATTCTTTAGCATCTGG
recA	Housekeeping gene	CGACTAATGTCTCAAGCACTAC
		CGAACATCACGCCAACTT
Ragg	Regulator of Aggregation; the ragg gene encodes a regulatory protein that influences biofilm formation by modulating the expression of genes involved in aggregation, such as asa1. Ragg functions as a transcriptional regulator, fine-tuning the levels of aggregation substance and other surface proteins, thereby affecting the structure and resilience of the biofilm.	CGTTGATAAAGCAGTTGAT
		TTGTAGTTGGTCTACTTCTT

RESULTS

Transmission electron microscopy (TEM) images of test nanomaterials, illustrated the spheroidal shape with nanosize of the test nanomaterials.

The cytotoxicity of nano-materials on human fibroblasts results were presented in (Fig. 2). The IC_{50} for the tested materials was determined from the plotted curves recording an IC_{50} of 30.74± 0.61 μg/ml (for PN), 446.99±11.76 μg/ml (for CHN), and 14.47± 0.25 μg/ml (for CN). One-way ANOVA revealed a significant difference ($p < 0.05$) between the test groups and the untreated control group. Interestingly, the CHN was the safest, followed by the PN as shown in figure (2).

The impact of materials on the inhibition of *E. feacalis* biofilm was investigated in the present work. Results revealed that the three tested materials recorded MBIC of 7.8 μg/ml as shown in figure (3A). Furthermore, PN presented the lowest MBEC (15.6 μg/ml) compared to the positive control (CHN; 31.25 μg/ml), however, CN recorded the highest MBEC (62.5 μg/ml) as demonstrated in figure (3B). Hence, PN was selected for further antibiofilm investigations.

SEM images of *E. faecalis* biofilm on coverslips in the absence or presence of PN were presented in figure (4). There were a large count of adhering *E. faecalis* bacteria appeared in clusters for untreated control sample with intact bacterial membranes and

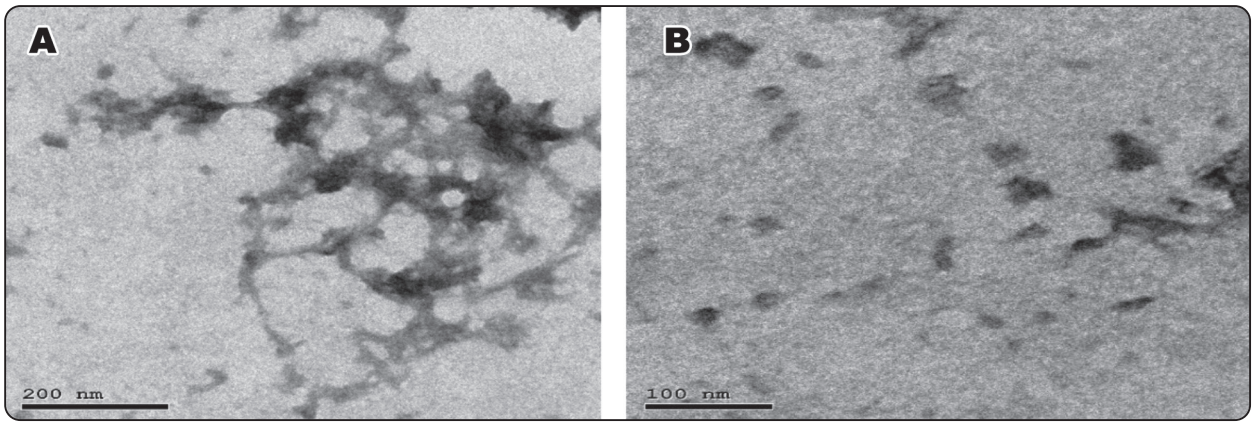

Fig (1) TEM images of prepared (A) PN and (B)CN.

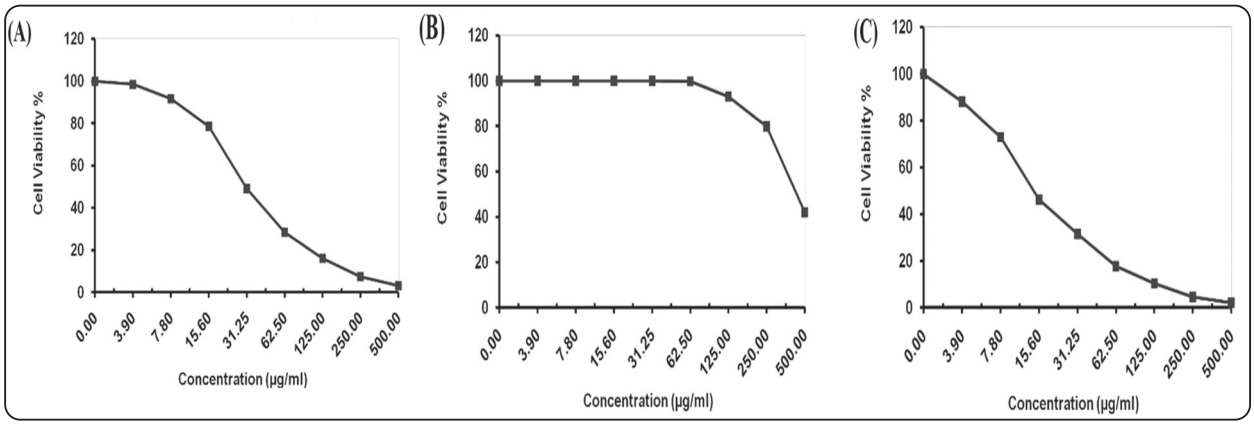

Fig (2) Cytotoxicity data of test nano-materials on human fibroblast showing the effect of (A) PN, (B) CHN, and (C) CN on the cells.

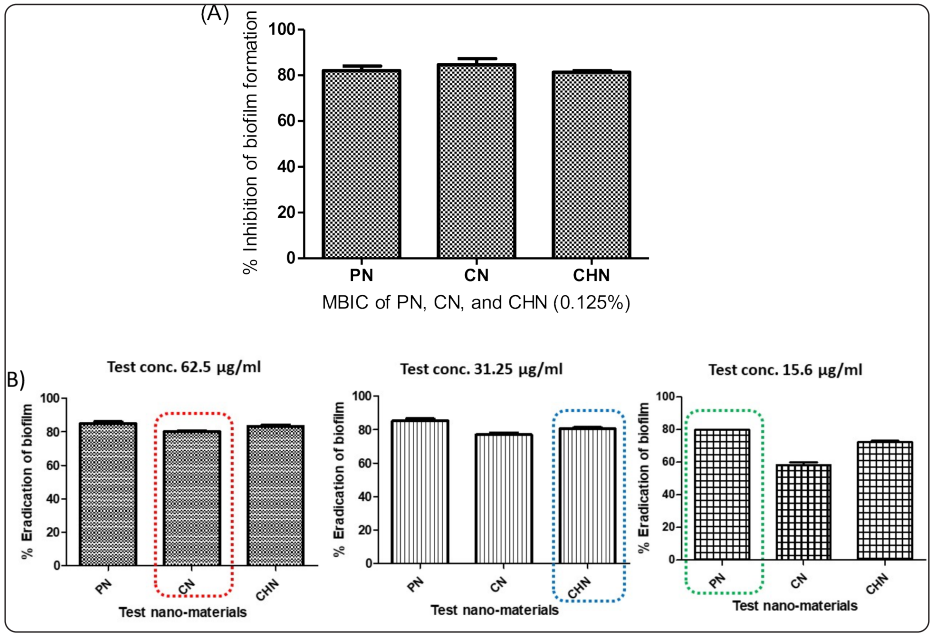

Fig. (3) Antibiofilm impact of herbal nanoparticles displaying (A) inhibition of *E. feacalis* biofilm formation by all test medicaments recording the same MBIC (7.8 μg/ml), and (B) eradication of *E. feacalis* pre-established biofilm by PN recording low MBEC (15.6 μg/ml) compared to CHN. Dotted boxes refer to MBEC for each nano-material.

cell walls, and rounded regular edges (figure 4 A and B). The other group treated with MBIC of PN (7.8 μg/ml) demonstrated irregularities in the outer surfaces (figure 4C). Additionally, the group treated with MBEC of PN (15.6 μg/ml) showed markedly reduced count of adhering *E. faecalis* bacteria with impaired and distorted cells showing rupture and leakage of the cytoplasmic components indicating severe damage (figure 4D).

The biofilm architecture of *E. feacalis* was further analysed using confocal laser scanning microscopy to better evaluate the antibiofilm activity of PN. Untreated *E. faecalis* control cells displayed well-structured and green-fluorescent biofilm indicating bacterial viability (figures 5A) after 24h incubation. The thickness of the untreated biofilm was 90 μm figures (5A and 6B). An increase in red fluorescence intensity (22.5%) was observed after 24h of incubation upon increasing the concentration of PN, denoting a progressive exponential decrease in the viability of the biofilm-forming bacterial cells. Interestingly, the thickness (60 μm) of the biofilm was markedly decreased (33.3%) following treatment with 7.8 μg/ml (figures 5B and 6B), however, this reduction in the biofilm thickness was dramatically observed (15 μm; 83.3%) after subjection to 15.6 μg/ml of PN as shown in Figures (5 C and 6 B). Values of fluorescence average in addition to the standard deviation (SD) designated in arbitrary units were presented in figure (6A), however, biofilm thickness measured in μm after and before treatment with PN was shown in figure (6B).

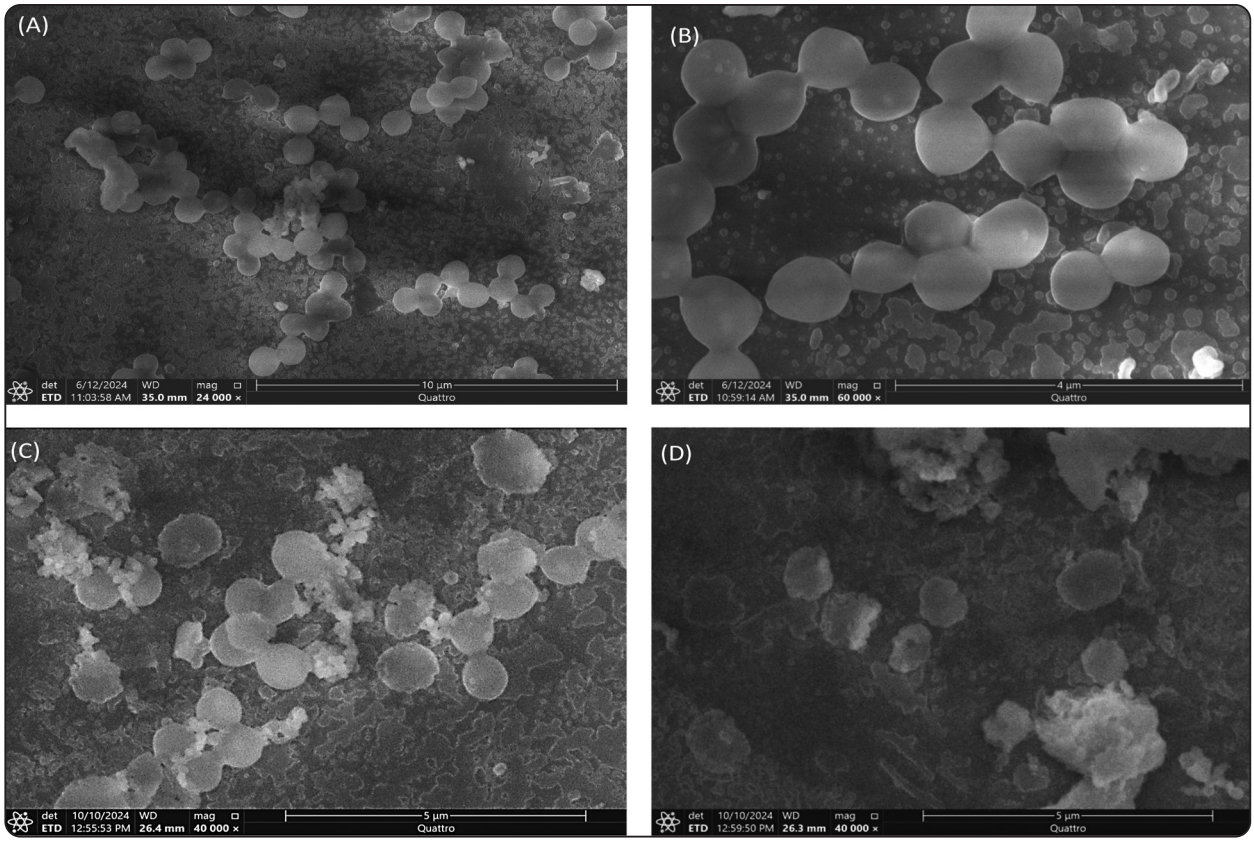

Fig. (4) SEM images of *E. feacalis* biofilm in the absence or presence of PN. Bacterial cells of untreated control seemed in clusters with normal rounded shape (A and B). Cell treated with MBIC of PN (7.8 μg/ml) were shown with outer irregularities as pointed by the arrows (C). Also, *E. faecalis* treated with MBEC of PN (15.6μg/ml) were detected in a low number with severe damage and intracytoplasmic leakage (D).

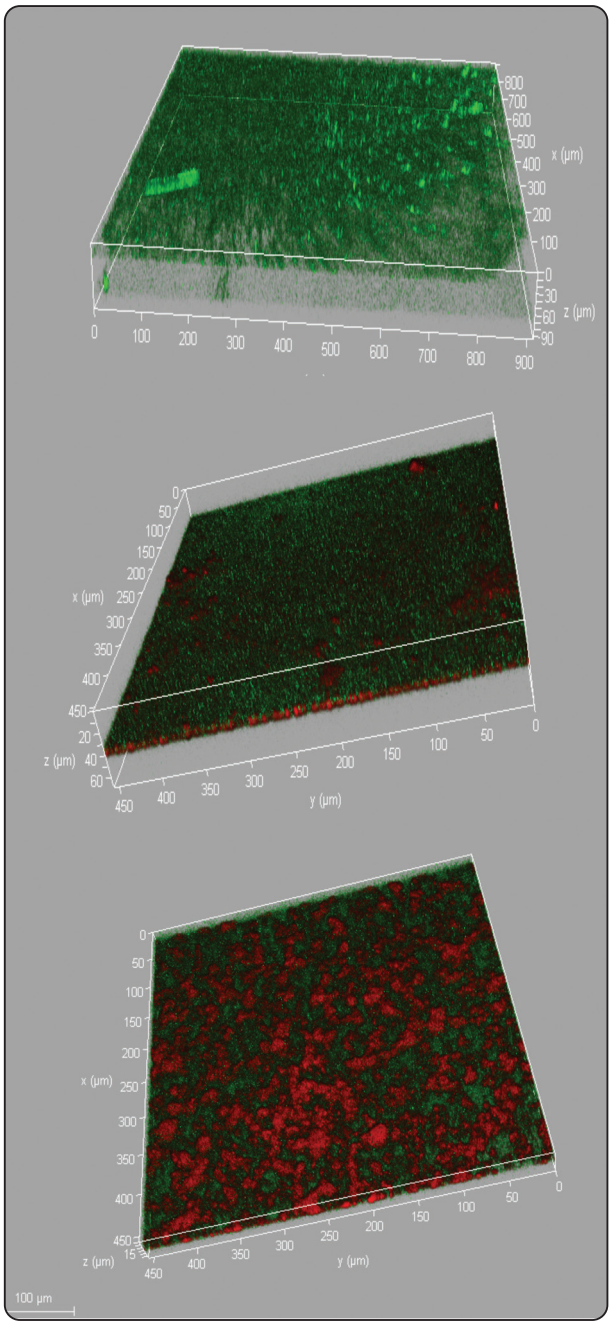

Fig. (5) Images of LIVE/DEAD-marked *E. faecalis* taken by confocal laser scanning microscope for 24-h-bacterial biofilm in the absence or presence of PN. (A) control untreated *E. faecalis* biofilm showed green fluorescence meaning that live bacteria were major and so absorbed the color of acridine orange stain, (B) bacterial biofilm treated with 1/2MBEC (7.8 μg/ml) displaying appearance of red-stained areas due to dead cells stained with propidium iodide, and (C) increased red fluorescence in the field after treatment with MBEC (15.6 μg/ml).

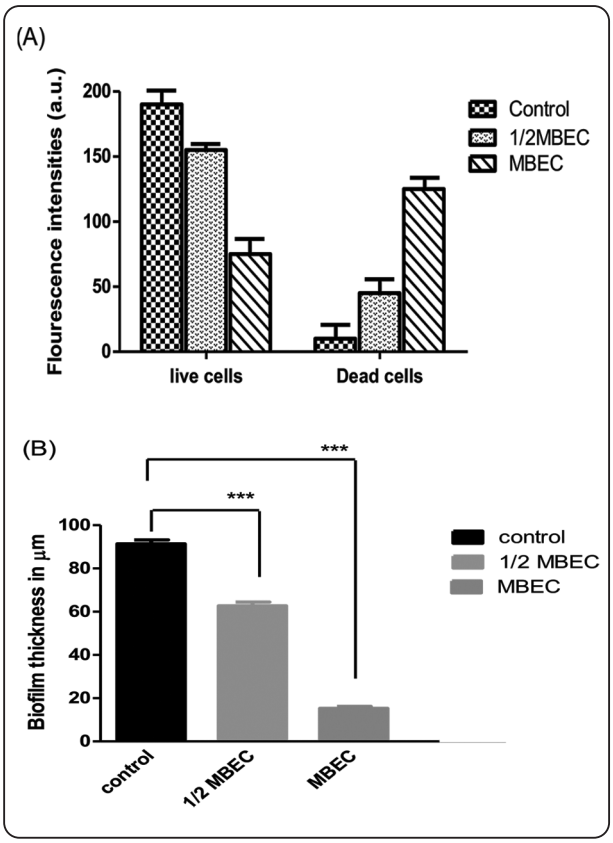

Fig. (6) (A) Values of fluorescence average in addition to the standard deviation (SD) designated in arbitrary units. (B) Biofilm thickness measured in μm after and before different treatment concentrations using PN. Statistical significance presented as asterisk above bars different from the untreated control biofilm ($P < 0.05$).

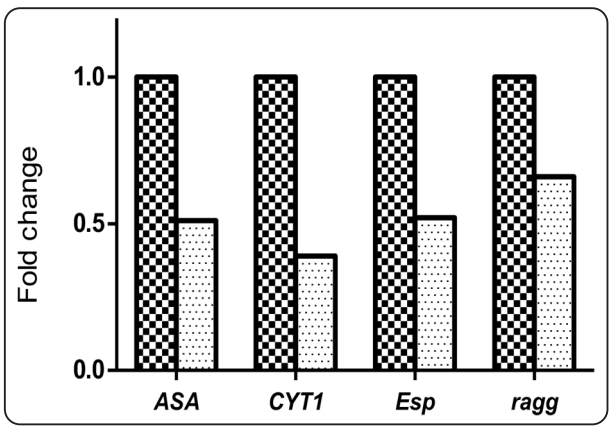

Fig. (7) Analysis of gene expression of *E. feacalis* in the absence and presence of 7.8μg/ml of PN.

Data revealed a marked reduction in the expression of *ASA, CYT1, Esp,* and *ragg* by 45 to 65% as shown in Figure (7).

DISCUSSION

The antibacterial efficacy of nano-herbal medications on *E. faecalis* biofilm was tested in this study, whether the effect on the formation of biofilm or the eradication of the pre-established biofilm. *E. faecalis* was chosen due to its high resistance to antibacterial agents since it has the ability of biofilm formation in root canals [19]. Accordingly, alternative agents with antibiofilm activity or even quorum-sensing inhibitory compounds are more recommended than traditional antimicrobial therapy [1-4]. Phytochemicals such as pomegranate and curcumin are known to be rich in antimicrobials with promising effects as antibiofilm and this is considered a part of their natural defense mechanisms suggesting them as valuable candidates for potential therapeutic use [15, 18].

Our results showed that the three tested herbal nano-materials recorded the same MBIC (7.8 μg/ml), however, PN recorded the lowest MBEC (15.6 μg/ml) on the pre-formed *E. faecalis* biofilm compared to the positive control (CHN; 31.25 μg/ml). Interestingly, both MBIC and MBEC of PN were safe (IC_{50}= 30.74 μg/ml) against normal fibroblasts. On the other hand, CN recorded the highest MBEC (62.5μg/ml) which was unsafe (IC_{50}=14.47 μg/ml) to be used for further studies. The activity of PN on *E. faecalis* biofilm could be attributed to the combined effect of pomegranate loaded in chitosan nanoparticles. First, pomegranate has a direct antibacterial effect particularly against Gram-positive bacteria due to the high content of tannins, such as anthocyanins and polyphenolic compounds (especially punicalagin). The polar regions of the microbial membrane is affected by the hydrophilic part of the tannin chemical structure, whereas a nonpolar inner section of the bacterial membrane is affected by the hydrophobic part; which cause membrane instability and subsequently substrates transport change inside the microbial cell [37]. Phenolic toxicity to the microbial cells due to biochemical reactions with sulfhydryl groups or through more nonspecific interactions with proteins causing function loss was also suggested by Naz et al. [38]. Moreover, substrates may be unavailable to microorganisms due to presence of phenols. In addition, phenols may interfere with bacterial protein secretions [39-40]. Second, chitosan is considered as an antimicrobial cation that acts on the cell membrane, The positive ions of chitosan could attach to the negative ions on the outer surface of *E. faecalis*-forming the biofilm causing cell lysis and intracellular electrolyte leakage (e.g., nucleic acid and glucose) [32-35]. These findings were also supported by the outcomes of SEM in the current study where surface irregularities, roughness, cellular damage, and leakage were observed. Similar conclusions were stated by Li and colleagues [27].

Regarding the impact of the best promising nano-material (PN) on the viability of *E. fecalis* cells in the mature biofilm developed on teeth slices, CLSM was used for evaluating this effect before and after treatment. The analysis of the bacterial biofilm architecture revealed the appearance of red-fluorescent areas (22.5%) following treatment with ½ MBEC (7.8 μg/ml) of PN. This is due to the presence of dead cells stained with propidium iodide dye. Furthermore, the red fluorescence was increased dramatically (62.5%) after using the MBEC (15.6 μg/ml). On the contrary, untreated *E. faecalis* control cells displayed well-structured and green-fluorescent biofilm (95%) due to staining with acridine orange indicating bacterial viability and recorded a thickness of 90 μm. Interestingly, the thickness (60 μm) of the biofilm was markedly decreased (33.3%) following treatment with 7.8 μg/ml, however, this reduction in the biofilm thickness was dramatically reduced (15 μm; 83.3%) after subjection to 15.6 μg/ml of PN. This was also in agreement with the study of Elshinawy and co-workers [19] who reported that Ch-NPs significantly diminished the total biofilm mass relative to the

control groups, exerting 97 and 94% decreases in both *E. fecalis*-single well as -mixed species biofilms, respectively. This may be due to the ability of nanoparticles to bind to calcium, and magnesium ions, which have a role in maintaining the integrity of the biofilm matrix [36].

At the molecular level, PN showed a marked downregulation in the quorums sensing genes regulating *E. feacalis* biofilm. The expression levels of *ASA, AYT1, Esp,* and *ragg* genes were reduced by 45 to 65%. These genes contribute distinct yet complementary functions that collectively support biofilm formation, maintenance, and resilience in *E. faecalis*. Their coordinated expression enhances biofilm stability, promotes genetic exchange, and improves the biofilm against host immune responses and antimicrobial agents, making *E. faecalis* a challenging pathogen in clinical settings [43]. Therefore, their downregulation by PN suggests its potential use as antibiofilm agent.

In conclusion, the study highlights the safety and efficacy profiles of CHN, PN, and CN in their potential applications against endodontic infections. CHN was used as a positive control and demonstrated the highest biocompatibility, marking it as the least cytotoxic among the tested materials, followed by PN and CN. All test agents exhibited comparable efficacy in preventing biofilm formation by *Enterococcus faecalis*, a common and resilient endodontic pathogen. Notably, PN loaded in chitosan showed superior efficacy in eradicating established biofilms, positioning it as a promising candidate for intracanal disinfection. The potent antibiofilm action of PN may stem from its ability to disrupt bacterial cell wall integrity, interfere with quorum sensing pathways, or inhibit the extracellular matrix synthesis essential for biofilm stability. Such mechanisms render PN a potentially effective agent in targeting persistent biofilms associated with endodontic infections, and its integration into endodontic treatment protocols could significantly improve therapeutic outcomes by addressing the challenges posed by biofilm-related microbial persistence.

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