Antibacterial effect of propolis nanoparticles against Enterococcus faecalis biofilm in the root canal

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Abstract
Background: To determine the antibacterial effect of propolis nanoparticles (PNs) as an endodontic irrigant against Enterococcus faecalis biofilm in root canal.

Methods: PNs were prepared by ultrasonication and the particle size distribution and polydispersity index were determined by dynamic light scattering using Zetasizer Nano S90. 210 extracted human teeth were sectioned to obtain 6mm of the middle third of the root. The root canal was enlarged to an internal diameter of 0.9mm. The specimens were inoculated with E. faecalis for 21 days. Following this, specimens were randomly divided into seven groups having 30 dentinal blocks in each group including group I: saline, group II: propolis 100µg/ml, group III: propolis 300µg/ml, group IV: propolis nanoparticle 100µg/ml, group V: propolis nanoparticle 300µg/ml, group VI: 6% sodium hypochlorite, group VII: 2% chlorhexidine. Dentine shavings were collected at 200 and 400 µm depths, and total numbers of CFUs were determined at the end of one, five, and ten minutes. The non-parametric Kruskal Wallis and Mann-Whitney tests were used to compare the differences in reduction of CFUs between all groups and probability values of P < 0.05 were set as the reference for statistically significant results. The scanning electron microscope and confocal laser scanning microscopy were also performed after exposure to PNs.

Results: PN300 was significantly more effective in reducing CFUs compared to all other groups (p <0.05) except 6% NaOCl and 2% CHX (p >0.05) at all-time intervals and both depths. At five minutes, 6% NaOCl and 2 % CHX were the most effective in reducing CFUs (p <0.05) however, no significant difference was found in between PN300, 6% NaOCl and 2 % CHX at 10 minutes (p >0.05). SEM images also showed the maximum reduction of E. faecalis with PN300, 6% NaOCl and 2% CHX (>90 %) at five and ten minutes. CLSM images showed the number of dead cells in dentin was highest with PN300 (>90%) compared to PN100 (>40%) and saline (all live cells).

Conclusion: PN300 was equally effective as 6% NaOCl, and 2% CHX in reducing E. faecalis CFUs after one minute, five and ten minutes at both depths.

Background
Primary objective of endodontic treatment of pulpal and periapical diseases is to eradicate microbial
infections from the involved root canal system. [1, 2]. Microorganisms in the root canal space can attach to each other and grow into biofilm as a microbial community on the dentin walls and make the disinfection challenging [3, 4]. Root canal disinfection can be achieved by mechanical and chemical means and irrigation with endodontic irrigant plays a crucial role [5] because it can reach areas with anatomical complexities including isthmus, ramifications, dentinal tubules of root canal system, where instrumentation cannot reach thereby, facilitate reduction of microbial biofilms [6, 7]. However, bacteria in mature biofilm can resist the action of antibacterial irrigants and are remarkably difficult to eradicate [8]. Enterococcus faecalis is one of the commonly isolated species that may play a role in persistent endodontic infections [9–11] due to inherent antimicrobial resistance, the ability to adapt to harsh environmental changes, and the ability to invade into the dentinal tubules where they are protected from endodontic irrigants and are therefore difficult to eliminate [12, 13]. A number of studies have evaluated the antimicrobial efficacy of endodontic irrigants such as sodium hypochlorite (NaOCl) [14], ethylene diamine tetra acetic acid (EDTA) [15], chlorhexidine (CHX) [16] and MTAD [17] in root canal treatment against E. faecalis biofilms [18, 19]. In general, the aim of any disinfection strategy is to reduce the bacterial load to a subcritical level so that the patient’s immune response allows healing by itself [12] but the endodontic research has always been focusing on developing methods/ endodontic irrigants that can completely remove the bacterial biofilm with minimum side-effects.

Though, a number of endodontic irrigants are being widely used in the treatment of biofilms however, the current trend is towards identification of natural products in disinfection, Propolis, a natural product has also been attempted as an endodontic irrigant in the recent past and shown to be effective against E. faecalis [20, 21] and its antibacterial effect has been attributed to its chemical composition [22]. There has been an increasing interest to use nanoparticles in clinical endodontics due to its enhanced drug stability, treatment efficacy and penetration power compared to a pure drug solution [23–25]. Nanoparticles with their unique physicochemical properties, such as ultrasmall sizes, large surface area/mass ratio, and increased chemical reactivity, have led research toward new prospects of treating and preventing dental infections [23]. Therefore, the aim of the present study
was to evaluate the antibacterial effect of propolis nanoparticle (PN) as an endodontic irrigant against *E. faecalis* biofilm in root canal dentinal tubules at depths of 200 and 400 μm and compare with different endodontic irrigants.

**Materials And Methods**

**Propolis, chemicals and reagents**

Malaysian propolis was collected from Pahang, Malaysia with the following geographical coordinates: north latitude 3.8126°, east latitude 103.3256° and height of 12 m above sea level. To study the content of Malaysian propolis, reversed phase high performance liquid chromatography (RP-HPLC) analysis was carried out. The flavonoids such as pinocembrin, kaempferol and quercetin were identified to be in the highest concentration in Malaysian propolis.

**Preparation of Ethanolic extracts of Malaysian propolis**

Propolis was manually cut into small pieces and a 20% (w/v) extract of propolis free from wax was prepared using 80 % ethanol under constant agitation in a rotary shaker (Certomat Model S II, Sartorius, Goettingen, Germany) at 200 rpm, 37°C for 48 hours. This was later centrifuged (Eppendorf Model 5810 R, Hamburg, Germany) at 3000 rpm for 15 mins, filtered through Whatman no.1 filter paper and subjected to distillation under reduced pressure using a rotary evaporator (Buchi Rotavapor R-215, Flawil, Switzerland) at the set pressure 175 mBar, temperature 52 °C and speed 95 rpm to remove the solvent. The ethanolic extract of propolis was then placed in a glass container and left for approximately 3 days for the residual solvent to evaporate; as a result, the extraction yield (Final weight/Initial weight x 100) was determined for extracts of propolis.

**Preparation of propolis nanoparticles**

Propolis nanoparticles were prepared by the ultrasonication method and compositions of the formulation are shown in Table 1. Propolis and tween 80 (stabilizer) were added to a conical centrifuge tubes containing distilled water. Then, the above mixture was briefly mixed in a vortex mixer for 1 minute followed by sonication for 20 minutes using a probe-type sonicator. To avoid thermal degradation of propolis during the sonication, formulation tubes were kept in an ice bath.

**Particle size analysis**
The particle size distribution and polydispersity index of the propolis nanoparticles were determined by dynamic light scattering using Zetasizer Nano S90 (Malvern, Worcestershire, UK) at fixed angle of 90°. The formulation was suitably diluted with distilled water and measured at 25°C. Data were collected after 2 minutes of equilibration time and averaged over three measurements (Figure 1).

Microorganism

A single colony of *E. faecalis* (ATCC 29212) was used in this study. The medium used was tryptic soy broth (TSB) (BD DifcoTM, NJ, USA).

**Dentine block specimens**

A total of 210 extracted intact human anterior single canalled teeth with complete root formation were selected for this study. The teeth were cleaned and stored in saline during all procedures to avoid dehydration. A middle third of the root (6mm in height) was obtained by sectioning the tooth below the cementoenamel junction and the apical part of the root using a slow speed diamond disc (Bredent®, Wittighausen, Senden, Germany) mounted on a milling machine under water cooling. The root canal was enlarged to an internal diameter of 0.9mm using Pesso Reamer no. 2 (Mani®, Utsunoniya, Tochigi, Japan) in a slow speed hand-piece (Kavo, Charlotte, North Carolina, USA) to standardize the internal diameter. The smear layer was removed using 5.25% NaOCl (Clorox®, Oakland, California, USA) and 17% EDTA (Calasept®, Nordiska Dental, Ängelholm, Skåne Country, Sweden) for two minutes with EndoActivator (Dentsply, Weybridge, Surrey, UK). The dentine blocks were thoroughly rinsed with sterile saline and autoclaved (LTE®, Oldham, Lancashire, UK) for 20 minutes at 121°C. The outer surfaces of the specimens were covered with nail varnish to prevent contact of the *E. faecalis* or any irrigant with the external surface. The dentin blocks were fixed to the petri dishes with wax but before that, the apical end of dentine blocks was blocked using a thin small square of sterilized parafilm (Parafilm M®, Brand, Wertheim, Baden-Württemberg, Germany) to prevent any softened wax from entering the canals.

Inoculation of dentinal blocks with *E. faecalis*

*E. faecalis* were suspended in 20.0 ml of TSB broth. The cell suspension was adjusted to match the turbidity of $1.5 \times 10^8$ CFU mL$^{-1}$ (equivalent to 0.5 McFarland standards). Five hundred microliters of *E.
faecalis suspension were transferred into the dentine blocks with the use of sterile 5.0mL syringes (Terumo®, Somerset, New Jersey, USA) with 30-gauge needles (Terumo, Somerset, New Jersey, USA) in a sterile laminar flow hood. The coronal part of the dentine blocks was then sealed immediately using parafilm (Parafilm M®, Brand, Wertheim, Baden-Württemberg, Germany). The dentine blocks were incubated at 37°C for 21 days, with renewal of E. faecalis every 3 days.

Disinfection of dentinal blocks

Following the inoculation period, 210 dentine blocks were randomly divided into seven groups (n = 30) according to the different endodontic irrigants: Group I: Saline, Group II: Propolis 100µg/ml (P100), Group III: Propolis 300µg/ml (P300), Group IV: Propolis Nanoparticle 100µg/ml (PN100), Group V: Propolis Nanoparticles 300µg/ml (PN300), Group VI: 6% Sodium Hypochlorite (6% NaOCl) (Calasept, Upplands Väsby, Sweden), Group VII: 2% Chlorhexidine (CHX) (Calasept, Upplands Väsby, Sweden). 5 ml of each endodontic irrigant was placed into the root canal using a 30-gauge side vented needle (Endo-EZE, Ultradent) Each group was further divided into three subgroups based on the time period for 1, 5 and 10 minutes.

Collection of dentinal shavings

At the end of the experimental periods, the dentine blocks were removed from the petri dishes and the canals were dried with sterile paper points. Samples of dentinal shavings were collected after one minute of exposure A1, B1, C1, D1, E1, F1 and G1, after five minutes of exposure for A2, B2, C2, D2, E2, F2, and G2 and after ten minutes of exposure A3, B3, C3, D3, E3, F3 and G3. Dentinal shavings were collected using Pesso Reamer (Mani®, Utsunoniya, Tochigi, Japan) size no. 4 (1.3 mm diameter) followed by size no. 6 (1.7 mm diameter), in a low speed handpiece (Kavo®, Charlotte, North Carolina, USA).

Antimicrobial assessment

The collected dentinal shavings were transferred into a micro-centrifuge tube (Axygen, NY, USA) containing 1ml sterile TSB broth. A sterile micro tip was used to take 0.1ml of broth containing dentinal shavings, transferred to another tube containing 0.9ml sterile TSB broth. The content of each tube was serially diluted from $10^{-1}$ until $10^{-4}$. 300µl of the diluted shavings was spread evenly using a
L-shaped glass rod and triplicated on three occasions. These plates were incubated for 24 hours at 37°C and the colonies were counted and readings were tabulated.

Total numbers of colony forming units were calculated for assessing the remaining vital viable microbial population. The SPSS computer software version 18.0 (SPSS Inc., Chicago, Illinois, USA) was used to perform statistical analysis. The values were analysed using non-parametric Kruskal-Wallis test and Mann Whitney U test to compare the reduction of *E. faecalis* between all intracanal medicaments. Probability values of *P* < 0.05 were set as the reference for statistically significant results.

**SEM Analysis**

Dentinal blocks (*n* = 3) were prepared as described in the methodology mentioned in materials and methods section. *E. faecalis* ATCC 29212 was cultured in 10 ml TSB broth supplemented with 8% sucrose (pH 7.4) with a minimal amount of xylitol (0–2%) at 37°C for 48 h and later incubated at 37°C for 24 h. After 4000 rpm centrifugation for 15 minutes, each of the cell pellet was washed three times with sterile phosphate buffered solution (PBS, 0.01 M, pH 7.2), re-suspended (O.D reading of 0.11 at 660 nm) in 100 ml of the respective growth medium and adjusted to a concentration of 0.5 McFarland (10⁸ cells/mL) before use. Five millilitres of TSB broth were mixed with equal weight of bacterial inoculum using sterilized syringes of sufficient volume to fill the root canal during 21 days’ period. After 21 days, endodontic irrigants were placed according to the groups mentioned above. Two parallel grooves were made onto the external surfaces, in a mesiodistal direction, to facilitate a split fracture. Final separation was made using chisel and hammer. All specimens were dehydrated in ascending grades of ethanol (35%, 65%, 85%, 95%, 2x100%, for 20 min in each) and immediately transferred to the pressure chamber of the critical point drying machine (CPD 30; Leica). The specimens were mounted on aluminium stubs with double-sided conductive tape, sputter-coated with 30 nm-thick layer gold/palladium (120 s) and examined using SEM (Philips/FEI XL30 FEG SEM) at an accelerated voltage of 5kV. Different magnifications and images were observed to evaluate the qualitative reduction of *E. faecalis*. Four-score scale system based on percentage of residual isolated
microbial cells was used to assess the microbial coverage on SEM images of the canal walls [26].

CLSM Analysis

After disinfection solution regimen, specimens were rinsed in 0.1wt% fluorescein for 24 h and rinsed with deionised water. Determination of bacterial viability was done using Live/Dead kit (BacLightTM kit L-13152; Molecular Probes, Inc., Eugene) with dyes applied for 5 min at a 1:1 ratio and examined using confocal laser scanning microscope (CLSM; Leica Fluoview FV 1000, Olympus, Tokyo, Japan) equipped with a 60 × /1.4 NA oil immersion lens using 480–500 nm argon/helium and a 633 nm krypton ion laser illumination both in reflection and fluorescence modes. This was adjusted to 473-nm laser emission for SYTO 9 and to 559 nm emission including propidium iodide. Reflected and fluorescence signals were detected with a photomultiplier tube to a depth of 20 μm and then converted to single-projection images for better visualization and qualitative analysis. This was maintained at a resolution of 512 pixels with a zoom factor of 1.0, giving a final pixel resolution of 0.41 mm/pixel. Stacks of fluorescent images obtained of the biofilm were examined using bioimage L software (v.2.0. Malmö, Sweden), which provided information on the structure of the biofilm, including green and red-stained bacteria volume on a two dimensional x-y section based on color segmentation algorithms written in MATLAB.

E. faecalis isolates from patients with failed root canal treatment (Antibacterial effect of PN as an endodontic irrigant in clinical samples)

Patients were selected from those who attended the clinic, with a need for non-surgical endodontic re-treatment. A detailed medical and dental history were obtained from each patient. Patients who have received antibiotic treatment during the last three months or have a systemic disease were excluded from the study. Ten teeth were included; all have been previously root filled and show radiographic evidence of apical periodontitis. Failure of root-canal treatment were determined on the basis of clinical and radiographical examinations. Tooth type, clinical signs and symptoms such as tenderness on percussion presence or absence of a sound coronal restoration, caries, sinus, swelling, periodontal status of the tooth, mobility, status of the root canal in terms of any exudate, timing and radiographic quality of the root-canal filling were recorded. After obtaining the consent the procedure was carried
out. An access cavity was made without the use of water spray under manual irrigation with sterile and by using sterile high-speed diamond bur. Root-filling material was removed by rotary instrumentation and K-files (Dentsply-Maillefer, Ballaigues, Switzerland) in a crown-down technique without the use of chemical solvent, accomplished by irrigation with sterile saline. A sterile pyrogen-free paper point (Dentsply-Maillefer, Ballaigues, Switzerland) was then introduced into the full length of the canal and retained in position during 60 seconds for sampling. Culture procedure was done using the selective \textit{E. faecalis} plates (Slanetz Bartley Agar (m-Enterococcus A.), Liofilchem, Italy) and the CFUs were grown. Thereafter, the grown \textit{E. faecalis} were suspended in 20.0 ml of TSB. The cell suspension was adjusted to match the turbidity of \(1.5 \times 10^8\) CFUs mL\(^{-1}\) (equivalent to 0.5 McFarland standards). One ml of \textit{E. faecalis} suspension was transferred into the Eppendorf tube and 50 microlitre of each irrigant according to these seven groups including group I: saline, group II: P100, group III: P300, group IV: PN100, group V: PN300, group VI: 6% NaOCl, group VII: 2% CHX. 50 microlitre of each endodontic irrigant was placed into 1 ml of \textit{E. faecalis} suspension into the Eppendorf tube for one minute, five and ten minutes. After this exposure, the content of each tube was serially diluted and this was spread evenly onto the agar plate using a L-shaped glass rod and triplicated on three occasions. These plates were incubated for 24 hours at 37°C and the colonies were counted, and readings were tabulated.

\textbf{Results}

The control group showed viable \textit{E. faecalis} at all experimental times, confirming the efficacy of the methodology.

Propolis nanoparticles size were observed in the range of 117.6 5.6 nm with Poly Dispersity index (PDI) of 0.277 0.011 (Table 1 and Figure1). The shape of nanoparticles was spherical in shape with a smooth surface as observed under transmission electron microscopy.

All the endodontic irrigants reduced CFUs significantly more than saline one, five, and ten minutes and at 200 and 400 \(\mu\)m depths (\(P < .05\)) (Table 2 and 3).

PN300 was more effective in reducing CFUs compared to saline, P100, P300, PN100 (\(p < 0.05\)) excluding 6% NaOCl and 2% CHX (\(p > 0.05\)) at all-time intervals and both depths (figure 4.6 and 4.7).
At five minutes, 6% NaOCl and 2% CHX were the most effective among all groups (p <0.05) however, no significant difference was found in between PN 300, 6% NaOCl and 2% CHX at 10 minutes (p >0.05). PN100 was more effective compared to saline and P100 at all-time intervals and both depths. However, there was no significant difference observed in between PN100 and P300 at one minute and five minutes at 200 and 400 µm depths. At ten minutes, PN100 showed more effectiveness than P300 at 400 µm depth. 6% NaOCl, CHX and PN 300 were more effective than PN100 at all-time intervals and both depths (p <0.05).

Mean reduction in CFUs was the highest at ten minutes followed by five minutes and minimum at one minute in all groups except saline at 200 µm (Figure 4) and 400 µm dentinal tubule depth (Figure 5). SEM images verified the presence of thick biofilm of bacteria when treated with saline. Maximum reduction of CFUs of >90% was observed with PN300, 6% NaOCl and 2% CHX at five and ten minutes. P100, P300 and PN100 showed the presence of bacteria (30-40%) after five minutes however, it reduced (10–20%) after ten minutes of exposure (Figure 6).

CLSM images showed the highest the number of dead cells (>90%) in dentin with PN300 compared to PN100 (>40%) and saline (no dead cells) after ten minutes of exposure (Figure 7).

Antibacterial effect of PN as an endodontic irrigant in clinical samples

Reduction in the number of CFUs was statistically significant in all groups compared to the control group (p<0.05). PN300, 6% NaOCl, 2% CHX showed no growth of E. faecalis after five and ten minutes. PN100, P100 and P300 completely eradicated E. faecalis after ten minutes (Figure 8).

Discussion

The eradication of bacteria by endodontic treatment from the root canal has been reported as difficult mainly due to the root canal complexity and biofilm formation [2]. The success of endodontic treatment depends on the chemomechanical disinfection that eliminates the vital or necrotic pulp tissue, kills microorganisms in the root canal system and disrupts the microbial biofilm thus eliminates the etiological factors responsible for endodontic infection. Therefore, the root canal instrumentation is always accompanied with copious irrigation to achieve chemical, mechanical and biological effects [27]. In this study, the extracted bovine tooth model developed by Haapasalo &
Orstavik was modified to include natural human teeth as specimens. This provided a better simulation to clinical settings to assess the antibacterial effectiveness of irrigants in the dentinal tubules [28]. Furthermore, Haapasalo & Orstavik model reported only the presence of bacteria in the dentinal tubules while in the present study, *E. faecalis* CFUs were quantified by inoculating in agar plates. Additionally, to standardise and ensure placing of constant number of bacteria during inoculation and intracanal medicaments, mid root dentin blocks of the root canal were prepared with uniform dimensions of 6.0mm height and 0.9mm diameter. The samples were tested at two depths of dentinal tubules, 200 µm and 400 µm, because previous studies have shown that dentin has an inhibitory effect on the antibacterial effectiveness of endodontic irrigants [29–31]. Therefore, the survival of the bacteria could be attributed to their invasion into the varying depths of dentinal tubules against irrigants [32, 33]. The present study determined the effective duration of various irrigants tested at three different time intervals because of their time-dependent antimicrobial effect [34, 35]. This characteristic could be useful in the clinical practice to efficiently disinfect the root canal system. *E. faecalis* was chosen as it has been one of the most prevalent (24% to 77%) microorganisms isolated from failed root canal cases [11, 36, 37], moreover, it can penetrate deep into dentinal tubules and adheres to host cells or abiotic surfaces leading to biofilm formation [33]. In this study, the effectiveness of endodontic irrigants were assessed against the 21 days’ mature biofilm because it has been shown that mature *E. faecalis* biofilms in dentin canals at 21 days are more resistant to endodontic irrigants than young biofilms [8]. The mechanism of resistance of the older biofilm is complex and may involve various mechanisms [38]. Quantitative analysis of bacteria in the dentine tubules was done to define a log reduction in CFU in infected dentine before and after the irrigation. CFU methodology has been widely used for microbiological analysis of bacteria inside the dentinal tubules. Although it was able to provide a reading of the bacterial colony that had invaded the dentinal tubules, it was unable to analyse spatial distribution and viability of the bacteria. Swimberghe et al [39] presented an overview of laboratory root canal biofilm model systems described in the endodontic literature and to critically appraise the various factors that constitute these models and they observed that the majority (86%) of the studies, a monospecies biofilm was
cultured. *E. faecalis* was the most frequently used test species in 79% of all studies and 92% of the mono-species studies. Human dentine was the most frequently used substratum in 88% of the studies, incubation times differed considerably, ranging from one to seventy days and the most common quantification method in 87% of the studies was bacterial culturing, followed by microscopy techniques.

In the present study, SEM was used to assess the presence or absence of bacteria in dentin after the placement of intracanal medicaments and irrigants because of its various advantages such as higher resolution, good depth of field, ability to image complex shapes and wide range of magnifications [40]. In addition, CLSM was used to differentiate live and dead bacteria [41] for saline, and PN groups. However, due to lack of funding it was not carried out in other groups.

In this study, all endodontic irrigants killed significantly more bacteria than saline used as a negative control. PN300 was more effective compared to all other groups except 6% NaOCl and 2% CHX at all-time intervals and both depths. This could be due to propolis nanoparticles having advantages of very small sizes, a large surface-area-to-mass ratio and very good reactivity leading to better penetration into the dentinal tubules than to only propolis [23,42]. Ethanol extracts of propolis has shown to have high antibacterial property mainly due to the presence of flavonoids like pinocembrin, quercetin and galangin that probably act on the microbial membrane or cell wall site, causing functional and structural damages [22, 43, 44]. Kayaoglu et al. [45], Al-Qathami et al. [46] and Jaiswal et al. [47] have also observed antibacterial property of propolis similar to sodium hypochlorite and CHX. At five minutes, 6% NaOCl and 2% CHX were the best among all groups however, no significant difference was found in between PN300, 6% NaOCl and 2% CHX at ten minutes. These results are in accordance with previous studies showing 6% NaOCl more effective against *E. faecalis* and disrupting biofilm [48–50]. Similarly, 2% CHX has also found to be effective against *E. faecalis* [51–53]. Chlorhexidine digluconate is a bisphenol compound that has a lower grade of toxicity compared to sodium hypochlorite and sustained action. It shows bactericidal action in high concentration and bacteriostatic in low concentration, primarily against gram-positive bacteria [49]. Although 2% CHX has been proven to promote higher antimicrobial effectiveness by reducing the bacterial load when
compared to 6% NaOCl, it is unable to disrupt biofilm [54].

PN100 was more effective compared to saline and P100 at all-time intervals and both depths however, 6% NaOCl, CHX and PN300 were better than PN100, this can be attributed to the higher concentration of propolis used in PN300. Propolis 100 was less effective than PN100 and PN300 at all-time interval and both depths and that can be explained due to their poor penetrability of propolis in the dentinal tubule comparing with propolis nanoparticles. Higher concentration of propolis, P300 could show almost similar antibacterial effect as PN100 except at ten minutes at 400 µm depth, this shows that higher concentration of propolis can be effective similar to lower concentration to propolis nanoparticles however propolis nanoparticles can penetrate deep into the dentinal tubules. Besides factors such as strength, time and frequency, the factor volume of an endodontic irrigant influences its antibacterial effect as demonstrated by Gazzaneo et al. [34] where high volume of an irrigant had a positive influence on intracanal disinfection. Therefore, to standardise the volume factor throughout the investigation five ml of each endodontic irrigant was used.

Antibacterial effect of PN against *E. faecalis* isolates from patients with failed root canal treatment also showed good results mainly due to the direct exposure of intacanal medicaments and irrigants instead of using the tooth model. Haapasalo et al. [55] demonstrated the effect of dentin on the antimicrobial properties of endodontic medicaments. They concluded that most of disinfecting agents can rapidly kill the microbes when tested in-vitro in a test tube but the effectiveness of the same agents can be weaker in the in-vivo conditions. This is mainly due to the interaction of endodontic disinfecting agents with dentin and other compounds such, serum proteins, hydroxyapatite, collagen, and microbial biomass.

This research project is one of its kind as PN have never been tested before. For further analysis, this study has used SEM for all groups and CLSM only for saline, PN100 and PN300. Due to lack of funding, CLSM could not be performed for other groups. This limitation can be addressed in future by conducting CLSM analysis for the remaining groups.

Future recommendations

The effectiveness of PN300 as an irrigant should be evaluated against polymicrobial biofilm and its
disruption in future studies.

To further strengthen the evidence, future animal studies and clinical trials are warranted.

Conclusions
PN300 was equally effective as 6% NaOCl and 2% CHX in reducing *E. faecalis* CFUs after one minute, five and ten minutes at both 200 and 400 μm. PN300 can be considered as an alternate irrigant.

Declarations
Ethics approval and consent to participate
This study was approved by the IMU Joint-Committee on Research and Ethics.

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Abbreviations
CFU Colony Forming Units
CHX Chlorhexidine
CLSM Confocal Laser Scanning Microscopy

**E. faecalis** Enterococcus faecalis

EDTA Ethylene Diamine Tetra Acetic acid

MTA Mixture of Tetracycline, Acid and Detergent

NaOCl Sodium Hypochlorite

P100 Propolis 100 μg/ml

P300 Propolis 300 μg/ml

PN100 Propolis Nanoparticle 100 μg/ml

PN300 Propolis Nanoparticle 300 μg/ml

SEM Scanning Electron Microscope

w/v weight per volume

TSB Tryptic Soy Broth

kV kilo Volt

v/v volume per volume
Declarations
Availability of data and materials
The data analyzed during this present study are available from corresponding author on request.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
AP conceived the original idea, designed and performed the experiment. HK, SR and AP supervised, contributed in the interpretation of the results and helped in writing the manuscript. All authors have read, reviewed and approved the manuscript.

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Tables
Due to technical limitations, tables are only available as a download in the supplemental files section.

Figures

![Statistics Graph (1 measurements)](image)

Figure 1

Particle size distribution intensity of propolis nanoparticles.
Figure 2

Mean reduction in CFU at 1, 5 and 10 minutes at 200 µm dentinal tubule depth.

Figure 3

Mean reduction in CFU at 1, 5 and 10 minutes at 400 µm dentinal tubule depth.
Comparison in between groups at 1, 5 and 10 minutes at 200 µm dentinal tubule depth.

Comparison in between groups at 1, 5 and 10 minutes at 400 µm dentinal tubule depth.
Figure 6

SEM images of all groups showing reduction in E. faecalis except saline group showing large amounts of bacteria (A- 5 minutes, B- 10 minutes).

Figure 7

CLSM of E. faecalis infected dentinal blocks treated by saline (control), PN100 and PN 300 after viability staining.
Comparison of CFUs count in-between PN and other endodontic irrigants at 1 minute, 5 and 10 minutes against E. faecalis isolates from patients with failed root canal treatment.

Supplementary Files
This is a list of supplementary files associated with this preprint. Click to download.
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