



Effect of *Azadirachta indica* Paste on *Lactobacillus rhamnosus* in Dentinal Tubules and Its Cytotoxicity for Dental Pulp Stem Cells

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ABSTRACT

Objectives: *Azadirachta indica* var. *siamensis* Valetton, also known as neem, possesses various beneficial biological properties, including antibacterial activity. Neem paste (NZ-paste) was developed to enhance its application. This study aimed to investigate the antibacterial activity of NZ-paste against *Lactobacillus rhamnosus* (*L. rhamnosus*), its penetration ability, and its cytotoxicity for human dental pulp stem cells (hDPSCs).

Materials and Methods: The active compounds of the neem extract were identified using high-performance liquid chromatography (HPLC). The rheological and physicochemical characteristics of 20mg/g NZ-paste were evaluated by pH measurement, penetration testing, and rheometer analysis. The antibacterial activity of NZ-paste against *L. rhamnosus* was assessed using agar disc diffusion and live/dead BacLight® assays in dentin, and confirmed by confocal laser stereomicroscopy (CLSM). The methyl thiazolyl tetrazolium assay was used to determine its cytotoxicity for hDPSCs.

Results: HPLC analysis identified azadirachtin, quercetin, nimbin, and nimbidin as the main bioactive compounds. NZ-paste had a smooth, fluid-like texture, was water-soluble, and exhibited a pH of 7.09. Although its inhibition zone was comparable to that of Ca(OH)₂, NZ-paste demonstrated superior antibacterial efficacy in the dentinal tubule invasion model, as indicated by the lowest live/dead ratio confirmed by CLSM. NZ-paste at concentrations up to 10mg/mL did not affect hDPSC viability.

Conclusion: The novel NZ-paste exhibits antibacterial activity against *L. rhamnosus*, with suitable penetration ability and favorable biocompatibility with hDPSCs at low concentrations.

Keywords: *Azadirachta*; Dental Caries; *Lactobacillus rhamnosus*; Microbial Viability

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INTRODUCTION

Dental caries is a chronic, multifactorial disease [1]. According to the World Health Organization, 2.514 billion people suffer from dental caries worldwide [2]. If left untreated, dental caries can extend to the pulp and periodontium, ultimately

resulting in tooth loss [3]. Calcium hydroxide [Ca(OH)₂] is the most commonly applied material for pulp protection because of its ability to promote dentinal bridge formation and exert antibacterial effects [4]. Its antibacterial activity is attributed to its ability to penetrate into

dental tubules up to approximately 300µm and to release hydroxyl ions that create a highly alkaline environment detrimental to bacteria. However, bacterial penetration into dental tubules can reach depths of at least 500µm [5,6]. Therefore, Ca(OH)₂ may be unable to eradicate bacteria residing in deeper layers of the dental tubules. *Lactobacillus rhamnosus* (*L. rhamnosus*) is frequently detected in cavities where Ca(OH)₂ has been used as the base material [7]. *L. rhamnosus* produces toxins that can diffuse through the dental tubules, resulting in pulp inflammation [8]. Thus, developing additional pulp lining materials may provide alternative treatment. The American Academy of Pediatric Dentistry recommends Ca(OH)₂, resin-modified glass ionomer, dentin bonding agents, mineral trioxide aggregate, and other biocompatible materials as liners [9]. However, these materials present certain limitations, including high cost, complex handling, and prolonged setting times; moreover, current evidence does not favor one material over another [10].

Natural products are becoming increasingly popular in health care. *Azadirachta indica* var. *siamensis* Valetton, also known as neem, has been used due to its therapeutic effects, i.e., wound healing, antioxidant, anti-inflammatory, and antimicrobial activities [11]. Previous studies have shown antibacterial activity of neem leaf extract against cariogenic bacteria, including *Streptococcus mutans*, and *Lactobacillus* species (*L. acidophilus*, *L. rhamnosus* and *L. casei*) [12-14]. Therefore, neem could be utilized as an alternative material for eliminating microorganisms in dental caries.

The study objective was to examine the antibacterial activity of an innovative paste containing neem leaf extract namely NZ-paste against *L. rhamnosus* as well as its physicochemical properties and biocompatibility with human dental pulp stem cells (hDPSCs).

MATERIALS AND METHODS

Neem leaf extraction and paste preparation:

In this in vitro study, fresh neem leaves were dried and ground into a coarse powder. Then, 30g of the powder was macerated in 95% ethanol (Merck, Germany) for 48 hours. The

solvent was evaporated using a rotary evaporator (BÜCHI Rotavapor R-205, Flawil, Switzerland) under reduced pressure, to gain the crude extract. Chemical compounds were identified using high-performance liquid chromatography (HPLC). A zinc oxide (ZnO) paste base was prepared using stearyl alcohol (KemAus™, Cherrybrook, Australia), sodium lauryl sulfate (KemAus™, Cherrybrook, Australia) polyethylene glycol (KemAus™, Cherrybrook, Australia), glycerol (Ajax Finechem pty Ltd., Taren Point, NSW, Australia), and distilled water. ZnO powder (KemAus™, Cherrybrook, Australia) was incorporated into the paste base at a ratio of 1:10 (w/w) [6]. The NZ-paste was then created by blending the ZnO paste with the neem extract, resulting in final neem extract concentrations of 20mg/g and 100mg/g.

HPLC:

The neem extract was re-dissolved in 80% methanol: hexane (Merck, Germany) (1:1) to determine its active compounds: quercetin, nimbin, and nimbidin. Analysis was conducted using a HPLC system (CBM-20Å, Shimadzu Corporation, Kyoto, Japan) equipped with a dual-gradient pump system (LC-20AT, Shimadzu Corporation, Kyoto, Japan, an autosampler (SIL-20A, Shimadzu Corporation, Kyoto, Japan), a UV detector (PD-10A, Shimadzu Corporation, Kyoto, Japan), and a column oven maintained at 35°C (CTO-20A, Shimadzu Corporation, Kyoto, Japan). Separation was achieved on a C18 column (Synergi 4u MA-RP 80A, 150×4.6mm, Phenomenex, Torrance, CA, USA). using gradient elution with solvent A (water) and solvent B (acetonitrile). The mobile phase solution flow rate was set at 1.0mL/min, and detection was carried out at 217nm. A 10µL aliquot of each sample was injected into the HPLC column. Active compounds were identified by comparing their retention times and UV spectra with those of reference standard. Quantification was based on the peak area of each compound. Azadirachtin identification was conducted with HPLC using a reverse phase Phenomenex Luna 5µ C18 (2) 100Å (250×4.60mm internal diameter) for chromatographic separations. The HPLC

mobile phase consisted of acetonitrile: methanol: 1% triethylamine (60:40:1) adjusted to a pH of 4 and filtered using a value stage vacuum pump (Model WP6122050, Millipore Corporation, Burlington, MA, USA). The flow rate was maintained at 1 mL/min, at room temperature, with detection at 210nm.

Agar disc diffusion test:

L. rhamnosus (ATCC 10863) was cultured in de Man, Rogosa, and Sharpe (MRS) broth (Difco™, Becton Dickinson, Sparks, MD, USA) under anaerobic conditions at 37°C for 24 hours. The bacterial density was set to 0.5 McFarland standard concentration using a spectrophotometer (Bio-Rad SmartSpec 3000UV/Vis, Bio-Rad Laboratories, Hercules, CA, USA) at 600nm. The bacterial suspension (200µL) was spread evenly onto MRS plates. Sterile paper discs (with 6mm diameter) were immersed in 0.2% chlorhexidine (CHX; Sigma-Aldrich, USA) as positive control. Standard treatments including 8mg of Ca(OH)₂ paste (UltraCal® XS, Ultradent Products, Inc., South Jordan, UT, USA), NZ-pastes containing 20mg/g and 100mg/g neem extract, and ZnO paste were applied on discs that were placed on the bacterial lawn culture and then incubated under anaerobic conditions at 37°C for 48 hours. The inhibition zone diameter was determined in millimeters. The test was repeated 3 times, with each test performed in triplicate [13].

Physicochemical property test:

The pastes' solubility, color, smoothness, pH, and viscosity were assessed through visual inspection, pH meter readings, and rheometer analysis. For rheometer analysis, ZnO paste, 20mg/g NZ-paste, or Ca(OH)₂ paste was placed on a parallel-plate rotor (20mm diameter). Oscillatory tests were performed at 1Hz with a fixed strain amplitude of 0.1% at 37°C. The data were continuously recorded every 5 seconds for 5 minutes, segment time (t_{seg}). The time dependent analysis evaluated the substance's flow behavior under applied stress. The storage modulus (G'), loss modulus (G''), and loss tangent ($\tan \delta$) were determined [15]. The pH of the substances was also tested on 1M of ZnO paste and 20mg/g NZ-paste solutions. The NZ-pastes were placed in tuberculin syringes and stored at 4°C for 6 months for subsequent reevaluation.

Tooth specimen preparation:

The experimental protocol was authorized by the Research Ethics Committee of Thammasat University, Pathum Thani, Thailand (approval no. 137/2564 and 105/2565). Sixty-five caries-free human premolars, extracted for orthodontic reasons, were included in this study. The tooth cavities (3mm width×4mm length×3mm depth) were created on the occlusal surfaces using a diamond fissure bur (Dentsply Maillefer, Ballaigues, Switzerland). The specimens were immersed in 17% ethylenediaminetetraacetic acid to remove the smear layer, followed by further immersion in 5.25% sodium hypochlorite (CU Dent, Bangkok, Thailand) for 4 minutes with ultrasonication. All specimens were then immersed in 0.9% saline for 10 minutes before autoclaving at 121°C at 15psi for 15 minutes [6].

Material penetration test:

Twenty specimens were randomly allocated to 4 groups (n=5). The test materials were Ca(OH)₂ and 20mg/g NZ-paste. Since ZnO paste and propylene glycol were difficult to recognize by color, 0.1mol/mL safranin O (Merck, Germany) solution was added to each material before application on the cavity floor. Each specimen was stored separately in a closed container and incubated for 48 hours at 37°C. The specimens were then longitudinally sectioned in half and examined under a stereomicroscope (Carl Zeiss 426126, Carl Zeiss, Jena, Germany) at ×15 magnification. To evaluate NZ-paste, Ca(OH)₂, ZnO paste, propylene glycol penetration into dentin, the penetration depth was measured from the cavity floor to the pulp chamber [6].

Dentinal tubule invasion and antibacterial test:

The remaining 45 tooth specimens were incubated in MRS broth containing 10⁸CFUs/mL of *L. rhamnosus* for 30 days. The culture medium was changed every 3 days [3,4]. The bacterial penetration was confirmed using a scanning electron microscope (JEOL JSM-6610LV, JEOL Ltd., Tokyo, Japan), at 15kV, and ×5000 magnification. The tooth specimens were randomly assigned to 5 groups of medications (n=9) i.e., no treatment (control), ZnO paste, 20mg/g NZ-paste,

Ca(OH)₂, and 0.2% CHX (positive control). Each specimen was placed in a sealed container and incubated for 48 hours at 37°C. The dentin samples were obtained using 5 sterile steel round burs of different diameters (ISO 012, 014, 016, 018, and 021), (Dentsply Maillefer, Ballaigues, Switzerland) corresponding to depths of 100-500µm [5]. The dentin debris was dislodged from each bur by vortexing in 1mL of phosphate buffered saline. The suspensions were centrifuged at 1,200rpm for 1 minute. The resulting supernatants were collected and centrifuged again at 12,000rpm for 2 minutes. After discarding the supernatant, the bacterial pellet was resuspended in 500µL of phosphate buffered saline. Subsequently, 1.5µL of the live/dead BacLight® staining solution (Molecular Probes™, Thermo Fisher Scientific, Eugene, OR, USA) was added and the samples were incubated in the dark for 40 minutes. The proportion of viable to dead microorganisms was determined by measuring the fluorescence intensity of SYTO 9 (green; excitation/emission 485/535nm) and propidium iodide (red; excitation/emission 485/642nm) using a microplate reader (DTX 880 Multimode Detector, Beckman Coulter, Brea, CA, USA) on 100µL of the stained bacterial suspension. The experiment was repeated in triplicate with 9 independent samples. The viability of the bacteria was confirmed at ×60 magnification using confocal laser scanning microscope (CLSM; Leica STELLARIS 5 inverse, Leica Microsystems, Wetzlar, Germany) [9].

Cytotoxicity test:

The hDPSCs (Poietics™, Lonza, Basel, Switzerland) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco™, Thermo Fisher Scientific, Grand Island, NY, USA) with 10% fetal bovine serum (HyClone™, Thermo Fisher Scientific, Logan, Utah, USA), 100IU/mL penicillin, and 100µg/mL streptomycin (Gibco™, Thermo Fisher Scientific, Grand Island, NY, USA) and 2.5mg/mL amphotericin B (Capricorn Scientific GmbH, Ebsdorfergrund, Germany) at 37°C 95% humidity, and 5% CO₂. Fourth to sixth passage hDPSCs (5×10³ cells) were seeded into 96 well plates (Nunc™, Thermo

Fisher Scientific, Roskilde, Denmark). The treatments were divided into 3 groups: untreated (control), treated with 20 mg/g NZ-paste, and ZnO paste, at various concentrations (80, 40, 20, 10, 5, 2.5, 1.25, 0.625, 0.325mg/mL). Five independent experiments were carried out with 3 replicates. All substances were diluted with medium, then filtered through a 0.2µm syringe filter (Minisart®, Sartorius AG, Göttingen, Germany) before use. The methyl thiazolyl tetrazolium assay (Sigma-Aldrich, St. Louis, MO, USA) was used to quantify cell viability after 72 hours of incubation, at a wavelength of 570nm. The percentage of cells was calculated relative to the control group [16].

Statistical analysis:

Data were presented as mean±standard error. Normal distribution of data was clarified using the Shapiro-Wilk test ($p>0.05$). Statistical analyses were conducted using one-way ANOVA, followed by the Tukey and Dunnett's post-hoc tests to determine between-group differences. A P value of less than 0.05 was considered statistically significant.

RESULTS

HPLC:

The neem extract contained bioactive compounds such as nimbin, quercetin, nimbidin and azadirachtin (Fig. 1).

Physicochemical properties:

The 20mg/g NZ-paste was matte green, and the ZnO paste was matte white. Both pastes exhibited a similar smooth texture and were miscible in water. The NZ-paste and ZnO paste had a pH of 7.09 and 7.69, respectively. When evaluated after 6 months of storage, the pH of the NZ-paste increased to 7.49, while the pH of the ZnO paste remained stable at 7.70.

Viscosity test:

The viscosity analysis showed that the 20mg/g NZ-paste exhibited the greatest Tan δ value, followed by the ZnO paste, which was close to 1, indicating that both pastes should be flowable. In contrast, Tan δ value of Ca(OH)₂ indicated that it was more viscous. After 6 months of storage, the Tan δ values of the ZnO and NZ-pastes decreased, indicating a transition to a fluid-gel consistency (Table 1).

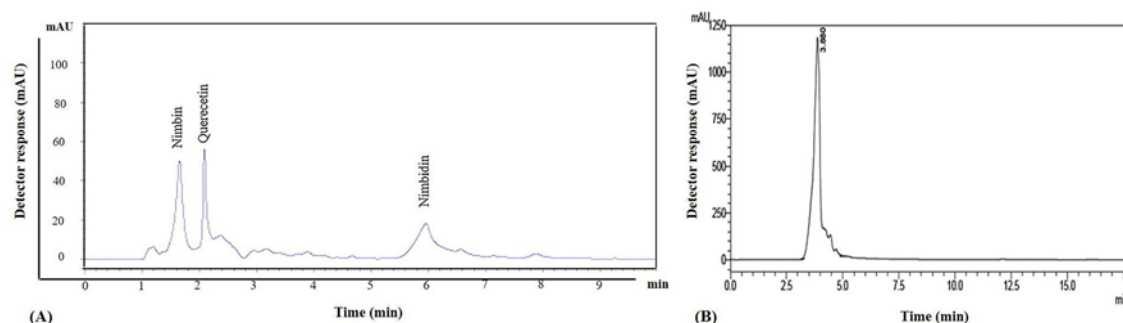


Fig 1. HPLC of neem extract: (A) Peaks corresponding to nimbin, quercetin, and nimbidin; (B) azadirachtin peak.

Table 1. Time-dependent rheological test results of pastes (triplicate samples)

Incubation time	Materials	G' (mean ± SE)	G'' (mean ± SE)	Tan δ (G' / G'') (mean ± SE)	Flowability level
1 month	ZnO paste	0.0075±0.0003	0.0067±0.0003	0.8876±0.0142	Fluid like
	20mg/g NZ-paste	0.0058±0.0020	0.0052±0.0003	0.8966±0.0140	Fluid like
	Ca(OH) ₂	0.0020±0.0006	0.0013±0.0005	0.5542±0.0765	Fluid-gel like
6 month	ZnO paste	0.0099±0.0002	0.0061±0.0002	0.6200±0.0079	Fluid-gel like
	20mg/g NZ-paste	0.0067±0.0003	0.0044±0.0001	0.6600±0.0154	Fluid-gel like
	Ca(OH) ₂	0.0034±0.0011	0.0021±0.0019	0.6176±0.3928	Fluid-gel like

ZnO: zinc oxide; NZ-paste: neem paste; Ca(OH)₂: calcium hydroxide; G': storage modulus; G'': loss modulus; tan δ: loss tangent; SE: standard error.

Antibacterial property:

All tested materials inhibited the growth and proliferation of *L. rhamnosus*. CHX had the widest inhibition zone (14.83±0.90mm); while there was minimal difference in inhibition zones between Ca(OH)₂ (6.27±0.20mm), ZnO paste (6.67±0.24mm), 100mg/g NZ-paste (6.89±0.86mm), and 20mg/g NZ-paste (7.06±0.95mm) groups. However, both NZ-paste concentrations inhibited bacterial proliferation more effectively than the ZnO paste and Ca(OH)₂. Therefore, 20 mg/g NZ-paste was the optimal concentration for subsequent investigations (Fig. 2).

Penetration test:

Propylene glycol exhibited the greatest penetration depth (3,855±463.9μm). The NZ-paste and ZnO paste penetrated into dentinal tubules to depths of 1,030±39.61μm, and 405.3±26.5μm, respectively, whereas Ca(OH)₂ showed a significantly lower penetration depth compared with all other tested materials (Fig. 3).

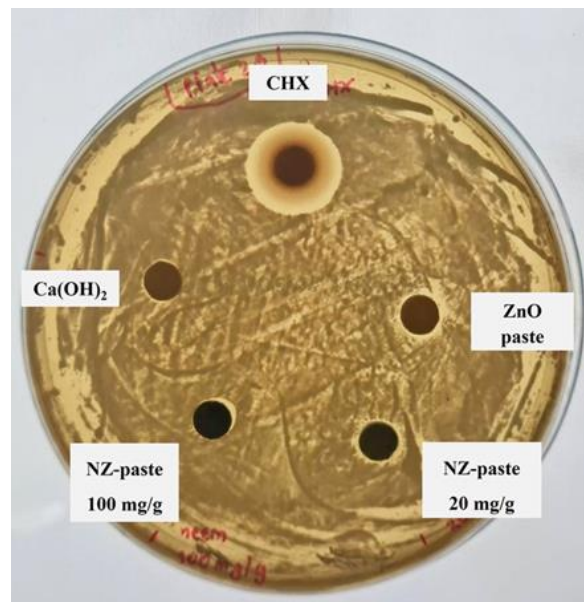


Fig 2. Disc diffusion inhibition zones. The highest zone of inhibition of *L. rhamnosus* belonged to 0.2% CHX followed by 20, and 100mg/g NZ-paste, ZnO paste, and Ca(OH)₂. NZ-paste: neem paste; Ca(OH)₂: calcium hydroxide; CHX: 0.2% chlorhexidine; ZnO: zinc oxide.

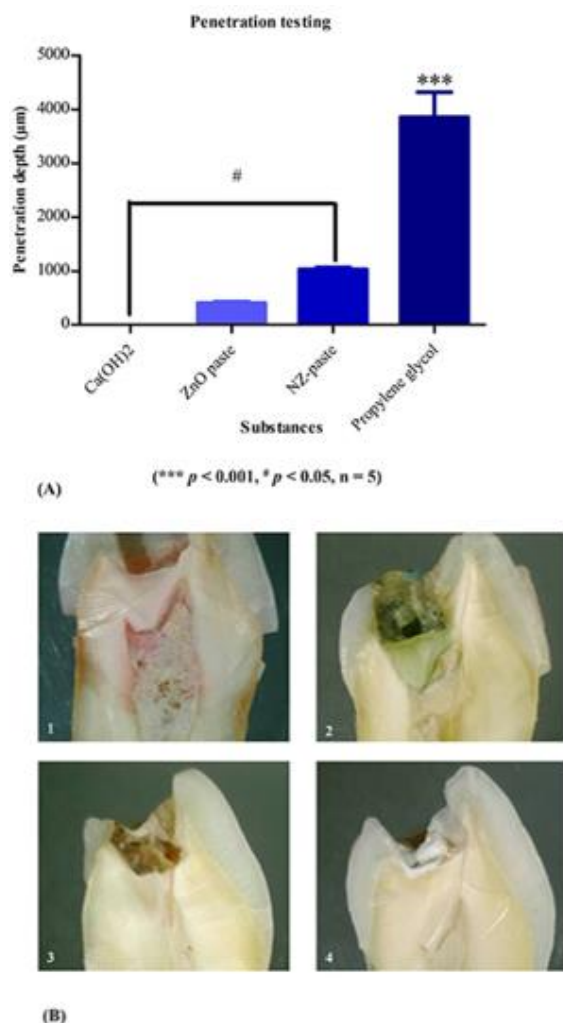


Fig 3. Penetration depth of materials into dentinal tubules. (A) Graph comparing penetration depths: propylene glycol demonstrated the greatest penetration, followed by 20mg/g NZ-paste, ZnO paste, and Ca(OH)₂. (B) Stereomicroscopic images: propylene glycol (B1) revealed the deepest penetration, followed by 20mg/g NZ-paste (B2) and ZnO paste (B3), whereas Ca(OH)₂ (B4) showed slight penetration. NZ-paste: neem paste; ZnO: zinc oxide; Ca(OH)₂: calcium hydroxide.

Dentinal tubule invasion:

After the incubation period, bacteria were not observed in the sterile specimens (Fig. 4A). In *L. rhamnosus* inoculated specimens, bacteria invaded the dentin to a maximum depth of 583.22µm (Fig. 4B-D).

Antibacterial test:

Although 0.2% CHX, ZnO paste, and 20mg/g NZ-paste demonstrated antibacterial activity toward *L. rhamnosus* throughout all depths,

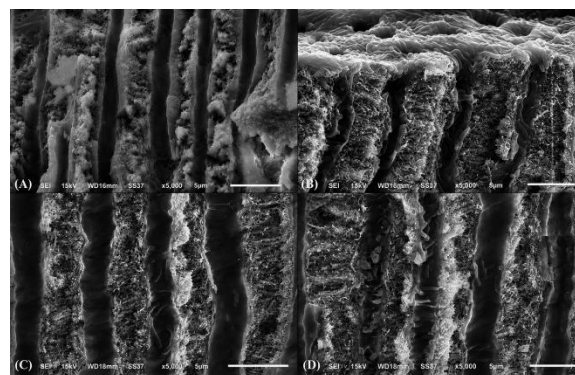


Fig 4. Scanning electron microscopic images showing bacterial invasion of dentinal tubules. (A) Sterile specimen showing no bacterial presence. (B-D) *L. rhamnosus* observed at depths of 175.07, 502.76, and 583.22µm, respectively.

Ca(OH)₂ treatment resulted in a number of viable bacteria similar to that observed in the untreated group. At a depth of 500µm, the 20mg/g NZ-paste showed the highest antibacterial activity among all tested materials (Table 2). The result was confirmed by CLSM (Fig. 5).

Cytotoxicity test:

The NZ-paste and ZnO paste were tested for cytotoxic effects on hDPSCs using the methyl thiazolyl tetrazolium assay. According to the findings, NZ-paste and ZnO paste at concentrations as high as 10mg/mL did not adversely affect the viability of hDPSCs, with cell growth ranging from 76.87±8.36 to 103±30.15 and 83.69±10.15 to 91.60±15.55, respectively. Furthermore, lower NZ-paste concentrations induced the proliferation of hDPSCs (Fig. 6).

DISCUSSION

This study demonstrated that the 20mg/g neem leaf extract paste (NZ-paste) has 3 main characteristics: (1) inhibiting the growth and proliferation of *L. rhamnosus*, (2) good penetration into dentinal tubules, and (3) satisfactory biocompatibility for hDPSCs. Our finding was consistent with a previous report regarding growth inhibition of *L. rhamnosus* by the neem leaf extract [12-14].

The 4 constituents found in neem extract that efficiently prevent bacterial growth and proliferation were identified as nimbidin, nimbin, azadirachtin, and quercetin [17-20]. The first three constituents have complex

Table 2. *L. rhamnosus* live/dead ratio after treatment (n=9)

Penetration depth (μm)	Live/dead ratio percentage (mean±SE)				
	No treatment	Calcium hydroxide	Chlorhexidine	20mg/g NZ-paste	ZnO paste
100	81.741±3.670	73.444±5.121	13.571±0.918 ^a	13.726±0.744 ^a	14.079±0.789 ^a
200	77.153±3.639	74.455±5.207	14.091±0.857 ^b	14.063±0.809 ^b	15.003±0.628 ^b
300	74.153±4.755	71.964 ± 3.644	15.161±1.164 ^c	14.501±0.536 ^c	14.971±0.694 ^c
400	69.868±5.180	66.225±4.375	17.686±2.035 ^d	15.051±0.901 ^d	16.564±1.040 ^d
500	69.605±3.939	71.195±4.846	20.960±2.962 ^e	15.825±1.002 ^e	19.026±1.565 ^e

^{a,b,c,d,e} p<0.001 when compared with the calcium hydroxide group; SE: standard error.

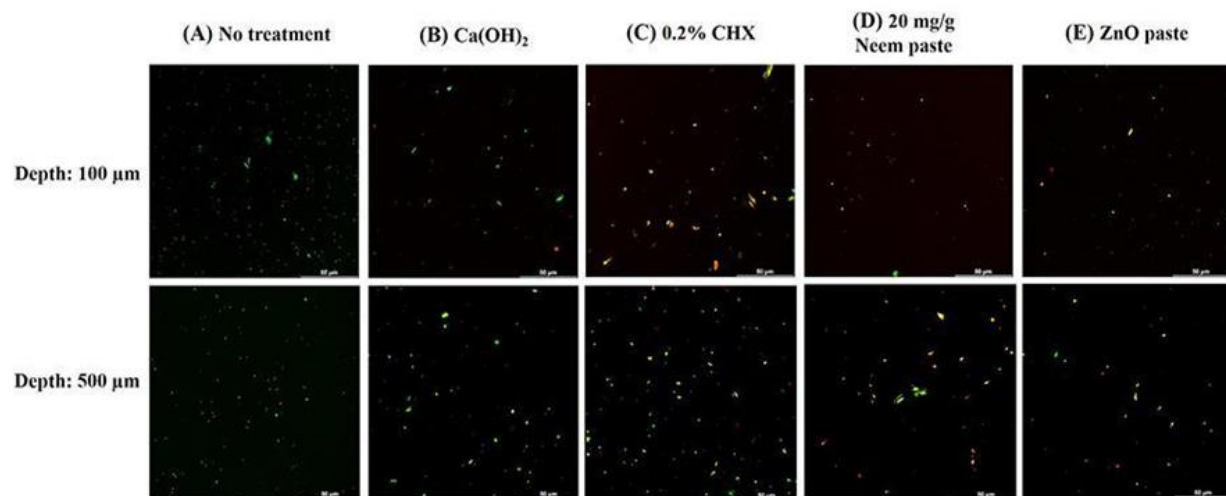


Fig 5. CLSM images (×60 magnification) showing live (green) and dead (red) bacteria within dentinal tubules at depths of 100μm and 500μm below the cavity floor. At 500μm depth, few live bacteria were observed in 0.2% CHX (C), 20mg/g NZ-paste (D), and ZnO paste (E) compared with Ca(OH)₂ (B) and no treatment (A). Ca(OH)₂: calcium hydroxide; CHX: 0.2% chlorhexidine; NZ-paste: neem paste; ZnO: zinc oxide.

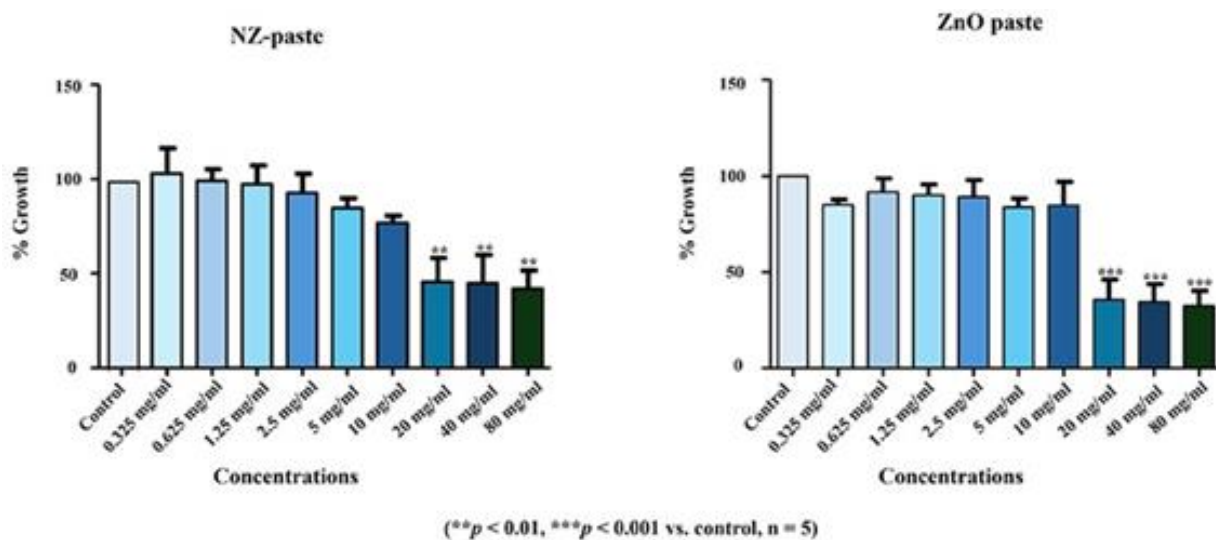


Fig 6. Graph of proliferation percentage of hDPSCs. (A) 20mg/g NZ-paste. (B) ZnO paste. The hDPSCs maintained their viability at more than 70% at concentrations ≤10mg/mL of either paste. In contrast, concentrations ≥20mg/mL of each paste significantly inhibited the proliferation of hDPSCs (less than 50%). NZ-paste: neem paste; ZnO: zinc oxide.

molecular structures that inhibit cellular oxidation reactions [21]. Quercetin provides 5 main bioactivities as follows: it prevents the growth and proliferation of various bacteria through mitochondrial dysfunction, enhances membrane permeability, disrupts nucleic acid synthesis, downregulates virulence factor expression, and inhibits biofilm formation [22]. Regarding chemical property, the pH of NZ-paste was within the normal pH range for human cell growth [23]. However, it is not suitable for growth and proliferation of *L. rhamnosus*, because the optimal growth and proliferation of *Lactobacilli* occur at a pH of 4.5–6.5 [24]. After 6 months, the physical properties of the NZ-paste remained constant over time, except the viscosity that increased, which may result from spontaneous dehydration. However, this characteristic of the paste still had no significant effect on its flowability. NZ-paste demonstrated greater penetration depth than $\text{Ca}(\text{OH})_2$ [5, 25]. This result was in agreement with the findings of Manca et al., [26] who found that neem oil had a higher viscosity after 14 days of storage.

Agar disc diffusion was performed for assessment of antibacterial activity. Our results exhibited inhibition zones independent of the dosage of neem. The antibacterial activity against *L. rhamnosus* for 20mg/g NZ-paste was found to be greater than 100mg/g. This particular result may be due to the fact that higher NZ-paste concentration results in a higher viscosity due to the decrease in polyethylene glycol, which could lead to a decrease in diffusion, thereby decreasing antibacterial activity compared to a lower concentration.

At all examined depths, $\text{Ca}(\text{OH})_2$ showed limited ability to disinfect dentin. This limitation may result from its high solubility and low penetration capacity. This result agrees with previous studies which showed that $\text{Ca}(\text{OH})_2$ can penetrate into dentinal tubules by less than 200µm. Consequently, *L. rhamnosus* can persist treatment with $\text{Ca}(\text{OH})_2$ [25, 27, 28]. According to a previous study, 2% CHX gel exhibits the strongest antibacterial activity in dentinal tubules at 400µm depth [28, 29], consistent with this finding that CHX

activity decreased at 500µm. Similarly, the growth inhibition activity of ZnO paste against *L. rhamnosus* is attributed to zinc ions that inhibit glucosyltransferase formation and glycolysis [30]. The ZnO nanoparticles have a penetration depth of approximately 300µm [31], while our study showed the effectiveness of ZnO paste by penetration to 400 µm depth. This could be because the ZnO paste contains polyethylene glycol as a carrier, which improves the diffusion of medications deep into the dentinal tubules and enhances antibacterial effects [32]. However, ZnO has been reported to have cellular toxicity for DPSCs [33].

The present findings suggest that 20mg/g NZ-paste may penetrate into the pulp chamber, warranting further investigation of its potential cytotoxicity. According to ISO 10993-5:2009, a material is defined non-cytotoxic when the cell number (viability) is more than 70% [16]. The present study indicated that the ZnO and 20mg/g NZ-pastes are safe for hDPSCs at concentrations up to 10mg/mL. Furthermore, the lower NZ-paste concentrations induced proliferation of hDPSCs probably through bioactive compounds. ZnO nanoparticles are significantly toxic to the macrophage cell line at 100µg/mL concentration [34]. However, ZnO nanoparticles containing neem leaf extract were safe at 125µg/mL implying that neem leaf extract may decrease the adverse effects of zinc ions on cells [35]. Additionally, azadirachtin promoted osteoblast proliferation, differentiation, and mineralization, and reduced the number of apoptotic cells in vitro and in vivo [36,37]. However, the high ZnO and NZ-paste concentrations inhibited the proliferation of hDPSCs.

Despite the value of the findings, this study has certain limitations. The present study focused on one single bacterial species; however, dental caries involves a complex microbial community organized as a biofilm. Therefore, future research should evaluate the antibacterial efficacy of neem paste against multispecies biofilms before drawing conclusions about its potential for caries treatment. The accuracy of SYTO 9 and PI staining may be affected by the presence of

cells with partially compromised membranes [38]. Hence, microbial culturing should be included as an additional quantitative method for assessing antimicrobial efficacy. Additionally, the procedure of collecting dentin samples may sometimes result in inconsistent amounts of dentin, potentially affecting the validity of results.

CONCLUSION

The present findings demonstrated that neem paste exhibits antibacterial activity against *L. rhamnosus*, with favorable penetration ability and satisfactory biocompatibility with hDPSCs at low concentrations. These results suggest that neem paste could be developed as an alternative therapeutic agent for managing dental caries.

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CONFLICT OF INTEREST STATEMENT

None declared.

GENERATIVE AI IN SCIENTIFIC WRITING

AI-assisted tools (ChatGPT, GPT-5.5, OpenAI) were used solely to improve the English language and grammar of the manuscript. No AI was used for scientific content generation, data analysis, interpretation, or conclusions.

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