Pharmacy Reports

https://doi.org/10.51511/pr.84

RESEARCH ARTICLE

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In vitro antibiofilm activity of eggshells derived nano-hydroxyapatite (nHA) against *Staphylococcus aureus* and *Streptococcus mutans*

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Abstract: Dental caries, a highly prevalent oral health condition worldwide, is primarily driven by the biofilm-forming abilities of *Staphylococcus aureus* and *Streptococcus mutans*. The interest in eggshell extracts has grown in recent years due to their potential benefits for oral health. Therefore, this study investigated the potential of nano-hydroxyapatite (nHA) derived from eggshells in combating bacterial infections and inhibiting biofilm formation by the selected cariogenic bacteria. The antibacterial activity of the nano-hydroxyapatite extract was initially assessed using the agar well diffusion method. Subsequently, biofilm inhibition was evaluated through crystal violet assays, and the disruption of biofilm structure was visualized under a light microscope. The findings indicated that the nano-hydroxyapatite extract lacked antibacterial activity in inhibiting the growth of both *S. aureus* and *S. mutans*. However, the extract demonstrated antibiofilm activity against mono-species biofilms, with observed disruption of biofilm formation upon treatment. As a result, nano-hydroxyapatite extracts derived from eggshells may hold potential as agents for inhibiting biofilm formation associated with dental caries.

Keywords: Biofilm, dental caries, eggshells, hydroxyapatite, oral pathogens

Introduction

Dental caries is a major chronic disease with a prevalence that is notably high among young people in Asia and Latin America, accounting for 70% of the global cases [1]. This oral disease is initiated by the presence of cariogenic bacteria on tooth surfaces, which metabolize sugars into acid, gradually demineralizing the tooth structure [2]. The process is instigated by heightened carbohydrate consumption, limited exposure to fluoride, and various psychological, behavioral, and social determinants, all of which collectively influence the progression of dental caries [3]. The onset of dental caries can also be triggered by the development of biofilm formed by organized clusters of bacterial cells adhering to the surface of a tooth [4]. Subsequently, this biofilm matures into plaque, leading to acid production and eventually developing dental caries.

Biofilm is a highly structured and well-organized community of microorganisms embedded within a supportive extracellular matrix [5]. The matrix serves as a vital framework that allows the biofilm to be attached to the surface of tooth enamel. It has been well documented that *Staphylococcus aureus* and *Streptococcus mutans* can form biofilms and produce acid as a metabolism by-product. These characteristics are deemed vital for their survival and virulence [6]. By forming biofilms, these bacteria are responsible for the infections linked to dental caries and contamination of various medical implants [7]. The formation of oral biofilms begins with the attachment of bacteria such as *S. mutans* to excrete extracellular polymeric substances

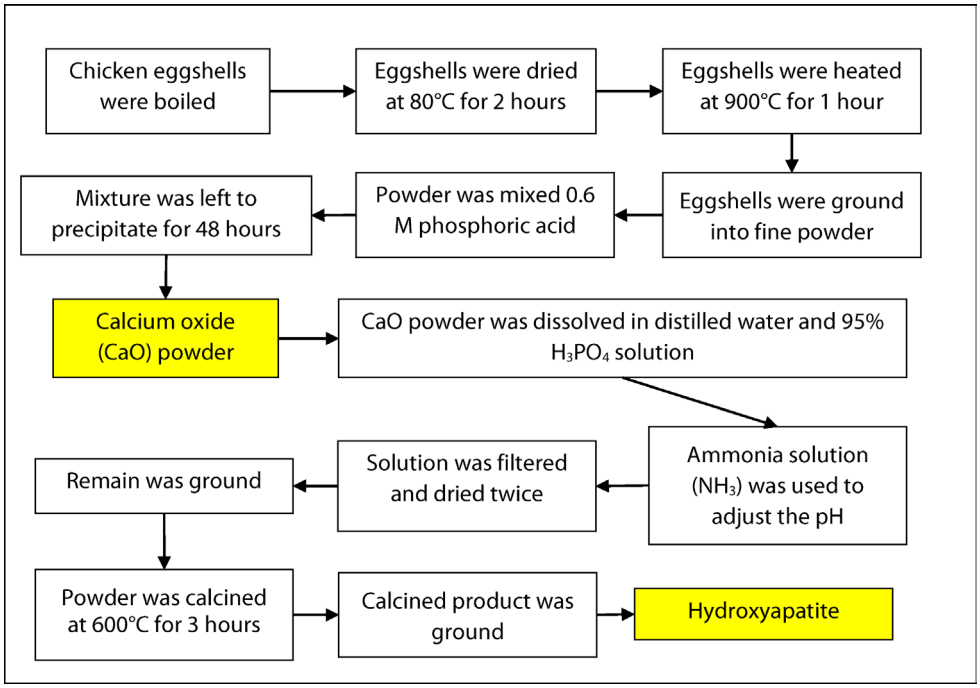

Figure 1. The detailed workflow of the nano-hydroxyapatite extraction process

(EPS) that act as a virulence factor [8, 9]. In addition, colonized bacteria provide a specific binding site for later bacteria colonization and facilitate the growth and maturation of biofilms. Oral biofilm contributes to tooth decay by corroding the tooth surface and breaking down sugars into acid within the biofilm it forms [10].

Hydroxyapatite (HA), a calcium and phosphate mineral, enables enamel to withstand stress and resist microbial infiltration [11]. When biofilm activity generates acidic by-products, the oral environment shifts to a low pH state, leading to hydroxyapatite breakdown and subsequent enamel demineralization [11]. This oral health concern particularly highlights the need for targeted interventions and preventive measures. Currently, preventative measures to prevent dental caries include synthetic compounds such as fluoride and chlorhexidine [12]. However, these compounds have been associated with side effects, including dental fluorosis and tartar formation [13]. In response to the increased need for combating biofilms, nanotechnology emerges as a promising alternative, especially nanotechnology that uses waste as a sustainable solution.

Eggshells are one of the solid wastes that produce approximately 250,000 tons per year [14] and are expected to produce up to 70,686 tons annually in Malaysia [15], making them a promising candidate for such utilization. The high calcium carbonate content in eggshells makes them an excellent source for hydroxyapatite extraction via nanotechnology. This extracted hydroxyapatite is frequently used in dental practices due to its biocompatibility and mineral composition, which mirrors that of natural dental enamel. HA is a calcium phosphate ceramic commonly used as a synthetic material for bone grafts, dental implants, socket preservation, dental cement, and oral reconstruction [16, 17]. With its unique nanostructure, HA potentially disrupts biofilm formation, thereby offering a novel avenue for combating microbial infections in both dental and medical contexts. Therefore, the study focuses on assessing the antibacterial and antibiofilm effects of nano-hydroxyapatite (nHA) synthesized from eggshells against *Staphylococcus aureus* and *Streptococcus mutans*, emphasizing its potential as a sustainable strategy for managing biofilm-related oral infections.

Methods

Nano-hydroxyapatite (nHA) extraction

The extraction of nano-hydroxyapatite was briefly illustrated in Figure 1. Chicken eggshells were first boiled and dried at 80°C for 2 hours. It was then heated at 900°C for one hour and ground into a fine powder. The powder was mixed with 0.6M phosphoric acid and left to precipitate for 48 hours before drying again to

obtain calcium oxide (CaO) powder. The powder was dissolved in distilled water, and a 95% ortho-phosphoric acid (H3PO4) solution was added. Ammonia solution (NH_3) was added before filtrating to adjust the pH between 9-12. The filtration and drying steps were performed twice to ensure thorough processing. The remaining material was then ground with a mortar and sieved, and the powder was calcined at 600°C for 3 hours to synthesize hydroxyapatite. Finally, the calcined product was ground again to produce a refined powder.

Bacterial strains

The stock culture of *S. aureus* (ATCC 25923) and *S. mutans* (ATCC 25175) was provided by the Centre for Medical Laboratory Technology Studies, Universiti Teknologi MARA. Throughout the experiment, the selected bacteria were cultured in Brain Heart Infusion (BHI) agar and BHI broth at 37°C for 24 hours.

Chemical and reagents

Distilled water and dimethyl sulfoxide (DMSO) from AMRESCO® were used as solvents in formulating the nHA suspension, which is attributed to their roles in stabilizing the suspension and facilitating penetration. The antibacterial analysis was performed using gentamicin 10µg (CN10) and chloramphenicol 30µg from OXOID™, while the biofilm analysis involved crystal violet (UCB), 95% ethyl alcohol (WESTLAB), and phosphate buffer saline (PBS) tablets, sourced from VWR® Life Science.

Culture media

The bacterial culture and identification were carried out using nutrient agar (NA) from OXOID™ and Columbia agar with 5% sheep blood (BA) from ISOLAC®. Antibacterial screening was performed with tryptic soy broth broth and Mueller-Hinton (MH) agar from BD BBL™, while brain heart infusion broth from MERCK was used for antibiofilm analysis.

Preparation of nHA suspensions

To prepare concentrations of 125 mg/mL and 250 mg/mL of nHA, 1250 mg and 2500 mg of nHA powder were dissolved in 10 mL of 10% DMSO, respectively.

Antibacterial analysis of nHA

To analyze the antibacterial ability of nHA, the agar well diffusion method protocol was used by Masri and Brown [18]. The bacterial suspension was first prepared by inoculating the *S. aureus* in TSB and standardized to 0.5 McFarland, giving a proximate 1 to 2 x 10^8 CFU/ml. The suspension was lawned onto MH agar, and 6 mm diameter wells were punched onto the agar using a sterile cork borer. Fifty microliters of different concentrations of nHA (125 and 250mg/ml) were loaded into the wells. Ten percent of DMSO and 10 µg gentamicin were used as a negative and positive control, respectively. Plates were incubated at ± 35°C for 24 hours, and the zone of inhibition was measured in mm after the incubation period ended. The procedure was repeated for *S. mutans* using 30 µg chloramphenicol for positive control.

Antibiofilm analysis of nHA

For evaluating the biofilm inhibitory effects of nHA suspension against *S. aureus* and *S. mutans*, an in vitro crystal violet assay was executed by the protocol established by Nassima, Nassima [19]. One hundred microliters was added into each well of sterile 96-well microplates. Two-fold serial dilutions of nHA (250, 125, 62.5, 31.25, 15.625 mg/ml) were done in the plates respectively. Then, 100 µl overnight culture of *S. aureus* that was previously prepared and adjusted to 0.5 McFarland was added into every well. Blank control (extract and broth), growth control (bacteria culture and broth), and media control (broth only) were also done in every microplate. The mixture was then incubated at ± 35°C for 24 hours. As the incubation period ended, the wells were rinsed with sterile PBS twice to remove any unbound cells and air-dried. The formed biofilm was stained with 100 µl of 0.1% crystal violet for 20 minutes, followed by rinsing with PBS and drying. Finally, the stained biofilms were solubilized with 96% ethanol before being measured with spectrophotometry at 570nm. The percentage of biofilm inhibition by nHA extract was calculated using the formula below:

$$\text{Biofilm Inhibition (\%)} = \frac{\text{OD growth control} - \text{OD sample}}{\text{OD growth control}}$$

Biofilm visualization by light microscopy

The visualization of the biofilm inhibition by nHA extract using a light microscope was performed according to Al-Ansari and Alkubaisi [20]. *S. aureus* and *S. mutans* were first cultured in LB broth and standardized to 0.5 McFarland. 2.3 ml of bacteria culture and 50 µl of nHA (125 and 250 mg/ml) extracts were added to six-well plates containing

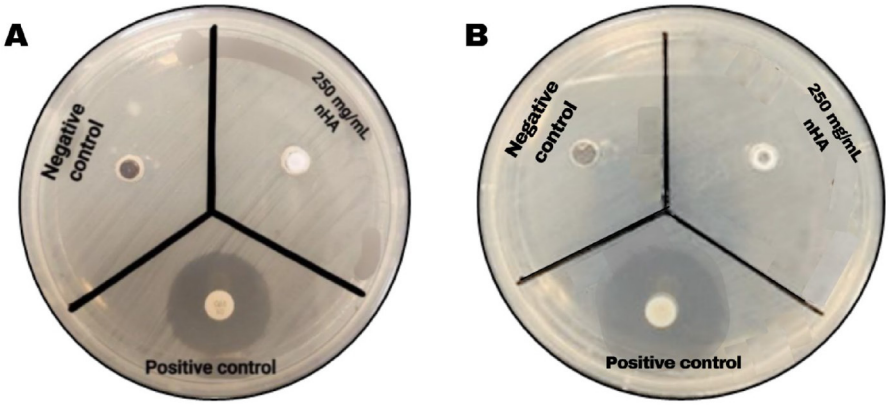

Figure 2. The zone of inhibition of 250 mg/ml nHA against A) *S. aureus* and B) *S. mutans*. 10% DMSO was used as the negative control, while 10 µg gentamicin and 30 µg chloramphenicol were applied as positive controls for *S. aureus* and *S. mutans*, respectively

glass coverslips. The plate was then incubated without shaking at ± 35°C for 48 hours. The biofilm that formed on coverslips was rinsed with PBS and stained with 0.2% crystal violet for 10 minutes. The process was followed by rinsing the stained biofilm with PBS before observing the biofilm structure under the microscope at 40x magnification.

Statistical analysis

SPSS Version 28.0 was used to perform statistical analysis, and all the experiments were repeated thrice. The independent T-test was used to quantify the biofilm inhibition for different concentrations of nHA extracts. The significance level was set at $p < 0.05$, and all the results were reported in mean ± standard deviation (SD).

Results

Antibacterial activity

The antibacterial analysis was conducted as a screening test to determine the antibacterial property of nHA against *S. aureus* and *S. mutans* using antibiotic susceptibility testing. In Figure 2, no inhibition zone was seen in both bacteria thus signifying that both bacteria are completely resistant to nHA.

Antibiofilm activity

Figure 3 shows the percentage of biofilm inhibition by nHA extracts against mono-species *S. aureus* and *S. mutans* biofilms. Serial dilutions were performed to obtain various extract concentrations. All concentrations of nHA (15.625, 31.25, 62.5, 125 and 250 mg/ml) inhibited the biofilm formation by both bacterial species. In addition, there was a significant difference between all concentrations and the untreated groups ($p < 0.001$). At the highest concentration (250 mg/ml) tested, nHA exhibited the most effective biofilm inhibition, reaching 92.29% for *S. aureus* and 95.53% for *S. mutans*. In contrast, nHA at 125 mg/ml showed a slightly lower but still significant inhibition of 91.41% and 92.69%, respectively. Even at the lowest tested concentration (15.625 mg/ml), the nHA extract exhibited a biofilm inhibition percentage surpassing 50% compared to untreated biofilm. The bar graph depicted a gradual increase in the percentage of biofilm inhibition by nHA against *S. aureus* and *S. mutans* with increasing concentration.

Visualization of biofilm inhibition

The results were substantiated by observing biofilms under a light microscope. Figure 4 illustrates a comparative analysis of biofilm structure using microscopy, showing the untreated biofilms and those of *S. aureus* and *S. mutans* treated with the highest two concentrations of nHA, 125 mg/ml and 250 mg/ml. Untreated biofilms were characterized by dense, intricately organized matrix, in contrast to the substantial thinning and structural disruption observed following treatment with 250 mg/mL nHA. The same destruction but with decreased intensity was also observed when the biofilm was treated with 125 mg/ml of nHA. This microscopic visualization supported the antibiofilm findings above, as the antibiofilm effects of the extracts could destroy the biofilm architecture.

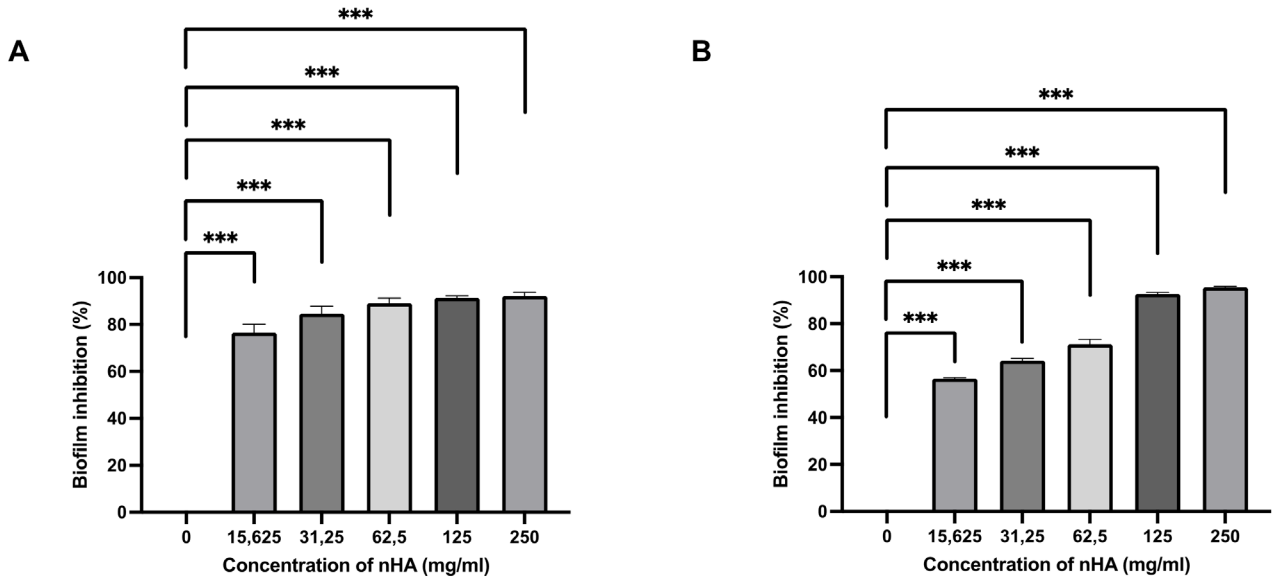

Figure 3. Percentage of biofilm inhibition by nHA against A) *S. aureus* and B) *S. mutans* biofilms. *** indicated significant differences when compared to untreated biofilm, 0 mg/ml (independent T-test, p <0.001)

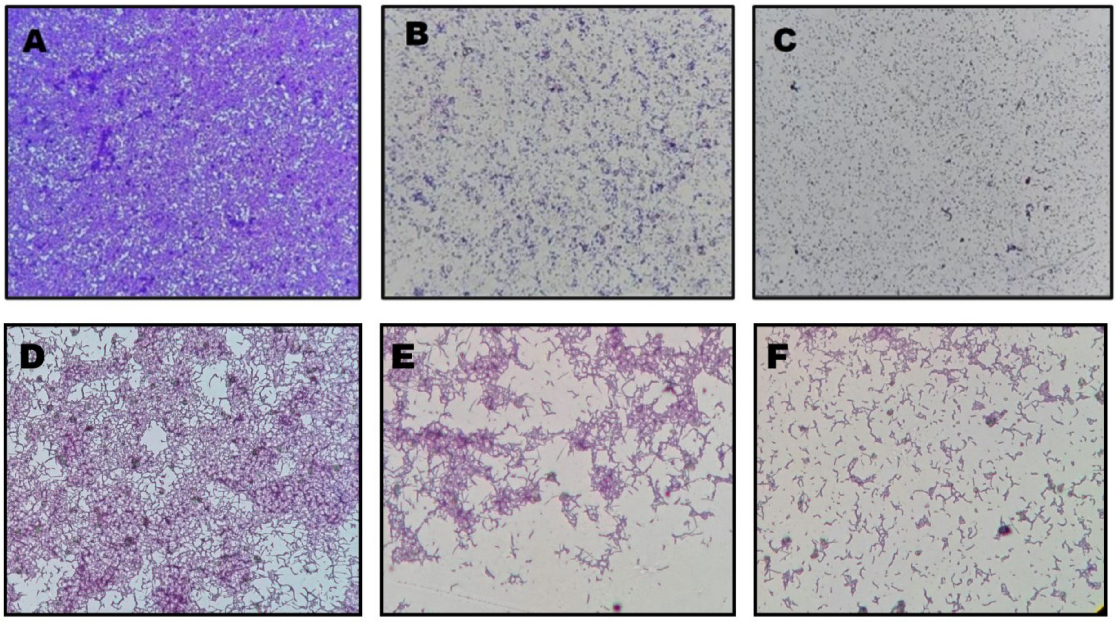

Figure 4. Visualization of biofilm structure of *S. aureus* biofilm between A) Negative control; B) 125 mg/mL of nHA; C) 250 mg/mL of nHA; and *S. mutans* biofilm between D) Negative control; E) 125 mg/mL of nHA and F) 250 mg/mL of nHA

Discussion

Dental caries represent a major chronic health issue, influenced by the dynamic interaction between host factors, plaque-forming bacteria, and dietary habits [21]. Key cariogenic bacteria, such as *S. aureus* and *S. mutans*, facilitate biofilm formation, accelerating dental caries' progression. The biofilms are encased in an extracellular matrix, which serves as a protective barrier against external environmental factors [22]. This study demonstrated that nHA effectively reduces the extent of biofilm formation by the mono-species *S. aureus* and *S. mutans*. These findings are consistent with structural visualizations showing disruption of the biofilm matrix with increasing nHA concentrations.

A recent study found that approximately 21.42% of individuals harbor significant levels of *S. aureus*

in their oral cavity, highlighting the importance of finding alternative methods to reduce its prevalence and maintain oral health [23]. Interest has grown in utilizing HA in dental care products due to its existing application in dental restoration. In this study, nano-sized HA synthesized through a wet precipitation method using eggshells was used at different concentrations. The antibacterial properties of the synthesized materials were assessed against *S. aureus* and *S. mutans* using the agar well diffusion method. The antibacterial analysis via AST showed that both *S. aureus* and *S. mutans* were completely resistant to the sample nHA, as evidenced by the absence of any zone of inhibition at the tested concentrations. The same finding was observed by Beyene and Ghosh [24], where the pure nHA failed to inhibit the growth of *S. aureus* whilst the addition of zinc oxide into the extract conferred antibacterial properties. It can be inferred that HA, in its unaltered state, exhibits no inherent antibacterial properties unless mixed or doped with other antimicrobial agents such as metal ions, silver, or zinc ions due to its ability to substitute calcium ions of nHA and damage the bacteria cell wall [25-28].

The ability of bacteria to form biofilms is crucial for their survival within the host, leading to severe and persistent infections, and *S. aureus* and *S. mutans* stand out for their remarkable biofilm-forming capacity [19, 29]. Hence, a potential strategy to address dental caries involves reducing and eradicating the formation of bacteria on the surfaces of teeth, specifically targeting plaque biofilm [30]. The present research also focused on assessing the ability of nHA derived from eggshells to combat *S. aureus* and *S. mutans* biofilms. This was accomplished by conducting *in vitro* crystal violet biofilm assays and analyzing the biofilm structure through light microscopy. The crystal violet-based assay is a commonly used technique to quantify bacterial biofilm inhibition by measuring the biofilm absorbance value of the crystal violet solubilization using a microplate reader. This study showed that, unlike antibacterial testing, HA possesses dose-dependent antibiofilm properties, wherein the antibiofilm effect is directly correlated with the concentration of the samples. All tested nHA concentrations exhibited significant differences compared to the untreated control groups (p <0.001). The HA has a protean hexagonal shape that can interfere with and prevent adherence of cariogenic bacteria [31]. HA creates a homogeneous and smooth surface through its enamel-like structure that diminishes irregularities, effectively reducing bacterial adherence and subsequent biofilm development. In addition, HA facilitates the creation of a uniform and polished surface on tooth enamel by promoting remineralization. This smooth enamel surface reduces the likelihood of adherence by *S. mutans*, thereby mitigating the initiation of biofilm formation on the tooth enamel [31].

Moreover, this study employed light microscopy to observe the structural development of *S. aureus* biofilms following treatment with nHA, providing additional confirmation to the *in vitro* crystal violet biofilm assay. Untreated specimens exhibited mature, compact, and highly cohesive biofilms, which were compared to the less dense structures observed in the treated samples. During colonization, planktonic cells adhere and secrete extracellular polymeric substances (EPS), which play a crucial role in promoting biofilm maturation [32]. The EPS matrix facilitates adhesion to surfaces, initiating and stabilizing biofilm formation [33]. Targeting EPS is therefore a key strategy for biofilm disruption. Microscopic analysis revealed a significant reduction in biofilm formation in the nHA-treated samples compared to the control group. The treated biofilm structures appeared less compact, with reduced surface coverage and a more dispersed appearance of both *S. aureus* and *S. mutans*. Despite the negative results in antibacterial activity, the findings indicate that nHA possesses notable antibiofilm properties. This suggests its potential mechanism involves disrupting and degrading the EPS matrix, thereby weakening the biofilm structure and promoting its dispersion.

The controlled *in vitro* environment used in this study represents a limitation, as it does not encompass the broader biological interactions that occur *in vivo*, including immune modulation, mechanical stress, and nutrient fluctuations. Additionally, this research focused solely on the short-term disruption of biofilms by nHA, leaving the long-term outcomes post-treatment unexplored. Furthermore, the possible cytotoxic effects of nHA were not assessed, which remains a significant concern that needs to be studied more thoroughly before advancing toward clinical applications.

Conclusion

The findings demonstrated that nHA extracts were ineffective in inhibiting the growth of both *S. aureus* and *S. mutans*, suggesting a lack of antibacterial activity. Fortunately, this study confirmed that nHA extracts

exhibit substantial antibiofilm activity against the mono-species biofilm structures of the targeted oral pathogens. The results indicated that nHA extracts derived from eggshells could effectively prevent dental caries by disrupting biofilm formation and architecture. This study suggests further investigation on nHA extracts to determine the suitability of the dosage and to evaluate the eggshells-derived extracts against dual- and multi-species biofilm. In addition, incorporating nHA into oral care formulations, including toothpaste, mouth rinses, and varnishes, offers a promising approach for long-term biofilm inhibition and preventing oral diseases.

Acknowledgment

This study is supported by Leave a Nest Grant Global Challenge Award 100-TNCPI/PRI 16/6/2 (065/2022) at Centre for Medical Lab Technology Studies, Faculty of Health Sciences, UiTM Puncak Alam.

Declaration of interest

There are no conflicts of interest in this study.

Author contributions

NFZ, NIAAA, NNS: the experimental procedures, data processing, and analysis. NAZ, FS, NM: study planning and provided supervisory support. NNS, NHT, FAH, SNMH: manuscript preparation, figure creation, data interpretation, and biofilm inhibition calculations. NM: synthesize the nano-hydroxyapatite powder. All authors actively participated in result interpretation and manuscript revision.

Received: October 29, 2024
Revised: December 24, 2024
Accepted: December 31, 2024
Published: December 31, 2024

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