

Unveiling the Potential of Eggshell Nanoparticles in Toothpaste Formulation: A Physicochemical Approach

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Research Article

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Abstract

Toothpaste commonly uses calcium carbonate as an abrasive; this study investigated eggshell-derived calcium carbonate nanoparticles (ENPs) as a sustainable formulation ingredient. Waste chicken eggshells were washed, thermally treated to generate a calcium oxide intermediate, dispersed and sonicated in water, and dried. The final recovered powder was identified as calcite-dominant CaCO_3 by XRD and FTIR and was characterised by XRF, SEM-EDS, FTIR and XRD. Fourteen toothpaste formulations varying ENP, peppermint-oil and clove-oil levels were generated using a response-surface/central-composite design structure, and inhibition-zone diameter against *Streptococcus* spp. was used as the response. Analysis of the 14 experimental runs gave a significant quadratic model ($F = 20.45$, $p = 0.0053$; $R^2 = 0.9787$; adjusted $R^2 = 0.9309$; predicted $R^2 = 0.7413$), with a negative ENP main effect and a positive ENP $\times$ peppermint-oil interaction. F12 showed the largest observed inhibition zone (22 mm). Scale-bar-calibrated analysis of the representative SEM micrograph produced apparent particle/agglomerate diameters of 29.7–268.9 nm (median 67.6 nm; mean 88.7 ± 64.8 nm), whereas the XRD Scherrer crystallite size was ~ 20 nm, indicating nanoscale crystallites within larger agglomerated structures. F12 had pH 8.2 ± 0.1 , moisture $2.5 \pm 0.3\%$ and solubility $28.4 \pm 1.2\%$, with Day-14 overall acceptance of 90% in the descriptive sensory assessment. The findings support formulation feasibility. Comparative clinical antimicrobial efficacy, validated colloidal particle size, biocompatibility, remineralisation and long-term shelf-life require dedicated comparator-controlled, particle-sizing, safety, dental-performance and stability studies.

Introduction

Oral health remains a major public-health concern, and dental caries, periodontal disease and dentin hypersensitivity affect large populations. Toothpaste serves both as a mechanical plaque-removal vehicle and as a carrier for active ingredients intended to support antimicrobial, desensitising and remineralising functions (Florea et al., 2023; Gümüştas et al., 2024; Sevagaperumal et al., 2024). Conventional formulations commonly contain calcium carbonate or silica abrasives; therefore, replacing part of this mineral fraction with a value-added material recovered from food waste is relevant to circular-economy formulation research.

Eggshell is a calcium-rich poultry-industry by-product composed predominantly of CaCO_3 , together with a smaller organic and trace-mineral fraction (Ahmed et al., 2022; Diningsih and Rohmawati, 2022; Assi and Shawkat, 2024). Its high calcium content has motivated conversion into calcium-based micro- and nanostructured materials for environmental and biomedical applications. In the present manuscript, the final calcite-dominant material recovered after thermal and aqueous processing is termed eggshell-derived calcium carbonate nanoparticles (ENPs), while recognising that particle-size validation requires more than crystallite-size estimation alone.

Eggshell-derived calcium materials have attracted interest in dentistry because eggshell can act as a calcium source and precursor for apatite formation. Previous studies have reported dentinal-tubule

occlusion or enamel/dentin remineralisation with eggshell-based or hydroxyapatite-containing systems (Abou Neel and Bakhsh, 2021; Juntavee et al., 2021; Hassan et al., 2023; Mkhize et al., 2023; Karthikeyan and Mahalaxmi, 2024; Shehani and Ramar, 2024; Latif et al., 2026). Related clinical and laboratory literature also supports the broader rationale for nano-hydroxyapatite toothpastes in remineralisation and hypersensitivity management (Amaechi et al., 2021a; Amaechi et al., 2021b; Polyakova et al., 2022; Wang et al., 2024; Guanipa Ortiz et al., 2024; Cocco et al., 2025; Amaechi et al., 2025). These findings provide literature-based rationale; enamel remineralisation, dentinal-tubule occlusion, pH-cycling, microhardness and hypersensitivity outcomes were outside the experimental scope of the present study.

Nanoscale calcium-carbonate systems can show high surface area and size-dependent interfacial behaviour, but 'crystallite size', dry-powder SEM feature size and hydrodynamic particle size are not interchangeable quantities. Reliable nanoparticle characterisation commonly combines electron microscopy with an orthogonal sizing method such as DLS or TEM and, for dispersions, zeta-potential measurement (Huang et al., 2020; d'Amora et al., 2020; Chuzeville et al., 2022; Torres-Mansilla et al., 2023; Shu et al., 2024; Ghaffari et al., 2024). Accordingly, the present work treats the SEM-based dimensions as semi-quantitative apparent particle/agglomerate sizes and the XRD value as crystallite size rather than claiming that the two measures are equivalent.

Eggshell-derived calcium nanomaterials have also been investigated for antimicrobial behaviour. Calcite CaCO_3 nanoparticles and related eggshell-derived calcium nanomaterials have shown antibacterial and antibiofilm effects in experimental systems, while peppermint-derived constituents can inhibit oral bacteria and biofilms (Al-Azzawi and Al-Kalifawi, 2023; Thakur et al., 2024; Lopes et al., 2024). Nanoparticle- or mineral-containing toothpastes are therefore reasonable candidates for agar-diffusion screening, but inhibition-zone magnitude should be interpreted against appropriate controls, as studies of dentifrices commonly include chlorhexidine or a commercial toothpaste as a positive comparator and water/saline or a base formulation as a negative control (Reddy et al., 2021; Klaophimai et al., 2024; Aliyyu et al., 2024).

Recent work has moved from materials characterisation toward oral-care applications, including eggshell-based dentifrice, enamel-remineralisation models and nano-hydroxyapatite toothpastes (Abou Neel and Bakhsh, 2021; Shehani and Ramar, 2024; Latif et al., 2026). However, fewer studies combine eggshell-derived calcium material, essential-oil variables, formulation optimisation, antimicrobial screening, physicochemical evaluation and short-term sensory observation in one workflow. This study therefore examines formulation feasibility rather than extending to clinical remineralisation, hypersensitivity treatment or safety outcomes outside the present experimental scope.

The current study aimed to prepare calcite-dominant eggshell-derived calcium carbonate material, characterise its physicochemical features, and incorporate it into toothpaste formulations. Design-Expert software was used for the response-surface design. ENP concentration, peppermint oil and clove oil were varied, and the formulation with the largest observed inhibition zone was subsequently evaluated for pH, moisture, solubility and short-term organoleptic properties. Response-surface methodology is an

established design-of-experiments approach for pharmaceutical formulation development when model adequacy and design limitations are reported transparently (Kumar et al., 2022; Podder and Mukherjee, 2024).

The study primarily addresses waste valorisation and formulation feasibility. It links eggshell processing, material characterisation and a multicomponent toothpaste design while identifying the analytical steps required before stronger biomedical claims can be made. In particular, validated particle sizing, cytotoxicity/mucosal-safety testing, controlled antimicrobial comparisons, enamel/dentin performance testing and longer-term stability studies remain necessary before the material can be described as biocompatible, clinically remineralising, desensitising, or suitable for commercial use.

Methodology

Preparation of Eggshell-Derived Calcium Carbonate Material

Waste chicken eggshells were washed with distilled water and immersed in 70% ethanol for disinfection. The shells were dried at 100 °C for 2 h and then thermally treated at 800 °C for 2 h in a muffle furnace. This thermal step can generate CaO as an intermediate from eggshell CaCO_3 . The calcined material was ground and sieved through a 100- μm mesh. One gram of powder was dispersed in 100 mL distilled water, stirred at 500 rpm for 30 min, sonicated at room temperature for 30 min and dried at 80 °C for 12 h. Because subsequent XRD and FTIR identified the final dried material as calcite-dominant CaCO_3 , the recovered product is referred to consistently as eggshell-derived calcium carbonate nanoparticles (ENPs) throughout the manuscript; CaO is used only for the thermal intermediate.

Characterisation of ENPs

X-ray Fluorescence (XRF)

The inorganic elemental composition of the recovered powder was assessed by X-ray fluorescence spectroscopy, with particular attention to calcium. XRF was used as an elemental/oxide-equivalent measurement and not as the sole basis for assigning the final crystalline phase; phase identity was instead interpreted from XRD together with FTIR and EDS.

Scanning Electron Microscopy (SEM)

Surface morphology was examined using a scanning electron microscope (SEM; JEOL JSM-6390LV). Powder was mounted on conductive carbon tape on an aluminium stub, gold-coated and imaged at 20 kV at multiple magnifications. The representative SEM micrograph was analysed by scale-bar-calibrated

digital segmentation to determine the apparent size distribution: 50 discernible particle/agglomerate features were measured after excluding obvious annotations and large connected background regions, and equivalent circular diameters were calculated. Because SEM thresholding of a dry, agglomerated powder is operator- and image-dependent, these values are reported as semi-quantitative apparent feature sizes rather than as a validated hydrodynamic particle-size distribution. The SEM measurements are interpreted as dry-powder apparent feature sizes rather than hydrodynamic or colloidal-size measurements. EDS was used to determine the principal elemental signals.

Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectra were recorded using a PerkinElmer Spectrum Two™ FT-IR spectrometer. The KBr pellet method was used to prepare samples by mixing 20 mg of spectroscopic-grade KBr with 2.0 mg of the nanoparticle powder and compressing the mixture into a transparent KBr pellet. The spectra were measured at ambient temperature (298 K) in transmittance mode, at 4 cm⁻¹ resolution over 500–4000 cm⁻¹ and with 16 scans.

X-ray Diffraction (XRD)

The crystalline structure and phase composition of nanoparticles were determined by XRD on the XPERT-PRO diffractometer with Cu K α -radiation ($\lambda = 1.5406 \text{ \AA}$). The full width at half maximum (FWHM) of the main reflection was used to estimate the crystallite size by applying the Scherrer equation.

Optimisation Using Response Surface Methodology

The toothpaste design used a 14-run central-composite structure generated in Design-Expert, with ENP concentration (A), peppermint oil (B) and clove oil (C) each represented at factorial and axial levels; the response was inhibition-zone diameter (mm). The 14 runs comprised eight factorial and six axial points without replicated centre-point or other replicate runs. Accordingly, pure error was not independently estimable and a formal lack-of-fit F-test was not calculable. The square-root-transformed response was fitted to the 14 tabulated runs using the full quadratic structure. Model adequacy was evaluated using overall F-test, R², adjusted R², PRESS-based predicted R², residual diagnostics and coefficient significance. The lack of replicated design points is reported as a design limitation, and model interpretation is restricted accordingly.

Toothpaste Formulation

Carboxymethyl cellulose was dispersed in hot distilled water and then allowed to swell in the hot water at room temperature to form a gel base (Mass 1) for each experimental run. The ENP powder was pre-mixed with sodium lauryl sulphate (SLS) to provide uniform dispersion before being added to Mass 1 to

form a homogeneous base (Mass 2). Humectants (glycerine and sorbitol) and thickeners (guar gum) were added to the formulation to increase moisture retention and smoothness, and to prevent phase separation, respectively. Peppermint and clove oils were added to act as flavouring/active agents, and distilled water was used to bring the formulation to 100 parts by weight. Homogenisation was performed to obtain a stable and shiny paste, which was subsequently stored and analysed in sterilised tubes.

Antimicrobial Analysis

All 14 toothpaste formulations were assessed for antimicrobial activity against *Streptococcus* spp. by agar-well diffusion. Blood agar plates were prepared using standard microbiological procedures, a standardised bacterial suspension was spread over the agar, uniform wells were produced with a sterile cork borer, and 10 µL of each toothpaste sample was introduced. Plates were allowed to diffuse and were incubated at 37 °C before inhibition zones were recorded. The antimicrobial assay was conducted without a positive comparator (for example, chlorhexidine or a commercial fluoride toothpaste) or a negative/base-paste control. Consequently, the assay supports only within-study comparison among the 14 formulations; it does not establish that the 22-mm zone is clinically superior or equivalent to an existing product (Reddy et al., 2021; Klaophimai et al., 2024).

Toothpaste Evaluation

Quality, stability and acceptability of the formulations selected by CCD were evaluated by organoleptic and physicochemical tests. The organoleptic tests were used to evaluate the sensory properties, and the physicochemical tests were used for quantitative pH, solubility and moisture content data.

Sensory (Organoleptic) Evaluation

The colour, odour and texture of the selected F12 toothpaste were observed at room temperature on Day 1, Day 7 and Day 14 using a 5-point hedonic scale (1 = very poor, 5 = very good), and overall acceptance was recorded as the percentage of panellists scoring ≥ 4 . The available sensory dataset comprised aggregate scores without panel size, individual-level ratings, or panellist selection/training metadata; therefore, the results are presented as descriptive short-term observations rather than inferential repeated-measures comparisons.

Physicochemical Tests

Physicochemical tests were conducted on the pH, moisture content, and solubility of the optimised formulation, all determined under ambient laboratory conditions:

- (i) pH value: the formulation was prepared as a 1:2 (w/w) suspension in distilled water and measured using a calibrated digital pH meter at 25 °C.
- (ii) Moisture content: determined by oven-drying a ~2 g sample at 105 °C to constant weight, expressed as percentage loss on drying.
- (iii) Solubility: determined by dissolving a known mass of paste in distilled water, stirring, centrifuging the supernatant, and drying; solubility was expressed as the percentage of total mass dissolved.

Results and Discussion

Synthesis of Nanoparticles

The eggshell material underwent washing, drying, crushing, calcination, aqueous dispersion/sonication and final drying (Figure 1). Calcination at 800 °C is described here as producing a CaO-rich intermediate rather than as the identity of the final powder. After the subsequent water-processing and drying steps, XRD and FTIR of the recovered material showed calcite-dominant CaCO_3 , while EDS showed Ca, O and C signals. The manuscript therefore standardises the final material as eggshell-derived calcium carbonate nanoparticles (ENPs) and uses 'CaO' only when referring to the thermal intermediate.

This distinction is important because eggshell-derived calcium phases can change during thermal, hydration and carbonation steps, and final phase assignment should rely on structural characterisation rather than on the starting composition or calcination step alone (Ahmed et al., 2022; Diningsih and Rohmawati, 2022; Assi and Shawkat, 2024). The present combination of carbonate FTIR bands and calcite XRD reflections supports a calcite-dominant final phase, while quantitative phase analysis and direct carbonation-kinetics measurements were outside the present scope.

Fourier Transform Infrared Spectroscopy (FTIR)

The results of FTIR exhibited the presence of various characteristic absorption bands (Figure 2a). A wide band in 2979-2512 cm^{-1} was attributed to the low-intensity vibrations of the C–H bonds of the remaining organic matter in the eggshell membrane. Asymmetric stretching of the carbonate group (CO_3^{2-}) was shown by a large band at 1795-1394 cm^{-1} , indicative of calcite/aragonite CaCO_3 , while sharp absorption peaks in the range 871-504 cm^{-1} indicated out-of-plane and in-plane bending vibrations of carbonate ions, confirming crystallinity. A large number of peaks in the fingerprint region (700–500 cm^{-1}) further confirmed the highly crystalline nature of the material.

The FTIR spectrum was consistent with a carbonate-containing material, with characteristic carbonate vibrations supporting calcite-dominant CaCO_3 . These assignments agree with reported spectra of eggshell-derived calcium carbonate and related calcium-carbonate nanomaterials (Ahmed et al., 2022; Assi and Shawkat, 2024; Chuzeville et al., 2022). Residual low-intensity organic-associated bands may

reflect incomplete removal of membrane-derived constituents; they do not by themselves establish biological compatibility or safety.

Scanning Electron Microscopy (SEM) and EDS Analysis

The SEM micrograph (Figure 2c) showed irregular quasi-spherical/sub-spherical features with visible clustering. Scale-bar-calibrated analysis of the representative micrograph generated the apparent size-distribution histogram in Figure 5 ($n = 50$ discernible features). Equivalent diameters ranged from approximately 29.7 to 268.9 nm, with a median of 67.6 nm, an interquartile range of 42.0–103.7 nm and a mean of 88.7 ± 64.8 nm. The broad right-tailed distribution and the presence of features above 100 nm indicate agglomeration and indicate that a uniform 10–100 nm size range is not supported by the image-derived distribution. These SEM-derived values are semi-quantitative two-dimensional measurements of dry features, not DLS-validated primary-particle sizes (Huang et al., 2020; d'Amora et al., 2020; Shu et al., 2024).

EDS (Figure 2b) showed Ca, O and C as the main detected elements (O 43.75 wt.%, Ca 28.41 wt.% and C 27.84 wt.%). This elemental pattern is compatible with a calcium-carbonate-rich material and is consistent with the carbonate bands in FTIR and calcite reflections in XRD. EDS is not a direct molecular-phase assay, so the phrase 'high-purity CaCO_3 ' is reserved for the combined interpretation of the structural and elemental data rather than being inferred from EDS alone.

X-ray Diffraction (XRD)

The XRD pattern (Figure 2d) showed several sharp and intense peaks, with the most prominent peak at $2\theta = 29.42^\circ$, corresponding to the (104) crystallographic plane of calcite. Additional reflections at approximately 23.08° , 36.04° , 39.46° , 43.21° , 44.67° , 47.51° and 48.43° were consistent with crystalline calcium carbonate, in agreement with reported eggshell-derived CaCO_3 characterisation (Ahmed et al., 2022; Assi and Shawkat, 2024). The average crystallite size estimated from the FWHM by the Scherrer equation was approximately 20 nm, and the reported crystallinity was about 85%. No distinct secondary-phase peaks were identified in the presented diffractogram.

The Scherrer crystallite size (~ 20 nm) does not represent the same quantity as the SEM apparent particle/agglomerate diameter. The substantially smaller crystallite estimate, together with SEM features extending above 100 nm, is consistent with larger particles or agglomerates being composed of multiple nanoscale crystalline domains. Accordingly, the study supports a nanocrystalline calcite component but cannot establish a monodisperse ~ 20 –100 nm colloidal particle population without TEM/DLS and zeta-potential data (Chuzeville et al., 2022; Ghaffari et al., 2024; Shu et al., 2024). This distinction moderates the nanoparticle claim while preserving the XRD evidence for nanoscale crystallites.

Response Surface Methodology (RSM) Optimisation of Antimicrobial Activity

The formulation variables—ENP concentration (A), peppermint oil (B) and clove oil (C)—were evaluated within the response-surface design to examine their association with square-root-transformed inhibition-zone diameter.

Model Selection

The 14 experimental runs were analysed across linear, 2FI and full quadratic models. The linear and 2FI models showed weak overall fit (overall $p = 0.1691$ and 0.2888 , respectively), whereas the full quadratic model was significant ($F = 20.45$, $p = 0.0053$) with $R^2 = 0.9787$, adjusted $R^2 = 0.9309$ and PRESS-based predicted $R^2 = 0.7413$ (Table 2). The quadratic model was therefore retained for exploratory interpretation, while the lower predicted R^2 and the absence of replicated centre points require cautious interpretation.

Quadratic response-surface models are useful when formulation behaviour is curved or involves interactions, but their credibility depends on adequate design support, replication and transparent diagnostics (Kumar et al., 2022; Podder and Mukherjee, 2024). Here, the quadratic terms substantially improved in-sample fit, but the design contained only 14 unique runs and no replicated centre points. Thus, the model is used to describe patterns within the investigated design space rather than as a fully validated predictive optimisation model.

ANOVA for the Quadratic Model

The square-root-transformed antimicrobial response was analysed using the quadratic model (Table 3). Overall regression sum of squares was 4.7248 with 9 df, residual sum of squares was 0.1027 with 4 df, and the model was significant ($F = 20.45$, $p = 0.0053$). R^2 was 0.9787 and adjusted R^2 was 0.9309; predicted R^2 from leave-one-out PRESS was 0.7413, and the residual standard deviation was 0.1602 (CV = 4.27%). Adequate Precision, calculated from the range of fitted responses relative to average prediction error, was 13.36 (>4), indicating an adequate signal within the observed design space. Term-level sums of squares in Table 3 are explicitly labelled partial (Type III) values and are therefore not expected to add to the overall model regression SS. A formal lack-of-fit test is reported as 'not estimable' because there were no replicated design points from which to calculate pure error.

ENP concentration (Factor A) showed the strongest linear association with the transformed response ($F = 66.08$, $p = 0.0012$), and the fitted coefficient was negative. Peppermint oil (B) was not significant ($F = 1.24$, $p = 0.3270$), and the clove oil main effect did not reach the 0.05 threshold ($F = 4.55$, $p = 0.0999$). Thus, the clove-oil main effect was not statistically significant at $\alpha = 0.05$.

Among interactions, ENP $\times$ peppermint oil (AB) was significant ($F = 29.50$, $p = 0.0056$), whereas AC ($p = 0.1423$) and BC ($p = 0.1834$) were not. The positive AB coefficient indicates that the combined effect was more favourable than expected from the two main effects alone within this fitted model. This statistical interaction should not be interpreted as a confirmed biological synergy without dedicated mechanistic and comparator-controlled experiments.

For curvature terms, C^2 was significant ($F = 9.74$, $p = 0.0355$), A^2 was borderline ($p = 0.0517$) and B^2 was not significant ($p = 0.0935$). Thus, only C^2 was statistically significant among the three curvature terms at $\alpha = 0.05$. The curvature nevertheless helps explain why a full quadratic model fitted the 14 observations better than simpler models, while the low residual degrees of freedom and non-estimable lack-of-fit test limit generalisation beyond the studied runs.

Regression Coefficients of the Quadratic Model

Regression coefficients are presented in Table 4 using coded factors. To make the intercept interpretable despite the absence of a centre-point run, the quadratic terms were mean-centred as ($A^2 - 0.9755$), ($B^2 - 0.9755$) and ($C^2 - 0.9755$). The resulting intercept (3.7528) represents the mean transformed response; coefficient signs and significance are unchanged by this centring.

The ENP main-effect coefficient was negative ($\beta = -0.3524$, $p = 0.0012$). Thus, within the coded design space and holding other model terms constant, increasing Factor A was associated with a lower square-root-transformed inhibition-zone response. Peppermint oil showed a small, non-significant negative coefficient ($\beta = -0.0484$, $p = 0.3270$), whereas clove oil showed a positive but non-significant linear coefficient ($\beta = +0.0925$, $p = 0.0999$).

The AB interaction was positive and significant ($\beta = +0.3076$, $p = 0.0056$). AC ($\beta = +0.1033$, $p = 0.1423$) and BC ($\beta = -0.0910$, $p = 0.1834$) were not significant. For mean-centred quadratic terms, A^2 was borderline ($\beta = +1.3866$, $p = 0.0517$), B^2 was non-significant ($\beta = +1.1079$, $p = 0.0935$) and C^2 was significant ($\beta = +1.5776$, $p = 0.0355$). VIF values were approximately 1.0 for linear/interaction terms and 1.2 for quadratic terms, providing no indication of problematic multicollinearity in the quadratic model.

Regression Equation in Coded Factors

Using mean-centred quadratic terms, the fitted coded-factor equation was: $\sqrt{Y} = 3.7528 - 0.3524A - 0.0484B + 0.0925C + 0.3076AB + 0.1033AC - 0.0910BC + 1.3866(A^2 - 0.9755) + 1.1079(B^2 - 0.9755) + 1.5776(C^2 - 0.9755)$. The negative A coefficient indicates the direction of the ENP main effect. Because no centre-point replicate was measured, this equation should be used for interpolation within the experimental design space rather than for strong extrapolative optimisation claims.

Response Surface Analysis

The response-surface plots (Figure 3) are consistent with the fitted coefficient directions: the most pronounced interaction was ENP $\times$ peppermint oil (AB, $p = 0.0056$), while AC and BC were non-significant. The negative ENP main effect and positive AB interaction mean that the influence of ENP concentration depends partly on peppermint-oil level rather than following a simple monotonic trend. Because only C^2 was conventionally significant among the three curvature terms and the design lacked replicated centre points, the surfaces are interpreted descriptively and should not be treated as externally validated optimisation maps.

Model Diagnostics

Residual diagnostics were reviewed for the quadratic model. The normal-probability plot of residuals (Figure 4) was broadly compatible with approximate normality, although two externally studentized residuals were about ± 3.5 and the residual degrees of freedom were only four. These features, together with the absence of replicate runs and the resulting non-estimable lack-of-fit test, warrant caution in using the model for strong predictive claims. The diagnostics support an exploratory fit to the observed design points rather than definitive model validation.

Antimicrobial Activity of Toothpaste Formulations

Antimicrobial screening showed measurable inhibition zones across the 14 experimental formulations, with observed values ranging from 8 to 22 mm. F12 produced the largest zone (22 mm), followed by F13 (20 mm), while F7, F9 and F14 each produced 18 mm. These values are reported as within-study observations rather than as external validation of the response-surface model or evidence of superiority to a commercial antimicrobial toothpaste.

Across the 14 formulations, F12 (Run 12) had the largest observed inhibition zone (22 mm; Table 1), followed by F13 (20 mm) and several formulations at 18 mm. F12 contained 1.0 g ENPs, 1.0 mL peppermint oil and 2.0 mL clove oil and was therefore selected as the best-performing observed formulation for subsequent physicochemical and sensory evaluation. The within-study ranking is compatible with the negative ENP main effect and positive AB interaction in the quadratic model. However, because the antimicrobial experiment lacked a commercial/chlorhexidine positive control and a base-paste negative control, the 22-mm zone cannot be interpreted as clinically superior, equivalent to a marketed dentifrice, or attributable specifically to ENPs rather than to the combined formulation (Reddy et al., 2021; Klaophimai et al., 2024; Ghaffari et al., 2024).

Organoleptic and Sensory Evaluation

The colour, aroma and texture scores of F12 were recorded descriptively over 14 days (Table 5). No visible phase separation was reported during this short observation window and the grey colour

remained visually unchanged. These observations support only short-term physical acceptability; they do not establish long-term shelf stability.

The three Day-1 attribute scores (3.0, 3.0 and 4.0) had an arithmetic mean of 3.33. Reported acceptance increased from 65% on Day 1 to 80% on Day 7 and 90% on Day 14, but this pattern is descriptive only. Because the available dataset contains aggregate scores without panel size, selection/training information or individual repeated ratings, statistical significance across days cannot be evaluated with Friedman or Wilcoxon procedures. The 14-day duration is also too short to support a general long-shelf-life claim.

Physicochemical Testing of the Optimised Formulation (F12)

The physicochemical properties of the optimised toothpaste formulation (F12) were determined by measuring its pH, moisture content and solubility (Table 6). These parameters are crucial factors for formulation quality, stability and suitability for oral application. The values obtained showed that the physicochemical properties of F12 were compatible with the recommended specifications for the formulation of toothpaste or toothpaste based on nanoparticles and herbal ingredients.

The pH of F12 was 8.2 ± 0.1 , indicating a mildly alkaline formulation. This value is reported as a physicochemical characteristic only; oral-mucosal compatibility remains to be established, so the pH result cannot be used to claim absence of irritation or biocompatibility. This distinction is particularly important because the formulation contains sodium lauryl sulphate (SLS), for which oral-mucosal irritation or desquamation has been reported in susceptible users (Kasi et al., 2022).

Moisture content was $2.5 \pm 0.3\%$. This low loss-on-drying value is useful for describing the tested batch, but it cannot by itself demonstrate microbiological preservation or long shelf life. Formal stability assessment would require longer real-time and/or accelerated storage with repeated physicochemical, microbiological, rheological and packaging-compatibility measurements.

The solubility of F12 was $28.4 \pm 1.2\%$, reflecting the combined contribution of water-soluble formulation components and insoluble mineral material. Taken together, the pH, moisture and solubility measurements provide a preliminary physicochemical profile of the selected formulation. They do not establish clinical safety, enamel remineralisation, dentin desensitisation, or commercial readiness; those outcomes require dedicated biological, dental-performance and longer-term stability studies.

Limitations and Interpretation Safeguards

Several limitations directly constrain interpretation. First, the 14-run response-surface design contained no replicated centre or duplicate design points, so pure error and a formal lack-of-fit test could not be

estimated. Second, antimicrobial testing lacked positive and negative comparator controls, preventing benchmarking of the 22-mm zone against existing products. Third, nanoparticle sizing was based on analysis of a representative dry-powder SEM image and XRD crystallite size; DLS, TEM and zeta-potential characterisation remain necessary for colloidal-size validation, and the SEM distribution includes agglomerates above 100 nm. Fourth, cytotoxicity and oral-mucosal irritation testing remains necessary before the formulation can be described as biocompatible or safe, particularly given the presence of SLS (Kasi et al., 2022). Fifth, enamel/dentin remineralisation and hypersensitivity benefits remain literature-based rather than experimentally established in this study (Guanipa Ortiz et al., 2024; Latif et al., 2026). Finally, sensory observations covered only 14 days and were based on aggregate scores without individual-level panel metadata, so acceptance trends are descriptive and do not establish statistically significant improvement or long-term stability.

Conclusion

Eggshell waste was converted into a calcite-dominant, nanocrystalline calcium-carbonate material and incorporated into toothpaste formulations, demonstrating a plausible circular-economy route from food waste to an oral-care formulation ingredient. In the response-surface analysis, ENP concentration had a negative main effect and the ENP × peppermint-oil interaction was positive, while F12 produced the largest observed inhibition zone (22 mm). SEM analysis showed a broad apparent particle/agglomerate distribution and XRD indicated ~ 20-nm crystallites, supporting nanocrystalline domains but also substantial agglomeration. The selected formulation showed acceptable preliminary physicochemical and short-term descriptive sensory characteristics. However, the present evidence does not demonstrate comparative clinical antimicrobial efficacy, validated colloidal nanoparticle size, biocompatibility, enamel remineralisation, dentin-hypersensitivity reduction, or long-term shelf stability. Controlled microbiology, DLS/TEM/zeta-potential analysis, safety testing, dental-performance studies and longer stability programmes are required before stronger clinical or commercial claims can be made.

Declarations

Conflict of interest:

The authors declare that there is no conflict of interest regarding the publication of this paper.

Funding declaration:

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Ethics declaration:

not applicable.

Author Contribution

S.N wrote the main manuscript text prepared all tables and figures 1-3 and reviewed the manuscript."

References

1. Abou Neel, E. A., & Bakhsh, T. A. (2021). An eggshell-based toothpaste as a cost-effective treatment of dentin hypersensitivity. *European Journal of Dentistry*, 15(4), 733–740. <https://doi.org/10.1055/s-0041-1729676>
2. Ahmed, N. S., Kamil, F. H., Hasso, A. A., Abduljawaad, A. N., Saleh, T. F., & Mahmood, S. K. (2022). Calcium carbonate nanoparticles of quail's egg shells: Synthesis and characterizations. *Journal of the Mechanical Behavior of Materials*, 31(1), 1–7. <https://doi.org/10.1515/jmbm-2022-0001>
3. Al-Azzawi, M. H., & Al-Kalifawi, E. J. (2023). Antibacterial and antibiofilm activity of calcite (CaCO_3) nanoparticles. *The Egyptian Journal of Hospital Medicine*, 90(2), 208–215.
4. Aliyyu, W. C., Riva, F. A., Anabel, S. M. P., Dwiandhono, I., Satrio, R., & Sari, D. N. I. (2024). Nano-hydroxyapatite toothpaste of rice field snail shell combined with basil leaf extract as a remineralizing and antibacterial agent to prevent dental caries. *Journal of Clinical and Experimental Dentistry*, 16(11), e1323–e1331. <https://doi.org/10.4317/jced.62073>
5. Amaechi, B. T., et al. (2021a). Anti-caries evaluation of a nano-hydroxyapatite dental lotion for use after toothbrushing: An in situ study. *Journal of Dentistry*, 115, 103863. <https://doi.org/10.1016/j.jdent.2021.103863>
6. Amaechi, B. T., Lemke, K. C., Saha, S., Luong, M. N., & Gelfond, J. (2021b). Clinical efficacy of nanohydroxyapatite-containing toothpaste at relieving dentin hypersensitivity: an 8 weeks randomized control trial. *BDJ Open*, 7, 23. <https://doi.org/10.1038/s41405-021-00080-7>
7. Amaechi, B. T., Tan, A. I., Noureldin, A. A. K., Kanthaiah, K., Holladay, E., Obiefuna, A. C., & Vijayaraghavan, M. (2025). In vitro evaluation of the ability of nanohydroxyapatite toothpastes to enhance remineralization of enamel caries lesion. *Journal of Dentistry*, 161, 106006. <https://doi.org/10.1016/j.jdent.2025.106006>
8. Assi, S. A., & Shawkat, S. M. (2024). Characterization of nano calcium carbonate extracted from eggshells. *South Eastern European Journal of Public Health*, 1681–1686. <https://doi.org/10.70135/seejph.vi.2185>
9. Chuzeville, L., Boury, F., Duday, D., Anand, R., Moretto, E., & Thomann, J. S. (2022). Eco-friendly processes for the synthesis of amorphous calcium carbonate nanoparticles in ethanol and their stabilisation in aqueous media. *Green Chemistry*, 24, 1270–1284. <https://doi.org/10.1039/D1GC03396D>
10. Cocco, F., Salerno, C., Wierichs, R. J., Wolf, T. G., Arghittu, A., Cagetti, M. G., & Campus, G. (2025). Hydroxyapatite-fluoride toothpastes on caries activity: A triple-blind randomized clinical trial. *International Dental Journal*, 75(2), 632–642. <https://doi.org/10.1016/j.identj.2024.09.037>

11. d'Amora, M., Liendo, F., Deorsola, F. A., Bensaid, S., & Giordani, S. (2020). Toxicological profile of calcium carbonate nanoparticles for industrial applications. *Colloids and Surfaces B: Biointerfaces*, 190, 110947. <https://doi.org/10.1016/j.colsurfb.2020.110947>
12. Diningsih, C. A., & Rohmawati, L. (2022). Synthesis of calcium carbonate (CaCO₃) from eggshell by calcination method. *Indonesian Physical Review*, 5(3), 208–215.
13. Florea, A. D., et al. (2023). Remineralization induced by biomimetic hydroxyapatite toothpastes on human enamel. *Biomimetics*, 8(6), 450. <https://doi.org/10.3390/biomimetics8060450>
14. Ghaffari, T., Daneshfar, P., Mosayebzadeh, A., Maleki Dizaj, S., & Sharifi, S. (2024). Inhibitory effects of chlorhexidine-loaded calcium carbonate nanoparticles against dental implant infections. *Bmc Oral Health*, 24, 1236. <https://doi.org/10.1186/s12903-024-05035-6>
15. Guanipa Ortiz, M. I., Gomes de Oliveira, S., de Melo Alencar, C., Aguiar, B., & Lima, F. H., D (2024). Remineralizing effect of the association of nano-hydroxyapatite and fluoride in the treatment of initial lesions of the enamel: A systematic review. *Journal of Dentistry*, 145, 104973. <https://doi.org/10.1016/j.jdent.2024.104973>
16. Gümüştas, B., Birant, S., & Şişmanoğlu, S. (2024). Ultrastructural investigation of the effect of toothpastes containing different remineralizing agents on demineralized enamel. *Journal of Esthetic and Restorative Dentistry*, 36(4), 663–672. <https://doi.org/10.1111/jerd.13150>
17. Hassan, N. M., Jafar, Z. J., & Abdul Latif, M. H. (2023). Nano-hydroxyapatite preparation for the remineralization of primary tooth enamel surface subjected to liquid medication: An observational study. *Health Science Reports*, 6(4), e1188. <https://doi.org/10.1002/hsr2.1188>
18. Huang, C., Lu, J., & Wang, J. (2020). Preparation and characterisation of calcium carbonate nanoparticles with controllable size and morphology using a colloidal gas aphrons technique. *Nanoscale Research Letters*, 15, 74. <https://doi.org/10.1186/s11671-020-03294-z>
19. Juntavee, A., Juntavee, N., & Hirunmoon, P. (2021). 'Remineralization potential of nanohydroxyapatite toothpaste compared with tricalcium phosphate and fluoride toothpaste on artificial carious lesions', *BioMed Research International*, 2021, 5588832. <https://doi.org/10.1155/2021/5588832>
20. Karthikeyan, B. S., & Mahalaxmi, S. (2024). Biomimetic dentin remineralization using eggshell derived nanohydroxyapatite with and without carboxymethyl chitosan: An in vitro study. *International Journal of Biological Macromolecules*, 270, 132359. <https://doi.org/10.1016/j.ijbiomac.2024.132359>
21. Kasi, S. R., Özcan, M., & Feilzer, A. J. (2022). Side effects of sodium lauryl sulfate applied in toothpastes: A scoping review. *American Journal of Dentistry*, 35(2), 84–88.
22. Klaophimai, A., Tosrisawatkasem, O., & Horsophonphong, S. (2024). Antibacterial effects of children's and adults' toothpastes containing different amounts of fluoride: An in vitro study. *Journal of Dental Research Dental Clinics Dental Prospects*, 18(1), 23–28. <https://doi.org/10.34172/joddd.40705>
23. Kumar, G., Virmani, T., Pathak, K., Kamaly, A., & Saleh, O., A (2022). Central Composite Design implemented azilsartan medoxomil-loaded nanoemulsion to improve its aqueous solubility and

- intestinal permeability: In vitro and ex vivo evaluation. *Pharmaceuticals*, 15(11), 1343. <https://doi.org/10.3390/ph15111343>
24. Latif, S. K., Fauziah, E., Budiardjo, S. B., & Latief, F. D. E. (2026). Chicken eggshell paste versus CPP-ACP for enamel remineralization: a pilot in vitro study using micro-computed tomography. *The Saudi Dental Journal*, 38(6), 86. <https://doi.org/10.1007/s44445-026-00176-6>
 25. Lopes, L. Q. S., et al. (2024). Chemical composition, cytotoxicity, antimicrobial, antibiofilm, and anti-quorum sensing potential of Mentha piperita essential oil against the oral pathogen Streptococcus mutans. *Journal of Toxicology and Environmental Health Part A*, 87(20), 824–835. <https://doi.org/10.1080/15287394.2024.2375731>
 26. Mkhize, S. C., Onwubu, S. C., Mokhothu, T. H., Mdluli, P. S., & Mishra, A. K. (2023). Comparative assessment of the remineralization characteristics of nano-hydroxyapatite extracted from fish scales and eggshells. *Journal of Applied Biomaterials and Functional Materials*, 21, 22808000231180390. <https://doi.org/10.1177/22808000231180390>
 27. Podder, S., & Mukherjee, S. (2024). Response surface methodology (RSM) as a tool in pharmaceutical formulation development. *Asian Journal of Pharmaceutical and Clinical Research*, 17(11), 18–25. <https://doi.org/10.22159/ajpcr.2024v17i11.52149>
 28. Polyakova, M., Sokhova, I., Doroshina, V., Arakelyan, M., Novozhilova, N., & Babina, K. (2022). The effect of toothpastes containing hydroxyapatite, fluoroapatite, and Zn-Mg-hydroxyapatite nanocrystals on dentin hypersensitivity: A randomized clinical trial. *Journal of International Society of Preventive & Community Dentistry*, 12(2), 252–259. https://doi.org/10.4103/jispcd.JISPCD_333_21
 29. Reddy, D., Selvan, A., Paul, S. T., & Azher, U. (2021). Antimicrobial efficacy of commercially available low-fluoride and fluoride-free dentifrices for children. *International Journal of Clinical Pediatric Dentistry*, 14(2), 183–186. <https://doi.org/10.5005/jp-journals-10005-1915>
 30. Sevagaperumal, A., Joel, J. R., & Periyasamy, S. (2024). Formulation and evaluation of characteristics, remineralization potential, and antimicrobial properties of toothpaste containing nanohydroxyapatite and nanosilver particles: An in vitro study. *International Journal of Clinical Pediatric Dentistry*, 17(6), 630–636. <https://doi.org/10.5005/jp-journals-10005-2855>
 31. Shehani, A. F., & Ramar, K. (2024). 'Comparative evaluation of the remineralizing potential of flaxseed paste, chicken eggshell paste and fluoride toothpaste on the enamel of primary teeth using scanning electron microscopy-energy dispersive X-ray analysis: an in vitro study', *Cureus*, 16. <https://pmc.ncbi.nlm.nih.gov/articles/PMC11162647/>
 32. Shu, W., Gong, J., Zhang, H., Zheng, F., Zeng, J., Wang, X., Qin, S., Zhang, C., Xue, H., & Dai, B. (2024). Nano-calcium carbonate with core–shell structure was prepared by dopamine chelation using Pluronic F-127 as template. *Particle & Particle Systems Characterization*, 41, 2300199. <https://doi.org/10.1002/ppsc.202300199>
 33. Thakur, P., et al. (2024). Synthesis of pectin and eggshell biowaste-mediated nano-hydroxyapatite, their physicochemical characterizations, and use as antibacterial material. *Applied Biochemistry and Biotechnology*, 196(1), 491–505. <https://doi.org/10.1007/s12010-023-04550-6>

34. Torres-Mansilla, A., Álvarez-Lloret, P., Fernández-Penas, R., D’Urso, A., Baldión, P. A., Oltolina, F., Follenzi, A., & Gómez-Morales, J. (2023). Hydrothermal transformation of eggshell calcium carbonate into apatite micro-nanoparticles: Cytocompatibility and osteoinductive properties. *Nanomaterials*, 13(16), 2299. <https://doi.org/10.3390/nano13162299>

35. Wang, Y., et al. (2024). Nano hydroxyapatite-silica with a core-shell structure for long-term management of dentin hypersensitivity. *iScience*, 27(12), 111474. <https://doi.org/10.1016/j.isci.2024.111474>

Tables

Table 1. Central-composite design matrix showing the factors (A: ENPs, B: peppermint oil, C: clove oil) and antimicrobial response.

Std	Run	A: ENPs (g)	B: Peppermint oil (ml)	C: Clove oil (ml)	Antimicrobial (mm)
10	1	2.3409	1.5	1.5	10
6	2	2.0000	1.0	2.0	13
7	3	1.0000	2.0	2.0	15
2	4	2.0000	1.0	1.0	8
12	5	1.5000	2.3409	1.5	8
3	6	1.0000	2.0	1.0	15
13	7	1.5000	1.5	0.6591	18
11	8	1.5000	0.6591	1.5	9
14	9	1.5000	1.5	2.3409	18
8	10	2.0000	2.0	2.0	15
4	11	2.0000	2.0	1.0	13
5	12	1.0000	1.0	2.0	22
1	13	1.0000	1.0	1.0	20
9	14	0.6591	1.5	1.5	18

Table 2. Model summary for the square-root-transformed antimicrobial response.

Source	Overall model p-value	Adjusted R ²	Predicted R ²
Linear	0.1691	0.1968	-0.2017
2FI	0.2888	0.2022	-0.0128
Quadratic	0.0053	0.9309	0.7413

Table 3. ANOVA for the quadratic model (response: $\sqrt{\text{Antimicrobial}}$). Term SS values marked * are partial (Type III) and are not additive; lack of fit is not estimable because no replicate runs were available.

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	4.7248	9	0.5250	20.45	0.0053
A–ENPs	1.6962*	1	—	66.08	0.0012
B–Peppermint oil	0.0320*	1	—	1.24	0.3270
C–Clove oil	0.1168*	1	—	4.55	0.0999
AB	0.7572*	1	—	29.50	0.0056
AC	0.0853*	1	—	3.32	0.1423
BC	0.0662*	1	—	2.58	0.1834
A ²	0.1932*	1	—	7.53	0.0517
B ²	0.1233*	1	—	4.81	0.0935
C ²	0.2501*	1	—	9.74	0.0355
Residual	0.1027	4	0.0257	—	—
Lack of fit	Not estimable	—	—	—	—
Pure error	Not estimable	0	—	—	—
Cor Total	4.8275	13	—	—	—

Model fit statistics: R² = 0.9787; adjusted R² = 0.9309; predicted R² = 0.7413; residual SD = 0.1602; mean transformed response = 3.7528; CV = 4.27%; Adequate Precision = 13.36.

Table 4. Regression coefficients in coded factors. Quadratic terms are mean-centred as A²–0.9755, B²–0.9755 and C²–0.9755.

Term	Coefficient	Std. Error	95% CI Low	95% CI High	VIF	p-value
Intercept	3.7528	0.0428	3.6340	3.8717	—	<0.0001
A–ENPs	−0.3524	0.0434	−0.4728	−0.2321	1.00	0.0012
B–Peppermint oil	−0.0484	0.0434	−0.1687	0.0720	1.00	0.3270
C–Clove oil	0.0925	0.0434	−0.0279	0.2128	1.00	0.0999
AB	0.3076	0.0566	0.1504	0.4649	1.00	0.0056
AC	0.1033	0.0566	−0.0540	0.2606	1.00	0.1423
BC	−0.0910	0.0566	−0.2483	0.0663	1.00	0.1834
A ²	1.3866	0.5054	−0.0167	2.7899	1.20	0.0517
B ²	1.1079	0.5054	−0.2954	2.5112	1.20	0.0935
C ²	1.5776	0.5054	0.1743	2.9809	1.20	0.0355

Table 5. Descriptive sensory evaluation of the selected F12 toothpaste formulation over 14 days.

Day	Colour	Aroma	Texture	Overall Acceptance (%)
Day 1	3.0	3.0	4.0	65%
Day 7	4.0	3.5	4.0	80%
Day 14	4.0	4.0	4.0	90%

Scale: 1 = very poor, 2 = poor, 3 = fair, 4 = good, 5 = very good. Overall Acceptance (%) = (panellists scoring ≥ 4 / total panellists) $\times$ 100. The available sensory dataset contained aggregate scores rather than individual-level repeated ratings; therefore, the table is descriptive and no inferential repeated-measures test is reported.

Table 6. Physicochemical test results for the optimised toothpaste formulation F12 (mean $\pm$ SD, n = 3).

Parameter	Unit / Method	Value
pH	Digital pH meter (1:2 w/w suspension)	8.2 $\pm$ 0.1
Moisture content	% (loss on drying at 105 °C)	2.5 $\pm$ 0.3
Solubility	% (water-soluble fraction)	28.4 $\pm$ 1.2

Figures

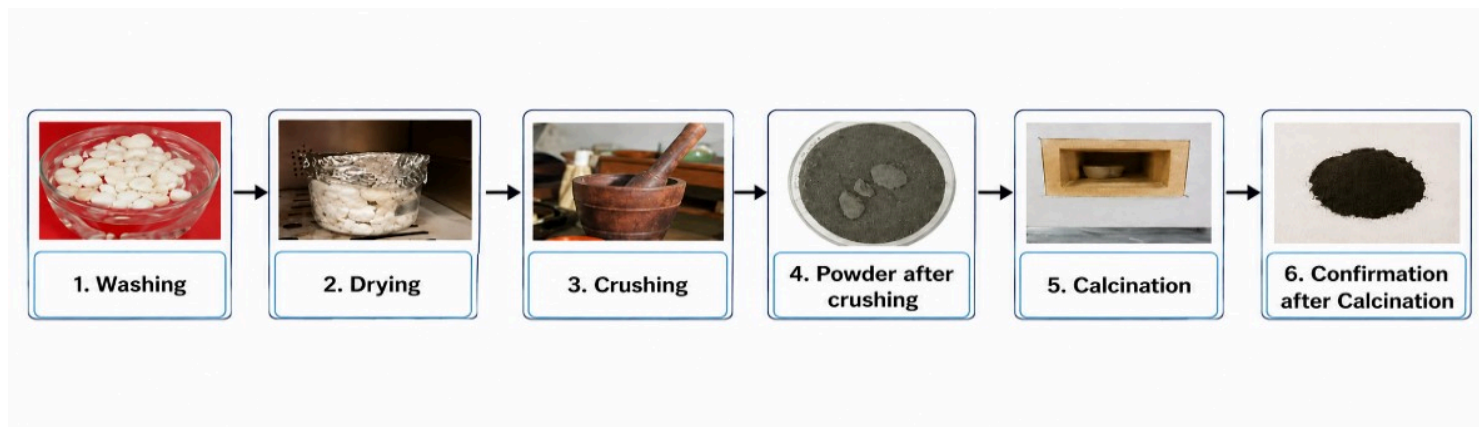


Figure 1

Sequential preparation process showing washing, drying, crushing, calcination to a CaO-rich intermediate, and recovery of the final calcite-dominant eggshell-derived calcium carbonate material after aqueous processing.

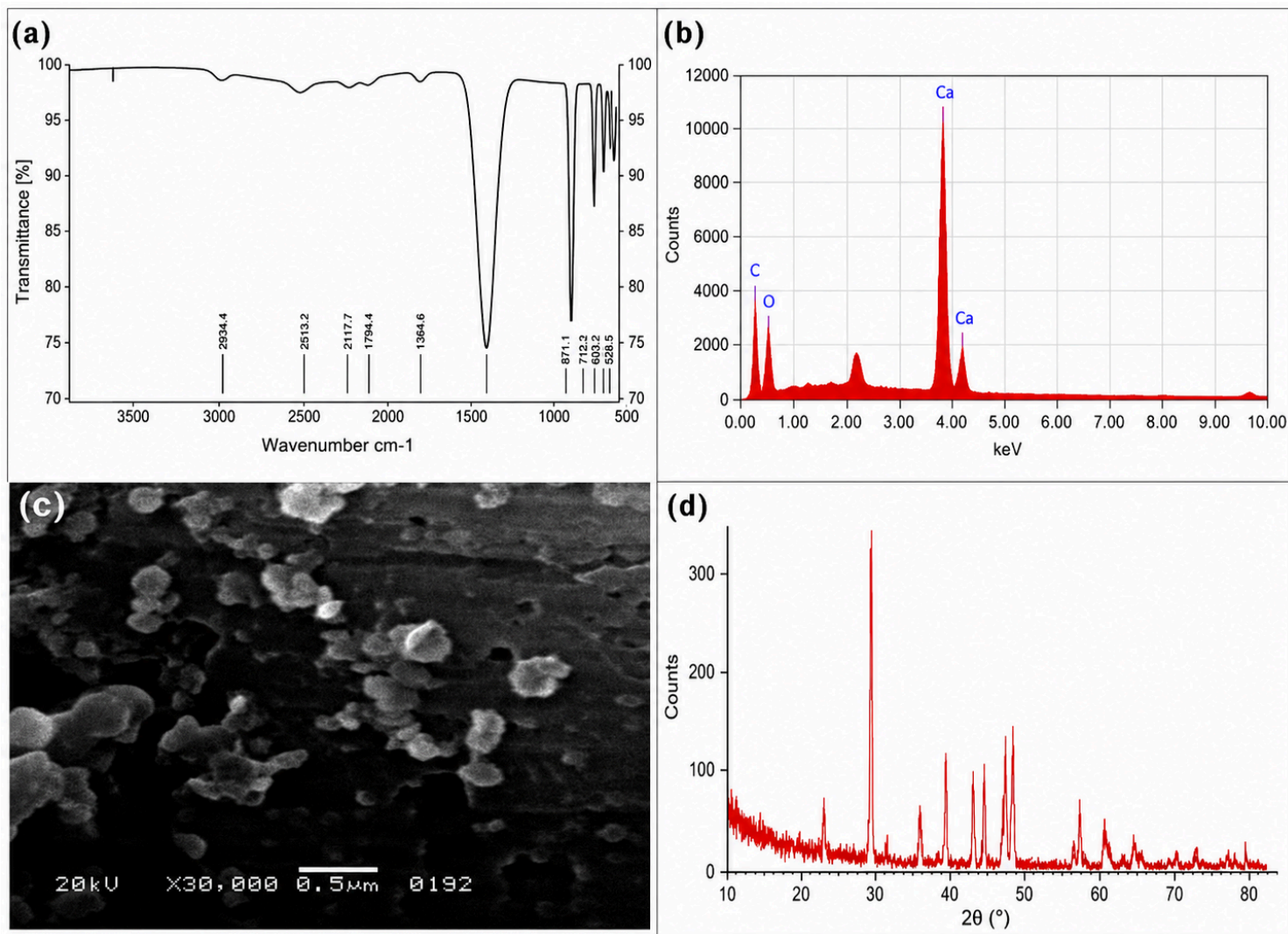


Figure 2

Characterisation of the final eggshell-derived calcium carbonate material: A) FTIR spectrum, B) EDS spectrum, C) SEM micrograph, and D) XRD pattern.

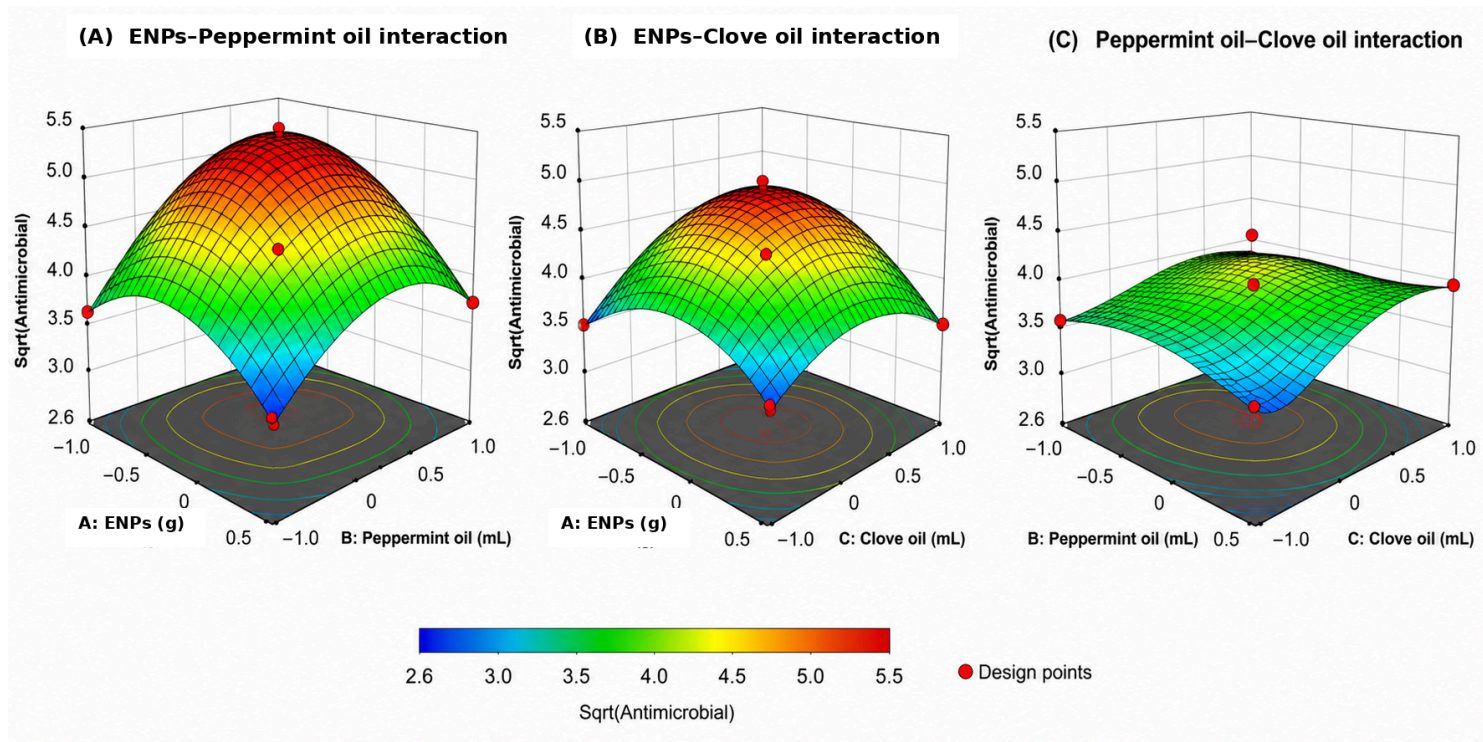


Figure 3

Response-surface plots showing the combined effects of ENPs, peppermint oil and clove oil on the antimicrobial response.

Sqrt(Antimicrobial)

Color points by value of
Antimicrobial:

2.828 4.690

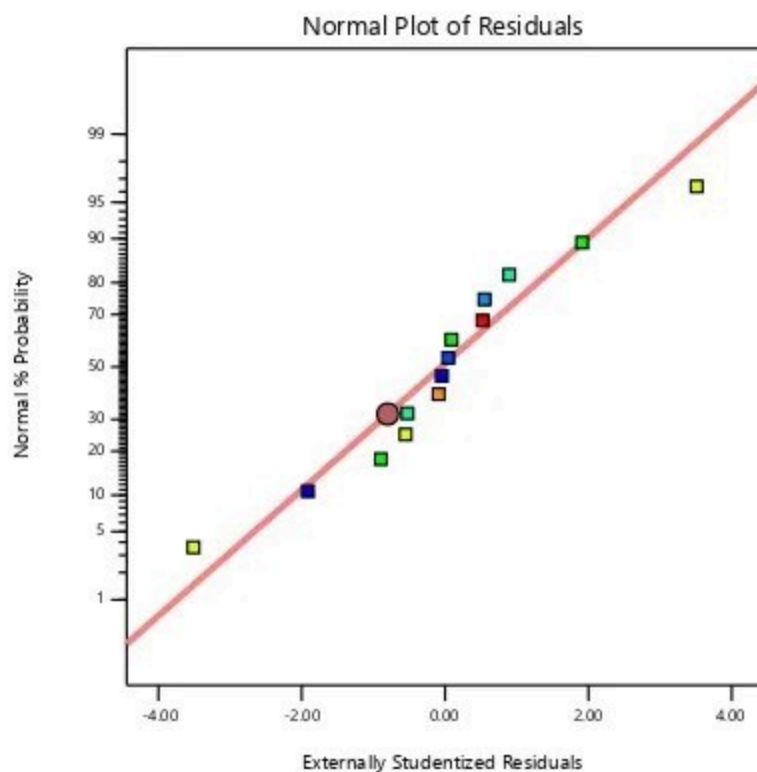


Figure 4

Normal-probability plot of residuals for the square-root-transformed antimicrobial response; interpreted cautiously because residual df = 4 and no lack-of-fit test was estimable.

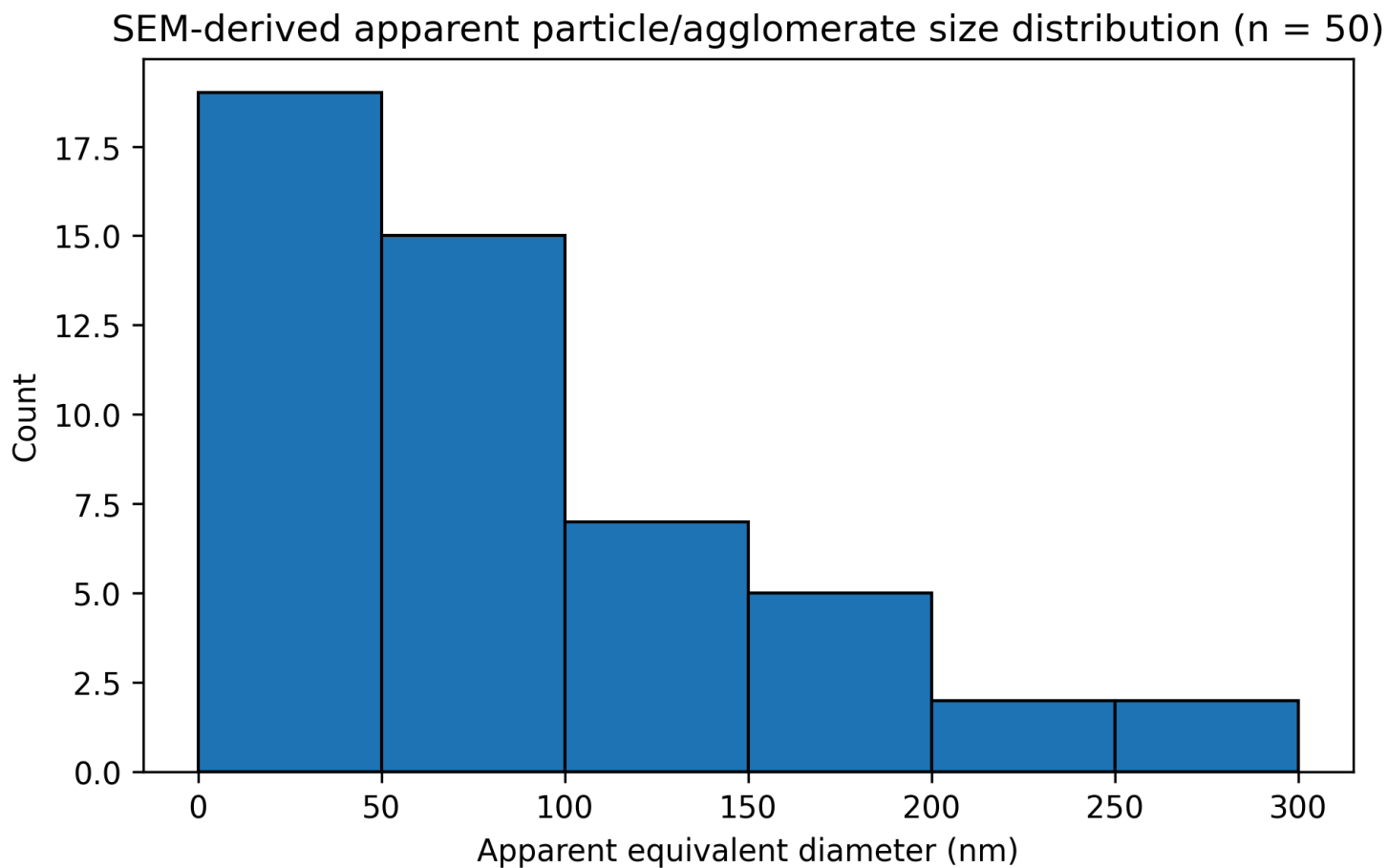


Figure 5

SEM-derived apparent particle/agglomerate size distribution from 50 digitally segmented features in the representative micrograph. Median = 67.6 nm; mean = 88.7 ± 64.8 nm; range = 29.7–268.9 nm. The plot is a semi-quantitative dry-image estimate and does not replace DLS/TEM or zeta-potential analysis.