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Remineralization Potential of Nanoemulsion-Based Amorphous Calcium Phosphate and Essential Oils on Demineralized Enamel: Invitro Study

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Abstract

Background: Dental caries remains one of the most prevalent chronic diseases worldwide, driven by a complex interplay between acidogenic bacteria, fermentable carbohydrates, and susceptible tooth surfaces. Traditional fluoride-based therapies are effective, but face concerns related to overexposure and antimicrobial resistance. Consequently, there is growing interest in biocompatible, natural alternatives such as amorphous calcium phosphate (ACP) and essential oils (EOs). This study evaluates the remineralization potential of thyme essential oil incorporating ACP nanoparticles in a novel formulated nanoemulsion on demineralized human enamel.

Methods: Sixty extracted human premolars and molars were demineralized and divided into three treatment groups (n=20) based on duration of exposure to the thyme nanoemulsion: 30 minutes, 8 hours, and 8 hours/day for 7 days. Remineralization efficacy was assessed through microhardness testing and scanning electron microscopy (SEM). Nanoemulsions were formulated with ACP, vitamin D3, and thyme essential oil and characterized using dynamic light scattering

Results: SEM and microhardness analyses showed time-dependent remineralization. Short-term exposure resulted in partial mineral deposition, while extended treatment (7 days) yielded near-complete enamel surface restoration. Thyme oil's antimicrobial properties likely contributed to a favorable remineralization environment by suppressing acidogenic bacteria. The nanoemulsion system enhanced the bioavailability and penetration of thyme oil and ACP nanoparticles.

Conclusion: The ACP-thyme oil nanoemulsion demonstrated significant remineralization capability, especially with prolonged application. This formulation provides a hopeful non-invasive way to fix early enamel damage by using both remineralizing and antimicrobial effects. Further clinical studies are recommended to validate its long-term efficacy and stability.

1 Introduction

Dental caries, one of the most widespread chronic diseases, continues to challenge clinicians and researchers despite decades of preventive measures and technological advancements. The dynamic interplay between acid-producing bacteria, fermentable carbohydrates, and susceptible tooth surfaces initiates the demineralization process, compromising enamel integrity. Left unchecked, this process leads to cavitation and potential tooth loss.[1] Although fluoride-based therapies have been pivotal in reducing caries prevalence, increasing awareness about fluoride overexposure and antimicrobial resistance has driven the search for safer, biocompatible, and equally effective remineralizing agents. [2]

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The shift in paradigm from restorative approaches to preventive and regenerative strategies has intensified research into nanotechnology and bioactive natural compounds. [3] Amorphous calcium phosphate nanoparticles (ACP-NPs) have emerged as a leading candidate for enamel repair, owing to their excellent biocompatibility and their ability to rapidly release calcium and phosphate ions—key minerals in enamel regeneration. Unlike crystalline hydroxyapatite, ACP offers greater solubility and reactivity, making it particularly suitable for early enamel lesion reversal. [4]

Complementing the mineralizing action of ACP, essential oils derived from medicinal plants have shown notable antimicrobial, anti-inflammatory, and antioxidant properties. Essential oils possess bioactive compounds capable of disrupting bacterial cell membranes and scavenging free radicals. [5-8] However, essential oils are typically hydrophobic and unstable in aqueous formulations. To overcome this limitation, the development of nanoemulsions has facilitated their dispersion, stability, and bioavailability. [9]

Zaher *et al.* [10] demonstrated that the addition of essential oils to calcium phosphate ($CaPO_4$)-coated titanium (Ti) dental implants will enhance corrosion resistance in simulated oral environments. The dental implant surface stability was greatly enhanced by a $CaPO_4$ coating, which consists of amorphous calcium phosphate nanoparticles (ACP) and is then combined with coriander, thyme, and cumin essential oils. Electrochemical tests like linear polarization and EIS results showed that essential oils and $CaPO_4$ coatings can improve dental implants' durability, bioactivity, and corrosion-resistant properties. These findings highlighted the potential of ACP-essential oil nanoemulsions as multifunctional bioactive agents capable of enhancing material performance.

Building on this success, the present study investigates the remineralization potential of thyme oil nanoemulsion on demineralized human enamel due to its superior remineralization effect demonstrated in previous investigations. Studies have shown that thyme-based nanoemulsions significantly enhance calcium and phosphate ion penetration into enamel lesions. [11-13]

Using microhardness testing and scanning electron microscopy (SEM), the present study evaluates the potential of ACP-essential thyme oil nanoemulsions to restore mineral content and enamel surface morphology. This investigation aims to bridge the gap between antibacterial protection and enamel regeneration, thereby positioning nanoemulsions as promising candidates for non-invasive caries management and preventive dental care.

2 Materials and Methods:

2.1 Material

2.1.1 Preparation of Amorphous Calcium Phosphate Nanoparticles and Nanoemulsions

ACP nanoparticles were synthesized via precipitation of calcium nitrate and potassium hydrogen phosphate, with PH adjustment to 8 using ammonium hydroxide. The precipitate was washed and dried at room temperature. Nanoemulsions were prepared by sonicating a mixture of ACP nanoparticles, paraffin oil, vitamin D3, and thyme essential oil using a Sonics Vibra Cell at 40°C (30s on/20s off) for two minutes to achieve nanoscale dispersion. [10]

The nanoemulsion formulation used in this study was originally developed and optimized by our research team in a previous investigation. This innovative formulation has been officially registered by the Egyptian patent office, EGPO (No. 20250321000004). The registration confirms the originality of the emulsion and establishes our ownership of the intellectual property associated with this formulation.

.1.2 Particle Size Characterization

Dynamic Light Scattering (DLS) via the NICOMP particle sizing system confirmed the average particle size and polydispersity index (PI). Thyme nanoemulsion demonstrated the mean particle sizes ranging from ~239 nm to ~640 nm with relatively low PI values, confirming good dispersion of the sample.

2.2 Sample Selection and Ethical Approval

2.2.1 Sample Size Calculation

According to Ghanim Rahman and Diab[14], a total sample size of 60 samples was sufficient to detect a power (1-β) of 80% at a significant level of 5% ($p<0.05$). The total samples (60 samples) were measured before and after demineralization, and then the samples were divided into three groups according to remineralization time, where each group was represented by 20 samples. The sample size was calculated according to G*Power software version 3.1.9.4. Where fS is the effect size, $\alpha = 0.05$, $\beta = 0.2$, and power = $1 - \beta = 0.8$.

2.2.2 Ethical Approval

A clarification letter was obtained from the Research Ethics Committee (REC) of the Faculty of Dentistry, MSA University, confirming post-conduction approval of the study. It was also confirmed that all patients attending the clinics had previously provided written informed consent—documented in their files—allowing the use of any clinical samples, extracted teeth, photographs, or radiographs for research purposes.

2.2.3 Sample Selection

A total of 60 freshly extracted human premolars and molars were collected from the Oral Surgery Department, following informed consent and in accordance with the ethical guidelines of MSA University. The teeth were stored in a 0.1% thymol solution at 4°C and used immediately.

Inclusion criteria:

- Intact teeth without visible caries, cracks, hypoplasia, or restorations.
- Premolars or molars extracted for orthodontic or periodontal reasons.

Exclusion criteria:

- Teeth with developmental defects, structural abnormalities, large restorations, carious lesions, or cracks.

2.3 Study Design

2.3.1 Sample Preparation and Demineralization

The teeth were thoroughly cleaned of any soft tissue debris and polished using non-fluoridated pumice. Each tooth was then embedded in an acrylic resin block, ensuring that the buccal enamel surface remained exposed. Baseline microhardness measurements were recorded on the sound enamel surfaces. Subsequently, the specimens were subjected to 37% phosphoric acid gel for one minute to simulate the formation of carious lesions.

2.3.2 Remineralization Treatment Protocol

Sixty teeth were demineralized, and for remineralization, teeth were divided into three groups (n = 20 each):

- Group A: Each tooth was immersed in thyme nanoemulsion for 30 minutes for short-term remineralization.
- Group B: Each tooth was immersed in thyme nanoemulsion for 8 hours for extended single-session remineralization.
- Group C: Each tooth was immersed in thyme nanoemulsion for 8 hours/day for 7 consecutive days for prolonged remineralization between treatments; samples were stored in artificial saliva at 37°C.

2.3.3 Artificial Saliva Composition

Artificial saliva used for specimen storage was *Xerostom*®, a commercially available formulation. Its composition includes purified water (aqua), xylitol, PEG-40 hydrogenated castor oil, propylene glycol, glycerin, betaine, *Olea europaea* (olive) fruit oil, panthenol, tocopheryl acetate (vitamin E acetate), allantoin, disodium phosphate, sodium phosphate, aroma (flavoring agents), hydroxyacetophenone, 1,2-hexanediol, caprylyl glycol, rebaudioside A, sucralose, *Carum petroselinum* (parsley) seed oil, citral, and limonene. The pH of the formulation was adjusted to 6.8 to approximate physiological oral conditions.

2.4 Sample Assessment

2.4.1 Microhardness Testing

Microhardness was assessed using a Vickers hardness tester under a 200 g load for 15 seconds:

- Before demineralization (baseline)
- After demineralization
- After each remineralization period (30 min, 8 hours, and 8 hrs/day x 7 days)

For each sample, **3 indentations spaced 100 μm apart** were made and averaged.

2.4.2 Scanning Electron Microscopy (SEM)

After completion of the remineralization, the specimens were rinsed gently with deionized water to remove any residual solution and air-dried. The enamel blocks were then sectioned to obtain flat surfaces when necessary and mounted onto aluminum stubs using conductive carbon adhesive tape to ensure proper fixation. To enhance electrical conductivity and minimize charging during imaging, the samples were sputter-coated with a thin layer of gold using a sputter coater for approximately 60 seconds. SEM analysis was subsequently performed under high-vacuum conditions at an accelerating voltage appropriate for enamel evaluation, and photomicrographs were captured at various magnifications to document surface characteristics, mineral deposition, and morphology changes across groups.

2.4.3 Statistical Analysis

The mean and standard deviation values were calculated for each group for each test. Data were explored for normality using Kolmogorov-Smirnov and Shapiro-Wilk tests; data showed parametric (normal) distribution.

The paired sample t-test was used to compare two groups in related samples. One-way ANOVA followed by a Tukey post hoc test was used to compare more than two groups in non-related samples. A repeated measure ANOVA followed by a paired sample t-test was used to compare more than two groups in related samples.

The significance level was set at $P < 0.05$. Statistical analysis was performed with IBM® SPSS® Statistics Version 25 for Windows.

3 Results

3.1 Scanning electron microscope results

3.1.1 Normal Enamel - Control Group

The SEM image shows sound enamel surfaces exhibited a characteristic smooth and continuous pattern with no visible porosities or defects. The enamel surface appeared dense and homogeneous, and prism outlines were subtle and intact. There was no noticeable loss of minerals. This microstructure is indicative of well-mineralized, healthy enamel. **(Fig. 1a)**

3.1.2 Demineralized Enamel

Following demineralization, SEM images showed clear surface degradation. Irregular depressions, porosities, and loss of interprismatic substance were visible. The surface became uneven with micropores indicative of mineral loss. These observations confirm the successful simulation of carious-like lesions. **(Fig. 1b)**

3.1.3 Remineralized Enamel

A. After 30 Minutes of Remineralization.

Initial mineral deposition was visible as isolated and scattered clusters of mineral particles adhering to the demineralized enamel surface. Although the general porosity of the surface was still apparent, some of the voids began to show partial occlusion by mineral deposits. The surface appeared heterogeneous, indicating that remineralization had started but was incomplete at this early stage. **(Fig. 1c)**

B. After 8 Hours of Remineralization

A more advanced stage of remineralization was noted. The previously scattered mineral clusters became more uniformly distributed across the surface. Numerous granular and globular mineral deposits were now seen filling surface irregularities and narrowing porosities. The surface was smoother compared to the 30-minute group, suggesting progressive mineralization. **(Fig. 1d)**

C. After 8 Hours/Day for 7 Days

At this stage, the enamel surface demonstrated significant remineralization. The mineral coating appeared nearly continuous, with most pores and surface depressions being filled or covered. The texture of the enamel resembled that of the control group, indicating near-complete restoration of enamel surface characteristics. Crystal formations were more densely packed and uniformly layered. **(Fig. 1e)**

3.2 Microhardness evaluation

3.2.1 Effect of Demineralization

A statistically significant difference was observed between the enamel before and after demineralization ($p < 0.001$). The mean value was highest in the untreated (control) enamel and lowest after demineralization, indicating a marked loss of mineral content. **(Fig. 2, Table 1)**

3.2.2 Effect of Remineralization after 30 Minutes

After 30 minutes of remineralization, the enamel showed a statistically significant improvement compared to the demineralized group ($p < 0.001$). However, the value remained significantly lower than the control enamel ($p = 0.003$). **(Fig. 2, Table 1)**

3.2.3 Effect of Remineralization after 8 Hours

Remineralization for 8 hours significantly improved enamel values compared to the demineralized group ($p < 0.001$), but no significant difference was found compared to the control enamel ($p = 0.785$). **(Fig. 2, Table 1)**

3.2.4 Effect of Remineralization after 7 Days

After 7 days, enamel values were significantly higher than the demineralized group ($p < 0.001$), and no significant difference was found between this group and the control enamel ($p = 0.964$), indicating near-complete remineralization. **(Fig. 2, Table 1)**

3.2.5 Comparison Between Remineralization Intervals

A statistically significant difference was found between the three remineralization time points (30 minutes, 8 hours, 7 days) ($p < 0.001$). The highest mean value was in the 7-day group, followed by 8 hours, and the lowest in the 30-minute group. **(Fig. 2, Table 1)**

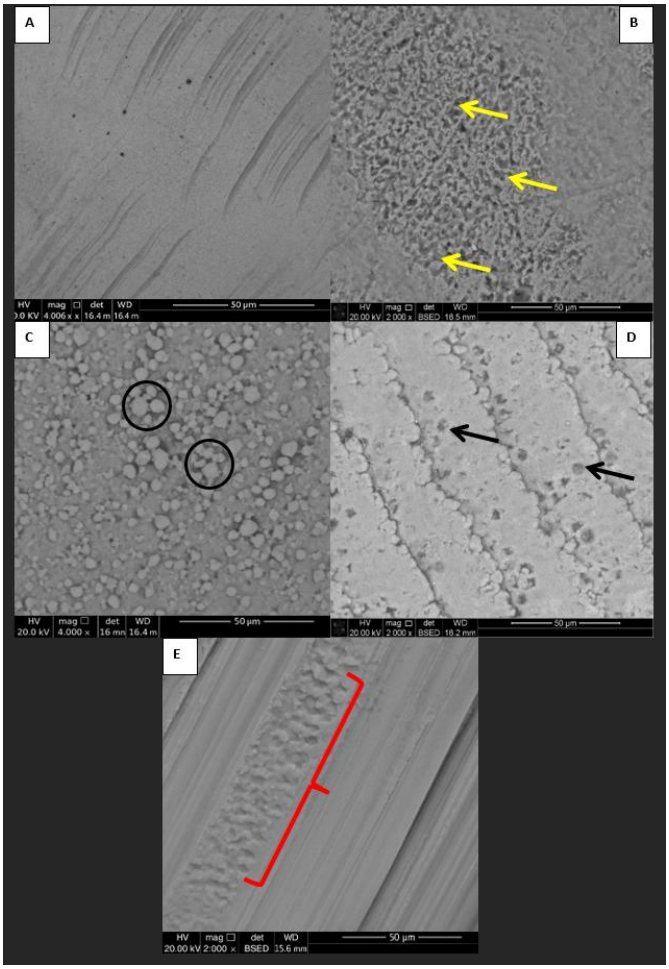

Figure 1. SEM image showing A) enamel surface appeared dense and homogeneous, and prism outlines were subtle and intact, B) enamel surface became uneven with micropores (yellow arrows) indicative of mineral loss, C) Initial mineral deposition was visible as isolated and scattered clusters of mineral particles adhering to the demineralized enamel surface (black circles), D) A more advanced stage of remineralization was noted. The previously scattered mineral clusters became more uniformly distributed across the surface, micropores are still noted (black arrows), E) the enamel surface demonstrated significant remineralization. The mineral coating appeared nearly continuous, with most pores and surface depressions being filled or covered, some areas are still uneven (red curly bracket)

Table 1. The mean, standard deviation (SD) values of Microhardness of different groups. (A repeated measure ANOVA followed by a Paired sample t-test) and (One-way ANOVA followed by a Tukey post hoc test)

	Before Demineralization	After Demineralization	After remineralization			*p-value*
			30 min	8 hours	7 days	
1	133.24 ±10.74	107.08±8.61	----------	----------	----------	<0.001*
2	133.24 ±10.74	107.08±8.61	124.58±2.66	----------	----------	<0.001*
			----------	132.56±2.76	----------	<0.001*
			----------	----------	134.17±3.17	<0.001*
3	----------	----------	124.58±2.66	132.56±2.76	134.17±3.17	<0.001*

Means with different small letters in the same column indicate significant difference. *; significant (p<0.05)

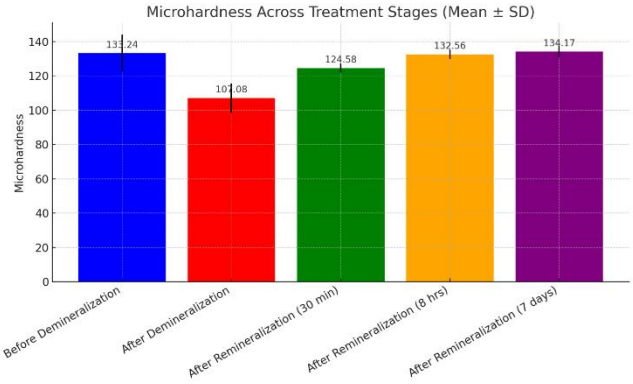

Figure 2. The bar chart represents the mean microhardness values (± standard deviation) across different treatment stages. Each bar represents a treatment stage, with error bars showing the standard deviation

4 Discussion

The present study demonstrated that nanoemulsion-based amorphous calcium phosphate (ACP) nanoparticles combined with thyme essential oil (EO) exhibit significant potential as a remineralization agent for demineralized enamel. Both microhardness measurements and scanning electron microscopy (SEM) analyses confirmed progressive mineral deposition and enamel surface restoration, which intensified with increased treatment duration.

Notably, mineral uptake was evident even after a short exposure period of 30 minutes, emphasizing the high solubility and reactivity of ACP. This is consistent with earlier reports by Cochrane *et al.*[15] and Fan *et al.*[16], who highlighted the amorphous state of ACP as a facilitator of rapid calcium and phosphate ion release—an essential prerequisite for early-stage enamel repair.

As the exposure duration increased, our findings revealed more uniform and extensive mineral deposition. The 8-hour and 7-day groups exhibited significant improvements in surface morphology and hardness, aligning with Karthikeyan *et al.*[17], who demonstrated that extended application of ACP allows deeper penetration of mineral ions and enhances lesion repair.

An important advantage of the current formulation lies in the incorporation of thyme essential oil. Recently interest has grown in natural bioactive agents for dental applications, largely due to concerns over fluoride overexposure and antimicrobial resistance. Essential oils such as thyme are recognized for their potent antimicrobial properties. This is supported by Bassolé and Juliani [18] and more recently by Mohamed

and Ashraf [19], who demonstrated that natural compounds could suppress acidogenic bacteria and support remineralization processes. In our study, the continuous remineralization observed over the 7-day treatment period may be partly attributed to this antimicrobial action, which likely minimized bacterial acid production and created a favorable environment for mineral deposition.

The remineralization potential of thyme oil has been supported by several recent studies emphasizing its dual antimicrobial and enamel-restorative effects. de Lima *et al.*[12] demonstrated that fluoride varnishes enriched with thyme essential oil significantly reduced enamel demineralization, attributing the effect to the enhanced antibacterial activity and the modulation of the local microenvironment that favors remineralization. Similarly, Sharma *et al.*[13] highlighted the promising role of thyme oil among natural products capable of aiding enamel repair, especially when incorporated into delivery systems that improve bioavailability.

Moreover, the nanoemulsion delivery system used in this study played a crucial role in ensuring the effective dispersion and bioavailability of the hydrophobic essential oils. Our results corroborate those of Salvia-Trujillo *et al.*[20], who demonstrated that nanoemulsions enhance the stability and solubility of essential oils, allowing for more uniform interaction with biological substrates such as enamel surfaces.

When comparing our findings to more traditional remineralization systems such as casein phosphopeptide-amorphous calcium phosphate (CPP-ACP), interesting parallels and distinctions emerge. For example, Dhinsa et al.[21] reported that CPP-ACP, silver diamine fluoride, and hydroxyapatite effectively remineralized early carious lesions. However, unlike our ACP-EO nanoemulsion, those systems lack antimicrobial action. The dual role of our formulation combining remineralization and bacterial suppression represents a valuable advantage in modern non-invasive caries management strategies.

Importantly, our results extend previous observations by demonstrating that ACP combined with essential oils within a nanoemulsion framework can achieve near-complete restoration of enamel surface morphology, as seen after the 7-day treatment. This level of recovery compares favorably to the findings of Tschoppe *et al.*[22], who used nano-hydroxyapatite pastes. However, our formulation offers the added benefit of antibacterial protection, suggesting a synergistic effect between mineralizing and bioactive natural compounds.

Despite these promising outcomes, certain limitations were observed. Although surface hardness significantly improved, complete restoration to baseline enamel hardness was not achieved, even after prolonged treatment. This finding is in line with Zhou *et al.* [23], who reported that while ACP can effectively fill surface irregularities, subsurface mineral density recovery remains challenging and may require additional ionic support or complementary agents.

Furthermore, it is necessary to acknowledge contrasting reports in the literature. Some studies have questioned the standalone remineralization efficacy of EO-based nanoemulsions. For example, Batista *et al.* [24], in their evaluation of eucalyptus EO-based nanoemulsions, reported potent antimicrobial activity but limited remineralization capability. Such findings indicate that while essential oils contribute positively to the antimicrobial aspect, they may not independently drive mineral regeneration as effectively as fluoride-based formulations.

In addition, the stability of essential oils within nanoemulsions is subject to external factors such as temperature, light, and storage conditions, which may influence their clinical performance. This was emphasized by Salvia-Trujillo *et al.* [20], who underscored the necessity for optimized formulations to maintain efficacy over time.

Our study underlines the potential of ACP-essential oil nanoemulsions as non-invasive agents for early enamel lesion management, especially in patients avoiding fluoride use or requiring adjunctive antimicrobial protection. However, being in vitro study, it does not account for complex oral conditions such as salivary flow, pellicle formation, and masticatory forces, which may impact the remineralization process. Future in vivo studies are warranted to validate the durability and bioactivity of the nanoemulsion system in clinical settings.

5 Conclusion

The findings of this in vitro study support the use of ACP-thyme essential oil nanoemulsions as a safe, multifunctional, and biocompatible remineralization agent. Further in vivo studies and clinical trials are warranted to validate these results and explore the full potential of this novel formulation for preventive and restorative dental applications

Authors' Contributions:

Heba Tarek Zaher designed study ideas, and she was responsible for practical work, data analysis, and data interpretation. **Samah Mohamed Kamel (SM Kamel)** designed the study, led the manuscript writing, and interpreted the data. **Sahar Ahmed Ali Fadlallah, Shaymaa Samir Mohamed Madeny and Sherif**

Mohamed Elhefnawy were responsible for supervision, data collection, analysis, and interpretation. **Marco Wagih Naiem Kaldas** was responsible for sample collection.
All authors have read and approved the manuscript.

Conflict of interest

The authors declare that they hold no competing interests

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