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**NANO-HYDROXYAPATITE AND HYDROXYCHAVICOL'S COMBINED
EFFECT IN *Streptococcus mutans* GROWTH**

Nurdiana Dewi^{1)*}, Beta Widya Oktiani²⁾, Deby Kania Tri Putri³⁾, Aura Amelia⁴⁾, Aisyah Nur Zahra⁴⁾

¹⁾Department of Pediatric Dentistry, Faculty of Dentistry, Universitas Lambung Mangkurat, Banjarmasin, Kalimantan Selatan, Indonesia

²⁾Department of Periodontia, Faculty of Dentistry, Universitas Lambung Mangkurat, Banjarmasin, Kalimantan Selatan, Indonesia

³⁾Department of Biomedics, Faculty of Dentistry, Universitas Lambung Mangkurat, Banjarmasin, Kalimantan Selatan, Indonesia

⁴⁾Undergraduate Study Program, Faculty of Dentistry, Universitas Lambung Mangkurat, Banjarmasin, Kalimantan Selatan, Indonesia

ABSTRACT

Background: Early childhood caries is an aggressive form of dental caries that affects children under six years old. The increasing prevalence of ECC caused by *Streptococcus mutans* highlights the need for effective antimicrobial agents. **Objective:** To evaluate the antibacterial activity of a combination of nanohydroxyapatite (nHAp) and hydroxychavicol (HC) against *S. mutans*. **Methods:** 100 mg/mL nHAp was combined with HC at 0.125, 0.25, 0.5, and 1 mg/mL concentrations. DMSO 5% was used as the negative control. *S. mutans* ATCC 25175 was cultured in Brain Heart Infusion (BHI) media. Minimum Inhibitory Concentration (MIC) was determined using a UV-Vis spectrophotometer to measure absorbance differences. Minimum Bactericidal Concentration (MBC) was established by plating onto BHI agar and counting the colony count. **Results:** The results showed that MIC occurred at a combination of nHAp 100 mg/mL + HC 0.125 mg/mL, while MBC was achieved at nHAp 100 mg/mL + 1 mg/mL HC, where no bacterial colonies were observed. Statistical analysis using ANOVA and Bonferroni post-hoc tests revealed significant differences in both absorbance reduction and colony counts between groups ($p < 0.05$), confirming the dose-dependent antibacterial efficacy of the nHAp-HC combination. **Conclusion:** These findings indicate that the synergistic action of nHAp and HC offers a promising strategy for controlling ECC, particularly by targeting the growth of *S. mutans*. Further, in vivo studies are recommended to explore clinical applicability and long-term safety.

Keywords: Antibacterial, Early Childhood Caries, Hydroxyapatite, Hydroxychavicol, *Streptococcus mutans*

***Correspondence :** Nurdiana Dewi; Faculty of Dentistry, Universitas Lambung Mangkurat, Jl, Veteran No. 128B, Banjarmasin 70249, South Kalimantan, Indonesia; Email: nurdiana.dewi@ulm.ac.id

INTRODUCTION

Early childhood caries is one of the most common oral health problems among children worldwide, caused by a complex interaction between pathogenic microorganisms such as *Streptococcus mutans* (*S. mutans*).^{1,2} *S. mutans* is the primary bacterium responsible for caries, producing organic acids from carbohydrate fermentation that lead to enamel demineralization.^{3,4} An effective approach to preventing caries involves using remineralization agents that also possess antibacterial properties to inhibit the growth of pathogenic bacteria.⁵

Nanohydroxyapatite (nHAp) is widely used in dental care because it supports tooth remineralization. As a remineralization agent, nHAp can replace lost minerals in the enamel structure because of its similarity to the mineral composition of human teeth. Additionally, its nanoscale particle size allows better penetration into the microscopic pores of demineralized enamel, restoring its strength and integrity.^{6,7} However, the

antibacterial effectiveness of nHAp against *S. mutans* remains limited, necessitating enhancement to provide optimal protection against caries.⁸

Hydroxychavicol, a phenolic compound derived from betel leaf (*Piper betel*), has been shown to possess strong antibacterial properties, including against *S. mutans*.⁹ Its antibacterial mechanism involves disrupting bacterial cell membranes, inhibiting enzyme metabolism and inducing oxidative stress, effectively suppressing the growth of pathogenic bacteria.^{10,11} This study aims to evaluate the effectiveness of the nHAp-HC combination in inhibiting the growth of *S. mutans*. By assessing this combination's antibacterial properties, the study seeks to determine its potential as an innovative approach for caries prevention through robust antibacterial protection.

METHODS

This study has been approved by the Research Ethics Committee of the Faculty of Dentistry, Lambung Mangkurat University (ULM), under ethical clearance No. 019/KEPKG-FKGULM/EC/VIII/2024, and employed an experimental design with four treatment groups, consisting of a negative control group and three treatment groups. Each treatment group was replicated five times to ensure reliability and reduce random variability. The nanohydroxyapatite (nHAp) used in this study was obtained from Indonesia's National Research and Innovation Agency. The nHAp was synthesized using Snakehead (*Channa striata*) fish bones as the raw material, with a final 100 mg/mL concentration. Hydroxychavicol (Fitopure, Indonesia) was prepared in four concentrations: 0.125 mg/mL, 0.25 mg/mL, 0.5 mg/mL, and 1 mg/mL. The negative control was a 5% DMSO solution (Himedia, India).

All media and all laboratory equipment were sterilized using an autoclave at 121°C for 15–20 minutes to eliminate potential contaminants. The bacterial inoculum was prepared by obtaining *S. mutans* ATCC 25175 from the Biomedical Laboratory of the Faculty of Dentistry, Universitas Lambung Mangkurat. *S. mutans* suspension was created using a sterile tube needle to remove grown *S. mutans* bacteria from agar media and then put them in a test tube with 1 mL of Brain Heart Infusion Broth (BHIB). The inoculated media was then incubated at 37°C for 18–24 hours to allow bacterial growth under optimal conditions. The reaction mixture was diluted by adding Brain Heart Infusion Broth (BHIB) and homogenized until 2×10^6 CFU/mL.

The MIC was determined using the broth microdilution method with varying concentrations of nHAp-HC combination. 96-well microplates were prepared, with each well containing 100 μ L of BHI broth. Test solutions (nHAp 100 mg/mL combined with HC at concentrations of 0.125, 0.25 mg/mL, 0.5 mg/mL, and 1 mg/mL) were added to the wells containing bacterial suspensions standardized to 1×10^6 CFU/mL. The microplates were incubated at 37°C for 18–24 hours. After incubation, bacterial growth was measured at 450 nm using a UV-Vis spectrophotometer. The MIC was defined as the lowest concentration of the test solution that resulted a significant reduction in bacterial growth compared to the negative control.

The absorbance difference (Δ Abs) before and after treatment was calculated to assess bacterial growth inhibition. Three independent measurements were taken within each replication to enhance data accuracy and consistency.

To determine the MBC, 10 μ L of the bacterial suspension from wells with concentrations above the MIC was transferred to the surface of a sterile agar plate using an aseptic streaking technique. The plates were incubated at 37°C for 18–24 hours in anaerobic conditions. The MBC was determined by transferring it to the surface of a sterile agar plate using an aseptic streaking technique. The number of colonies in each treatment group was counted. The lowest concentration of the test agent that resulted in no visible bacterial colonies was recorded as the MBC.

The one-way ANOVA was performed to determine if there were statistically significant differences in the Δ Abs and colony number. Bonferroni test was used to identify which specific groups significantly differ from each other. The significance level was set at $p < 0.05$ to indicate that any observed differences were statistically significant.

RESULT

The study results are summarized in Table 1, which presents the differences in absorbance (Δ Abs) of *S. mutans* cultures before and after treatment, and Table 2, which display the colony count of *S. mutans* after treatment. The treatment given in this study was nHAp in combination with HC at various concentrations.

Table 1. Absorbance Differences (Δ Abs) of *S. mutans* cultures before and after treatment

Treatment group	Δ Abs	P value
Negative control	0.41 ± 0.03	
nHAp 100 mg/mL + HC 0.125 mg/mL	-0.90 ± 0.01	
nHAp 100 mg/mL + HC 0.25 mg/mL	-0.85 ± 0.02	0,00
nHAp 100 mg/mL + HC 0.5 mg/mL	-0.95 ± 0.02	
nHAp 100 mg/mL + HC 1 mg/mL	-1.02 ± 0.02	

Based on Table 1, the negative control exhibited increased absorbance, indicating active bacterial growth. In contrast, the treatment groups containing nHAp combined with HC demonstrated a progressive reduction in absorbance values. A decrease in absorbance was observed in the group treated with HC 0.125 mg/mL; however, an increase in absorbance again in the combination with HC concentration 0.5 mg/mL. The combination of 100 mg/mL nHAp and 1 mg/mL HC resulted in the highest reduction of absorbance. These findings suggest a dose-dependent inhibitory effect on *S. mutans*.

Table 2 shows a gradual reduction in the number of *S. mutans* colonies was observed with increasing hydroxychavicol (HC) concentrations. At a concentration of 0.25 mg/mL, microbial growth was still observed on agar media. The number of microbial colonies decreased at a concentration of 0.5 mg/mL, and no bacterial growth was detected on agar plates at a 1 mg/mL HC concentration, indicating bactericidal activity at this concentration. These findings indicate that a combination of 100 mg/mL nHAp and 1 mg/mL HC concentration represents the MBC.

Table 2. Colony number of *S. mutans* after treatment

Treatment group	Colony counts ($\times 10^6$ CFU/mL)	p Value
nHAp 100 mg/mL + HC 0.25 mg/mL	1074.80 ± 510.99	
nHAp 100 mg/mL + HC 0.5 mg/mL	$223,20 \pm 182.13$	0,00
nHAp 100 mg/mL + HC 1 mg/mL	0	

The Bonferroni post-hoc analysis revealed significant differences between groups in both the absorbance difference and the colony count results ($p < 0.05$).

DISCUSSION

The results of this study indicate that the combination of nHAp and HC has a potential synergistic effect in inhibiting and killing *Streptococcus mutans*, a key bacterium responsible for dental caries, especially early childhood caries, with MIC nHAp 100 mg/mL + HC 0.125 mg/mL and MBC nHAp 100 mg/mL + HC 1 mg/mL. The antibacterial action of nHAp is primarily based on its electrostatic interaction with the negatively charged components of the bacterial cell wall, such as teichoic acids and lipopolysaccharides (LPS).^{12,13}

nHAp nanoparticles possess a high surface area, which allows them to interact effectively with bacterial cell membranes.¹⁴ Once nHAp comes into contact with the bacterial membrane, the nanoparticles can disrupt the integrity of the membrane, leading to leakage of cellular contents, such as proteins, ions, and other molecules crucial for bacterial survival. This disruption of the bacterial cell membrane results in cell death or inhibition of bacterial growth.¹⁵ Additionally, nHAp has been shown to induce reactive oxygen species (ROS) production, which further damages bacterial cells. ROS, such as superoxide anions and hydrogen peroxide, can cause oxidative stress by damaging the cell's DNA, proteins, and lipids, leading to cellular dysfunction and death. These combined effects contribute to the antibacterial efficacy of nHAp.¹⁶

On the other hand, HC, as a phenolic compound, exhibits potent antibacterial activity through several mechanisms, making it a promising antimicrobial agent.¹⁷ HC exerts its effects through direct membrane disruption and induction of oxidative stress in bacterial cells.¹⁸ Like other phenolic compounds, HC can embed itself into the bacterial cell membrane due to its lipophilic nature.¹⁹ This embedding disrupts the membrane's integrity by altering its fluidity, leading to leakage of essential cellular components such as ions and metabolites, thus compromising bacterial cell function and survival.^{9,20}

Upon entering bacterial cells, HC undergoes oxidation, which produces ROS like hydrogen peroxide, superoxide anions, and hydroxyl radicals.²¹ These ROS are highly reactive and can damage the

bacterial cell by attacking cellular macromolecules such as proteins, lipids, and DNA. This oxidative damage disrupts key cellular processes and leads to cell death. The increased ROS production also triggers bacterial defense mechanisms, such as the activation of repair systems, but these mechanisms are often overwhelmed, leading to cell mortality.²²

Phenolic compounds, including HC, are known to have antimicrobial properties due to their ability to act as electron donors in redox reactions. The hydroxyl group (-OH) in the phenolic structure can form hydrogen bonds with bacterial cell components, thereby disrupting enzyme activity and protein function.¹⁷ The phenolic group can also interfere with bacterial metabolic processes by binding to enzymes, inhibiting their activity, and preventing bacterial growth.²³ HC is a naturally derived compound, and its use in antimicrobial formulations can be considered safer and more biocompatible than synthetic antibiotics or other phenolic compounds that may have higher toxicity to human cells or tissues.²⁴ This makes HC an attractive option for developing dental and oral care products, as it can provide antimicrobial benefits without posing significant risks to human health.¹⁷

These findings indicate that the synergistic action of nHAp and HC effectively inhibits the growth of *S. mutans*, likely through mechanisms such as bacterial cell membrane disruption and the generation of reactive oxygen species. The study concludes that concentration nHAp 100 mg/mL + HC 0.125 mg/mL effectively inhibits bacterial growth, while concentration nHAp 100 mg/mL + HC 1 mg/mL is optimal for achieving bactericidal effects. This approach offers a promising strategy for controlling dental caries, by directly targeting the primary etiological agent. Further, in vivo studies are recommended to evaluate its clinical applicability and long-term safety.

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