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Green synthesis of $Ca(OH)_2$ nanoparticles from chicken eggshell waste as antibacterial agent

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ABSTRACT

Chicken eggshell waste, though abundant in $CaCO_3$ that can be converted into various products like calcium hydroxide nanoparticles (NPs), has received limited attention. This study aimed to synthesize calcium hydroxide NPs from eggshell waste and evaluate their antibacterial properties against *Staphylococcus aureus* and *Escherichia coli*. The researchers employed a top-down approach with thermal decomposition to produce environmental-friendly calcium hydroxide NPs, and conducted in vitro tests to assess their antibacterial activity, with and without exposure to sunlight. The minimum inhibitory concentration and disc diffusion methods were used to evaluate the antibacterial effects. X-Ray diffraction analysis confirmed the synthesis of calcium hydroxide NPs. Further, a paired T-test showed that the calcium hydroxide NPs exhibited significant antibacterial activity against both bacterial through photocatalytic mechanisms, in comparison to their performance without such techniques. The utilization of green synthesis techniques to produce materials with potential antimicrobial applications presents significant developmental opportunities for the future.

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1. INTRODUCTION

Infectious diseases caused by infections from pathogenic bacteria are known to cause significant challenges in global public health [1]. In addition, various factors have been reported to be associated with the increasing number of cases, such as misuse of antibiotic drugs, improper medical diagnostics, and use of antibiotics without a prescription. The accumulation of these harmful practices facilitates the adaptation of bacteria, such as *Staphylococcus aureus* (*S. aureus*), and *Escherichia coli* (*E. coli*) [2]–[4]. *S. aureus* is the primary causative agent of pneumonia and respiratory tract infections, surgical sites, prosthetic joints, cardiovascular infections, and bacteremia nosocomia. Meanwhile, *E. coli* is a significant contributor to the incidence of intestinal and extraintestinal diseases in humans [5], [6]. According to a 2019 case report, outbreaks of infectious diseases originating from *S. aureus*, *E. coli*, and many other bacteria caused approximately 7.7 million death cases globally [7].

In response to these challenges, several studies have developed several antibacterial that can prevent infection caused by pathogenic bacteria. Serafin *et al.* [8] conducted antimicrobial tests using antibiotic from hybrid peptide compounds, comprising ranatensin and dermorfin known as LENART01, against strains of *E. coli* K12, R1-R4. The results of the minimum inhibitory concentration (MIC) test at a concentration of 0.321 µg/mL ±0.011 showed the ability of LENART01 as antibacterial. Altamimi *et al.* [9] also conducted a

MIC test using ceftobiprole antibiotic, with an MIC50/90 concentration of 1/1.5 mg/L. These results showed the ability of ceftobiprole to function as antibacterial against methicillin-resistant *S. aureus* (MRSA).

In addition to developing various antibiotic, previous reports have also produced different nanoparticles to treat infections caused by pathogenic bacteria. Afandy *et al.* [10] reported that silver nanoparticles (Ag) synthesized using reducing agents from green tea extract could function as antibacterial against *E. coli* using MIC methods. The results obtained were characterized by the absence of turbidity in liquid bacterial cultures, and the concentration of nanoparticles used was 12.8 mg/mL. Another study explored the synthesis of silver nanoparticles (Ag) based on calcium hydroxide ($Ca(OH)_2$) using leaf-reducing agents *Andrographis panikulata* and *Ocimum sanctum Linn*. The results of antibacterial tests against S. aureus bacteria showed the emergence of a bacterial growth inhibition zone of 10.56 mm, with the use of 25 µL nanoparticles [11]. In addition, the both methods used for nanoparticles production are known as green synthesis techniques due to the use of bioactive ingredients from various sources, such as plants, microorganisms, agricultural waste, and eggshell waste [12].

According to a study by Djayasinga *et al.* [13], waste content of purebred chicken eggshell has a crystalline phase, consisting of calcium carbonate ($CaCO_3$) at a concentration of 72.29%. This crystalline phase has been shown to have the potential to be used as antibacterial through various sewage treatments [14], [15]. Despite it is potential, there are limited studies on the use of purebred chicken eggshell waste for synthesizing calcium hydroxide nanoparticles, and the ability of nanoparticles as antibacterial. Therefore, this study aimed to synthesize calcium hydroxide NPs from purebred chicken eggshell waste with 2 calcination stages and assess their potential as antibacterial agent against *S. aureus* and *E. coli* with sunlight energy exposure. The two calcination stages were used to obtain the desired structure and nanoscale size of the crystal phase. According to Ran *et al.* [16], irradiation of sunlight in the form of electron emission to nanoparticles, such as $Ca(OH)_2$, can initiate reduction-oxidation process in the conduction band (C_B) and valence band (VB) regions. This process, in turn, leads to the production of reactive oxygen species (ROS) products that can cause microbial cell death.

2. METHOD

2.1. Bacterial cultivation and collection of chicken eggshell waste

S. aureus and *E. coli* bacteria were obtained from the Department of Medical Laboratory Technology of Poltekkes Kemenkes Tanjungkarang in Indonesia. Furthermore, the bacterial culture materials were in liquid form, namely brain heart infusion broth (BHIB), nutrient broth (NB), and Muller Hinton Agar (MHA). Furthermore, chicken eggshell waste in this study was obtained from fried rice traders in Bandar Lampung City in Indonesia.

2.2. Design study

This research method is a pure experiment with carried out antimicrobial susceptibility testing to antibacterial properties of Calcium hydroxide NPs with irradiation of sunlight and without irradiation on both bacteria. The dependent variable in this study was testing antibacterial properties of calcium hydroxide NPs against *S. aureus* and *E. coli* bacteria, which was carried out with the disc diffusion method (qualitative), and quantitative testing using the MIC method. The independent variable in this study was calcium hydroxide NPs, which showed characteristic properties in the form of peaks at 2θ (°) at 17.82°, 28.54°, and 33.92°, as determined through analysis using X-ray diffraction instrumentation. In addition, the measurement scale was nominal, and nanoparticles size was calculated using the Debeye-Scherer equation, which had a nanometer (nm) measurement unit [17].

Performance indicators from the application of both antibacterial testing methods were qualitatively characterized when there was a clear zone around calcium hydroxide NPs in MHA bacterial cultures that had been overgrown with bacteria [18]. Based on quantitative assessment using the MIC method, the performance indicator of calcium hydroxide NPs as antibacterial was marked when there was no turbidity in the bacterial culture liquid [19]. The measurement scale of the both antibacterial testing methods in this study was a nominal scale using the measurement technique namely qualitative testing method where the clear zone results around calcium hydroxide NPs in MHA bacterial culture (diffusion) was measured in mm. Furthermore, the quantitative testing method was carried out by observing the optical density of the bacterial culture, which corresponds to the concentration of the bacteria. The bacterial growth inhibition was measured based on the MIC, which was expressed in units of micrograms per milliliter (µg/mL) [20].

2.3. Tools and materials

Laboratory equipment used were biosafety cabinet (BSC) model BSC-1300IIB2-X, porcelain mortar, X-Ray Diffraction XRD--6000 Schimadzu, muffle furnace model LentonUAF 16/10, electrical balance model

BEL MW 333i, micropipette, incubator model Glotech GTLI-9082A, and turbidimeter model TU-2016. Bacterial culture among others; BHIB, NB, and MHA.

2.4. Synthesis of calcium hydroxideNPs

Waste material was washed, dried, and grounded using a porcelain mortar until it became a fine powder. In addition, the powder was calcined using muffle furnace instrumentation with a two-stage technique, where the first stage was carried out until it reached a temperature of 600 ºC. The temperature was then maintained for 10 hours, and the process was stopped when it reached room temperature. The second stage of calcination was continued until the temperature reached 900 °C, which was maintained for 10 hours, and at both stages, the temperature increase was set to 5 °C/minute [21]–[24].

2.5. Testing calcium hydroxideNPs as antibacterial through disc diffusion test method, and MIC method dilution test

The 2 types of test bacteria were inoculated in BHIB bacterial culture for 15 minutes, and the turbidity was measured using turbidimeter instrumentation at a wavelength of 850 nm. Furthermore, the 2 samples were inoculated in each of the 2 MHA culture dishes using sterile cotton swabs. The inoculated plates were then allowed to stand for 15 minutes to allow the bacteria to adhere to the agar surface. All these microbiological procedures were performed inside the biosafety cabinet to maintain a sterile environment [25].

2.5.1. Disc diffusion test method

Bacteria that had grown on the BHIB bacterial culture were taken using sterile cotton to inoculate the MHA bacterial culture and allowed to stand for 15 minutes [26]. In addition, a small amount of calcium hydroxide NPs was placed on the MHA culture surface, and the plate was closed. All these procedures were carried out in the BSC room. The bacterial culture plate was allowed to stand for five minutes on sun exposure, while some plates were not exposed to the sun for comparison. All bacterial plates were incubated at 37 °C for 24 hours using an incubator [27].

2.5.2. MIC method dilution test

A certain amount of calcium hydroxide NPs was weighed and mixed into a bacterial culture NB solution. The solution was then diluted with various concentrations ranging from 1,000, 500, 250, 125, 62.5, 31.3, and 15.6 µg/mL using a micropipette based on the procedures in previous studies [28]. In addition, the 2 types of bacteria were mixed with each solution of calcium hydroxide NPs with various concentrations, and irradiated by sunlight for five minutes, while some were not irradiated for comparison. The plates were subsequently incubated at 37 ℃ for a duration of 24 hours following the procedures [29].

2.6. Data analysis

Analysis using the paired T-test on 20 replicate samples to observe the inhibition zone of calcium hydroxide NPs 1,000 µg/ml against *S. Aureus* and *E. Coli* bacteria with two treatments (with and without exposure to sunlight) [30]. Meanwhile, Analysis of study data was carried out on purebred chicken eggshell powder after calcining based on peak-peak data 2θ (°) characteristics obtained from measurement using X-ray diffraction (XRD) instrumentation. Analysis of the Rietveld method was carried out using Rietica software version 4.2 to determine the goodness of fit (GoF) value and weight percent of each crystal phase [31]. The study also determined the size of calcium hydroxide NPs crystal phase formed using the Debeye-Scherrer equation.

3. RESULTS AND DISCUSSION

The disc diffusion method was used in testing antibacterial activity to determine the real effect of calcium hydroxide nanoparticles at a concentration of 1,000 µg/mL. Tests were carried out on *S. aureus* and *E. coli* bacteria which had been grown on MHA media, then exposed to sunlight for 5 minutes and without sunlight. The results showed that calcium hydroxide nanoparticles had a significant inhibitory effect on *S. aureus* and *E. coli* bacteria, with or without exposure to sunlight. The results of this study are in accordance with previous research, which found that calcium hydroxide nanoparticles have potential antibacterial properties against various bacterial strains, including *S. aureus* and *E. coli*. Observations were also made by measuring the size of the inhibition zone for bacterial colony growth.

3.1. Bacterial cell death

Figure 1 representation of antibacterial properties test results of calcium hydroxide NPs dilution method (MIC). Figure 1(a) represents the results of the antibacterial property test of calcium hydroxide nanoparticles using the Minimum Inhibitory Concentration method, where in the image the tested calcium

hydroxide nanoparticles NPs did not inhibit bacterial growth, as evidenced by the turbidity observed in the reaction tubes, indicating that the bacteria can proliferate.

The MIC test results presented in Figure 1(b) are the results of the antibacterial property test using the calcium hydroxide nanoparticle dilution method to determine the Minimum Inhibitory Concentration, where the reaction tubes containing nutrient broth medium have been inoculated with one of the test bacteria, then mixed with 1,000 µg/mL of calcium hydroxide NPs, and then exposed to sunlight for 5 minutes, so that no turbidity is visible, indicating total inhibition of bacterial growth. Additionally, Tables 1 and 2 present the results of observations on the growth of the two bacterial species, *Escherichia coli* and *Staphylococcus aureus*, tested for each concentration of $Ca(OH)_2$ NPs in the MIC test treatment. The antimicrobial properties of the calcium hydroxide NPs were found to be concentration-dependent, with complete inhibition of bacterial growth observed at concentrations of 1,000 and 500 µg/mL for both bacterial species

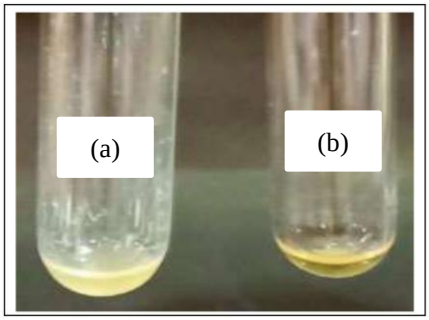

Figure 1. Representation of antibacterial properties test results of calcium hydroxide NPs dilution method (MIC): (a) it looks cloudy, indicating that bacteria grow because there is no intervention by $Ca(OH)_2$ NPs and (b) looks clear because there is no bacterial growth due to the intervention of $Ca(OH)_2$ NPs as an antibacterial

Table 1. Results of the antibacterial properties test for calcium hydroxide NPs by MIC method with exposure to sunlight

Types of bacteria	Concentration of calcium hydroxide NPs (µg/mL)						
	1,000	500	250	125	62.5	31.3	15.6
S. aureus	Clear	Cloudy	Cloudy	Cloudy	Cloudy	Cloudy	Cloudy
E. coli	Clear	Cloudy	Cloudy	Cloudy	Cloudy	Cloudy	Cloudy

Note: Clear means that calcium hydroxide NPs can act as an antibacterial agent, and vice versa for cloudy

Table 2. Results of the antibacterial properties test for calcium hydroxide NPs by MIC method without exposure to sunlight

Types of bacteria	Concentration of calcium hydroxide NPs (µg/mL)						
	1,000	500	250	125	62.5	31.3	15.6
S. aureus	Cloudy	Cloudy	Cloudy	Cloudy	Cloudy	Cloudy	Cloudy
E. coli	Cloudy	Cloudy	Cloudy	Cloudy	Cloudy	Cloudy	Cloudy

Note: Clear means that calcium hydroxide NPs can act as an antibacterial agent, and vice versa for cloudy

The antibacterial properties of calcium hydroxide NPs were further tested by a disc diffusion test using 1,000 µg/mL Calcium hydroxide NPs applied to the surface of the MHA culture on which the test bacteria had grown. In this disc diffusion test method, 20 replicates of the test were performed on each test bacterium. The presentation of the disc diffusion test results as shown in Figure 2 assessment of antibacterial efficacy using the disc diffusion assay with and without solar radiation exposure, whre Figures 2(a) and 2(b) does not show any bacterial growth inhibition zone, while Figures 2(c) and 2(d) show that there is a bacterial growth inhibition zone. In addition, Tables 3 and 4 provide information on the results of the paired t-test analysis.

The ability of calcium hydroxide NPs to function as antibacterial was primarily due to the influence of sunlight, thereby facilitating photocatalysis. In addition, photocatalysis caused a photoelectric effect that allowed oxidation-reduction reactions to occur, where sunlight hit calcium hydroxide NPs in MHA and NB bacterial cultures. Sunlight caused e- (electrons) in the atomic structure of calcium hydroxide NPs in the valence band (V_B) to move towards the conduction band (C_B). The displacement of e- led to the occurrence of a charged hole (+) denoted with h^+, while the conduction band was negatively charged because it was already occupied by e- in the valence band [32], [33].

Electrons in the conduction band (C_B) were also subjected to a reduction reaction process with O_2 from the air contained in MHA and BHIB cultures to further form ROS, consisting of oxygen anion radicals ($\bullet O_2^-$) and superoxide radicals (•HO). In the valence band region (V_B), h^+ underwent oxidation reactions with water vapor to form ROS in hydroxyl radicals ($\bullet H_2O$). This ROS product triggered damage to the bacterial cell wall, leading to intra-cell components being pumped out of the cell. The process disrupted the chemical reaction mechanism, which led to the death of bacterial cell contained in MHA and NB cultures [34]–[36]. ROS attacked hydrophobic residues of amino acids, contributed to the breaking of peptide bonds, and disrupted the function of these proteins. Carbonylation was another feature of proteins that underwent oxidative damage. Carbonylated proteins formed aggregates that could not be chemically altered and degraded through proteasomes, causing a permanent loss of their function [37]–[40].

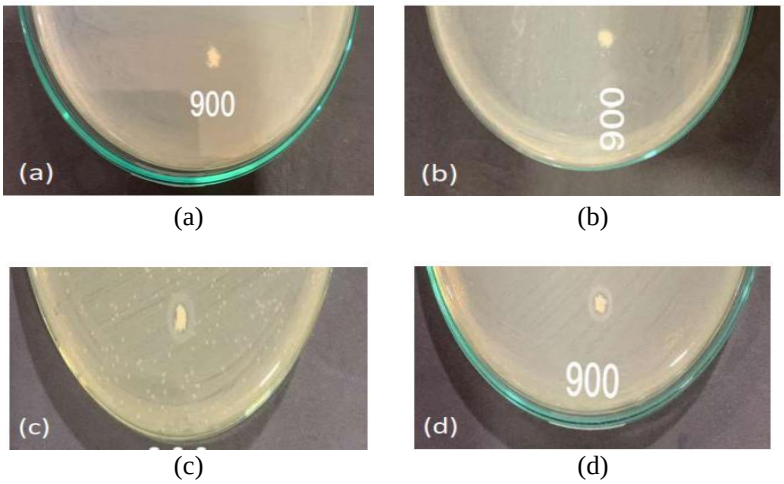

Figure 2. Comparison of antibacterial properties test results using the disc diffusion method with and without exposure to sunlight; (a) without exposure to sunlight, the *S. aureus* bacteria continued to grow, marked by the absence of a clear zone, (b) without sunlight shining on $Ca(OH)_2$ NPs, *E. coli* bacteria are able to grow, as evidenced by the absence of a clear zone, (c) under sunlight exposure to $Ca(OH)_2$ NPs, *S. aureus* did not grow, as indicated by a clear zone, and (d) when $Ca(OH)_2$ NPs were exposed to sunlight, *E. coli* failed to proliferate, as evidenced by a clear zone

Table 3. T-test paired two sample for means (*S. aureus*)

Test parameters	A	B
Mean	1.45	0.01
Variance	0.260526316	0.000947368
Observations	20	20
Pearson correlation	0.301511345	
Hypothesized mean difference	0	
df	19	
t Stat	12.3718607	
P(T<=t) one-tail	7.72371E-11	
t Critical one-tail	1.729132812	
P(T<=t) two-tail	1.54474E-10	$\mathbf{p_{value}<0.05}$
t Critical two-tail	2.093024054	

Note: A: application of calcium hydroxide NPs with exposure to sunlight for five minutes; B: Without exposure to sunlight

The data for the inhibition zone measurements are shown in Table 5. The results of the analysis using the paired T-test on 20 replicate samples to observe the inhibition zone of calcium hydroxide NPs 1,000 µg/ml against *S. aureus* and *E. coli* bacteria with 2 treatments (with sunlight and without sunlight) showed very good results. significant, namely that crystal phase of calcium hydroxide NPs with a concentration of 1,000 µg/ml exposed to sunlight for 5 minutes has inhibitory power against *S. aureus* and *E. coli* bacteria. Meanwhile, the calcium hydroxide NPs with a concentration of 1,000 µg/ml, which was not exposed to sunlight, had no inhibitory effect on *S. aureus* or *E. coli* bacteria.

Table 4. T-test paired two sample for means (*E. coli*)

Test parameters	A	B
Mean	1.3	0.005
Variance	0.221052632	0.0005
Observations	20	20
Pearson correlation	-0.150187852	
Hypothesized mean difference	0	
df	19	
t Stat	12.21723866	
P(T<=t) one-tail	9.56226E-11	
t Critical one-tail	1.729132812	
P(T<=t) two-tail	1.91245E-10	p_{value}**<0.05**
t Critical two-tail	2.093024054	

Note: A: Application of calcium hydroxide NPs with exposure to sunlight for 5 minutes; B: Without exposure to sunlight

Table 5. Results of observations of the inhibition zone of calcium hydroxideNPs 1,000 µg/ml against *S. aureus* and *E. coli* bacteria with two treatments

Repetition	Zone of inhibition of calcium hydroxide NPs against bacteria (mm)			
	Sun exposure (5 minutes)		non exposure to sunlight	
	S. aureus	*E. coli*	*S. aureus*	*E. coli*
1	1	2	0.1	0
2	1	1	0	0
3	2	1	0	0
4	1	2	0	0
5	2	1	0	0
6	2	2	0	0
7	1	1	0	0
8	1	1	0	0.1
9	1	1	0	0
10	1	1	0	0
11	2	2	0	0
12	2	1	0	0
13	2	1	0	0
14	2	1	0	0
15	1	1	0.1	0
16	1	1	0	0
17	1	2	0	0
18	2	1	0	0
19	2	1	0	0
20	1	2	0	0

Tables 3 and 4 show the results of statistical tests that there is a very significant difference in inhibitory power between the treatment of calcium hydroxide NPs with a concentration of 1,000 with five minutes of sunlight on *S. aureus* bacteria compared to the treatment of calcium hydroxide NPs at a concentration of 1,000 µg/ml without sunlight with a p-value=1.91×10^{-10} (p-value<0.05). The results of the statistical test for inhibition of *E. coli* bacteria also showed a very significant difference between the treatment of calcium hydroxide NPs at a concentration of 1,000 µg/mL exposed to sunlight for five minutes and the treatment of calcium hydroxide NPs at a concentration of 1,000 µg/mL without sunlight, with a p-value=1.54×10^{-10}.

3.2. Characteristic analysis of calcium hydroxide NPs

The analysis results of purebred chicken eggshell powder after two-stage calcination showed that there was a crystal phase of calcium hydroxide NPs from a peak of 2θ° at 17,82°; 28,54°; 33.92°. At these positions, these peaks were similar to the results of previous reports [41]. The quantitative analysis results of the presence of calcium hydroxide NPs are presented in Figure 3. The results showed that the GoF values of calcium hydroxide NPs is 1. In addition, the GoF value obtained in this study met the reference value, namely GoF<4 [42], [43]. The percentage of calcium hydroxide crystal phase was also obtained at 57.25%, with full wide half maximum (FWHM) value of 0.74 at 2θ (°) 33.92°. Based on FWHM data and peak 2θ (°), these data were needed for determining the crystal phase size of calcium hydroxide NPs using the Debeye-Scherrer formula according to previous references [44].

$$D = \frac{k\,\lambda}{\beta\,cos\theta} \quad (1)$$

Where, D=crystal phase size (nm), k=form constant valued at 0.9-1, λ=X-ray wavelength used=1,542 nm, β = Measurement result of half the maximum peak width height (in radians, $\alpha\,^\circ\,\frac{\pi}{180^\circ}\,x\,FWHM$, $\alpha\,^\circ$ =Bragg angle)

$$D = \frac{0.9.1{,}542\ nm}{\beta\ (0.74).cos\theta\ (\frac{33.92}{2})} = 11.23\ nanometer\ (nm)$$

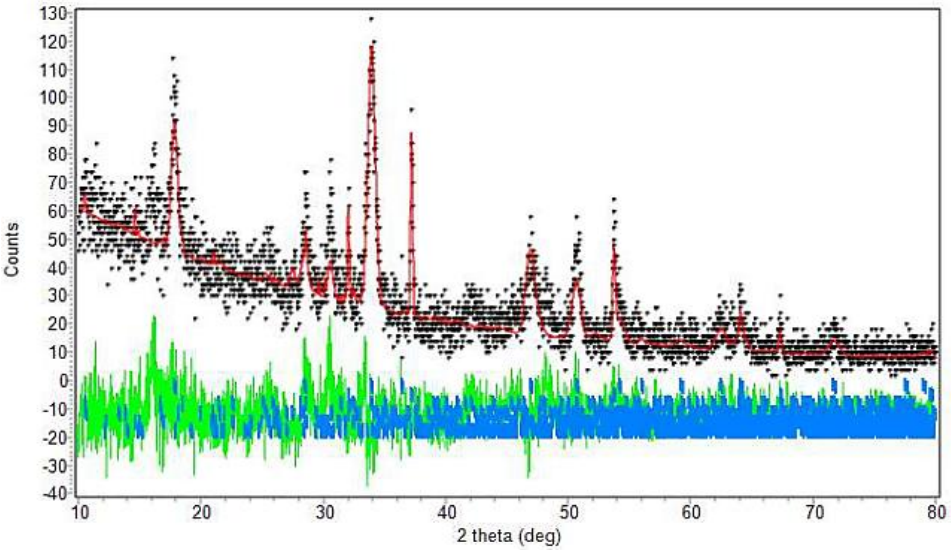

Figure 3. Diffractogram of calcium hydroxide NPs from chicken eggshell waste

4. CONCLUSION

In conclusion, this study successfully converted purebred chicken eggshell waste into calcium hydroxide NPs, which had a size of 11.23 nm with antibacterial properties. Antibacterial ability of calcium hydroxideNPs was activated using sunlight for five minutes against *S. aureus* and *E. coli* bacteria. The statistical test results show that there is a very real difference in inhibitory power between the treatment using calcium hydroxide NPs as an antibacterial at a concentration of 1,000 µg/ml with five minutes of sunlight against *S. aureus* bacteria compared to the treatment with calcium hydroxide NPs at a concentration of 1,000 µg/mL without sunlight with a p-value=1.91×10^{-10} (p-value<0.05). On the other hand, the results of statistical tests on *E. coli* bacteria with the same treatment and concentration of calcium hydroxide NPs obtained a p-value=1.54×10^{-10}.

This study has a limitation in that it did not thoroughly investigate and elucidate the mechanism underlying cell death triggered by the intervention of calcium hydroxide nanoparticles. Additionally, further research is required to optimize the mass of chicken eggshell powder utilized, the duration of exposure, and the exploration of photocatalysis energy sources beyond just sunlight. Synthesis of calcium hydroxide NPs was a technique known as green synthesis due to the conversion of purebred chicken eggshell waste into a more useful product as an antibacterial. This research has converted purebred chicken eggshell waste into calcium hydroxide NPs measuring 11.23 nm with the ability as an antibacterial agent activated using sunlight irradiation for five minutes against *S. aureus* and *E. coli* bacteria. Sunlight is necessary for the photocatalysis process to take place. Photocatalysis triggers calcium hydroxide NPs in forming ROS, and then ROS causes damage to bacterial cell membranes and disruption of protein synthesis, especially deoxyribonucleic acid (DNA).

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