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Antibacterial Effectiveness of Synthesized Copper Nanoparticles by Ultrasonication Assisted Method

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ABSTRACT
Antibiotic resistance among pathogenic bacteria has become a problem in the medical community. Copper nanoparticles (CuNPs) have become one method of combating antibiotic resistance in bacteria. The antibacterial activity of Synthesized Copper nanoparticles (CuNPs) from sigma 774103-5G against various species of harmful bacteria, including *Staphylococcus aureus* FNCC 0047, *Escherichia coli* FNCC 0091, and *Salmonella typhimurium* FNCC 0134, was determined in this research. CuNPs were optimized by ultrasonication in order to enhance the release of Cu ions into the environment. CuNPs ranging in size from 60 to 80 nm were employed. CuNPs' bactericidal impact can be assessed using a variety of methods, including the disk diffusion test, the Minimum Inhibitory Concentration (MIC), and the Minimum Bactericidal Concentration (MBC), and it can conducted with ultrasonification or non-ultrasonification respectively. The ultrasonication treatment was found to be efficient in boosting the antibacterial activity of all of the microorganisms examined. CuNPs demonstrated the best antibacterial activity against *Staphylococcus aureus* FNCC 0047, with a 23.7 mm inhibition zone diameter and a 37.5 g/mL MIC. The ultrasonication treatment was chosen for the preparation of CuNPs samples before further testing with the Alkaline Phosphatase Assay method, and the results showed that alkaline phosphatase enzyme as an indicator of cell damage was mostly detected in *Staphylococcus aureus* FNCC 0047 with a 4 hour exposure time. Membrane Integrity Staining with fluorescence microscopy viewing further revealed that cell damage increased as CuNPs exposure time increased.

Keywords: *Antibacterial, Copper Nanoparticles, Membrane integrity, Resistance.*

1. INTRODUCTION

Microbial resistance rises as a result of antibiotic overuse and misuse [1], [2]. *Staphylococcus aureus* is a methicillin-resistant bacteria [3], while *Escherichia coli* and *Salmonella typhimurium* are other harmful bacteria that are antibiotic-resistant [4], [5]. Because resistance to the bacterium that causes the disease cannot be easily overcome by using other antibiotics, which can actually improve their resistance, nanoparticles are one method that can be utilized to combat bacterial resistance to antibiotics [6].

Nanoparticles are increasingly being used for microbial control due to several factors that influence the level of toxicity, one of them is the ratio of the size of nanoparticles to their surface area in huge volumes, it provides for enhanced absorption and stability in physiological media by facilitating cellular internalization of nanoparticles in bacteria or other cells [7]. Copper Nanoparticles (CuNPs) have a schematic toxicity, such as the formation and release of metal ions into the environment [8], the production of reactive oxygen species (ROS) [9], CuNPs induce structural and mechanical damage to membranes, as well as the modification of DNA cations, which leads to denaturation and inactivation of proteins in cells [10], [11].

Bacterial cell membranes contain a negative charge at biological pH due to an abundance of carboxylic groups, which permits positively charged CuNPs to attach strongly to the cell membrane [12]. The creation of holes around CuNPs on the membrane surface can cause structural damage [13], and the release of metal ions dissolved in suspension plays a significant part in the toxicity process, notably in bacteria [14].

Metal ions cross the bacterial membrane, attach to phospholipids, and the presence of CuNP can interact with the amino acid cysteine and other thiol-containing compounds. Furthermore, due to increased membrane permeability, metal ions can cause cell leakage [15]. The surface oxidation process of CuNPs causes the release of metal ions [14], and it is well known that the larger the surface area of CuNPs, the effective it is at releasing metal ions [16].

Increasing the effectiveness of CuNPs before testing for antibacterial activity is very important because of the potential for physicochemical changes that can reduce the surface area of CuNPs, for example, When in solution, agglomeration states and changes in surface charge can occur [17]. These modifications may have a substantial influence on the observed toxicological response [18].

The state of the dispersed CuNPs is particularly significant in preparing samples for toxicological research in order to determine the biological response, because unstable and agglomerated nanoparticle dispersions in in vitro or in vivo investigations might result in an incorrect estimate of nanoparticle toxicity [17], [19]. In this study using the help of ultrasonication techniques to break up agglomerates [17], the process was carried out in order to obtain dissolved CuNPs with a maximum surface area and optimally dispersed.

Departing from such condition, the aim of this research was to determine the antibacterial activity of Synthesized Copper Nanoparticles (CuNPs) with ultrasonication and non-ultrasonication treatment against pathogenic bacteria, *Staphylococcus aureus* FNCC 0047, *Escherichia coli* FNCC 0091, and *Salmonella typhimurium* FNCC 0134. The method that conducted in this study were disk diffusion test, MIC and MBC. To determine and reinforce the results of antibacterial activity testing, the Membrane Integrity Staining and Alkaline Phosphatase Assay procedures were used sequentially.

2. METHODS

The material used in this research is Synthesized Copper Nanoparticles (CuNPs) from Sigma 774103-5G with particle sizes ranging from 60-80 nm. The bacteria used in this research were obtained from the Food and Nutrition Laboratory of Microbiology, Postgraduate Universitas Gadjah Mada which included *S. aureus* FNCC 0047, *E. coli* FNCC 0091, and *Salmonella typhimurium* FNCC 0134. The research was carried out in the Laboratory of Microbiology, Postgraduate Biotechnology, Universitas Gadjah Mada (UGM), Yogyakarta, Indonesia.

2.1. Visualization of the Structure and Size of Synthesized Copper Nanoparticles (CuNPs)

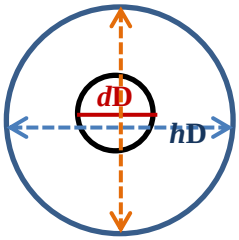
CuNPs were suspended in DI (Deionized) Water and SEM (Scanning Electron Microscope) imaging was used to assess the influence of Ultrasonication on CuNP agglomeration. This assay was divided into two treatments: ultrasonication for 15 minutes and non-ultrasonication in order to evaluate the difference between the two structures in nanoparticles.

2.2. Determination of Antibacterial Activity Using Disk Diffusion Test

S. aureus FNCC 0047, *E. coli* FNCC 0091, and *S. typhimurium* FNCC 0134, were cultivated in Mueller Hinton Broth (MHB) liquid culture and then quantified using a UV-Vis spectrophotometer to an absorbance of 0.5 at a wavelength of 600 nm, yielding a cell number of 1.5x108 cfu/mL (0.5 McFarland).

Bacterial liquid cultures were streaked into Mueller Hinton Agar (MHA) media with sterile cotton buds. After placing paper discs on agar media, 20µl of CuNPs samples from the ultrasonication and non-ultrasonication stages were added to each disc paper. The CuNPs sample was added in concentrations ranging from 5, 10, 20, and 40 mg/mL. Positive control was chloramphenicol 1 mg/mL, and negative control was DI water. Antibacterial activity was determined after 24 hours of incubation at 37°C using the Equation (1) for calculating the Inhibitory Zone

Diameter [20], where *v*D is vertical diameter, *h*D is horizontal diameter, and *d*D is disc diameter (Fig 1).



$$IHZ = \frac{(vD - dD) + (hD - dD)}{2} \quad (1)$$

Figure 1. The formula for diameter of inhibition zone

2.3. Minimum Inhibitory Concentration and Bactericidal Concentration Determination

The Minimum Inhibitory Concentration (MIC) and Bactericidal Concentration (MBC) were determined using the standard method with minor modifications [21]. CuNPs were prepared using ultrasonication and non-ultrasonication at various concentrations, including 37.5, 75, 150, 300, 600, 1200, and 2400 (ppm or µg/mL) in 2 mL of MHB medium. Then, 20µl of bacterial culture with a bacterial concentration of 10^3-10^4 cfu/mL was added. Three replications were used for each treatment. The growth of each treatment was seen and measured using a UV-Vis spectrophotometer at a wavelength of 600 nm after 24 hours of incubation at 37°C.

The next test was MBC, using agar plating method or spreading 100 µl of liquid medium from MIC treatment on plate agar medium and incubated at 35ºC for 24 hours [21]. The MIC value is defined as the lowest concentration of a CuNPs or antibiotic that inhibits an organism's growth [22]. Then the MBC value was obtained from the lowest concentration of nanoparticles which killed 99.9% of bacteria [23].

2.4. Alkaline Phosphatase assay

The test was carried out using the Alkaline Phosphatase Assay (DALP-250) protocol kit of the Bioassay Systems brand, with the addition of *p*-nitrophenyl phosphate as a substrate which was hydrolyzed by ALP into a yellow product, namely *p*-nitrophenol + phosphate, measurements using an ELISA Reader with a maximum absorbance of 405 nm. The reaction rate is directly proportional to the enzyme activity [24]. CuNPs used was a concentration of 1 x MIC for each bacterium, with variations in CuNPs exposure time 0, 4 and 24 hours. The Alkaline phosphatase (ALP) activity in the sample (IU/L) calculate using the Equation (2).

$$ALP = \frac{(OD_{sample\,t} - OD_{sample\,0}) \times Reaction\ vol}{(OD_{calibrator} - OD_{H2O}) \times Sample\ vol \times t} \times 35.3 \quad (2)$$

2.5. Membrane Integrity Assays

The bacterial membrane integrity test was performed using the fluorometric method and the Merck Millipore Live/Dead double Staining (QIA76-100TESTCN) Kit. CuNPs exposure times of 0, 4, and 24 hours were tested on the three bacteria using a concentration of 1 x MIC.

3. RESULT AND DISCUSSION

This study employed two Gram-negative pathogenic bacteria, *E. coli* FNCC 0091 and *S. typhimurium* FNCC 0134 (Fig 2a, b), as well as a Gram-positive bacteria, *S. aureus* FNCC 0047. (Fig 2c). The copper nanoparticles (CuNPs) used were synthesized and ranged in size from 60 to 80 nm and resistivity 1.673 µΩ-cm, 20°C.

Visualization of CuNPs using SEM (Scanning Electron Microscope) showed the difference in the characteristics of CuNPs in samples with ultrasonication, the structure of CuNPs was clearly visible, whilein non-ultrasonication samples agglomeration occurred and CuNPs could not be seen clearly (Fig 3).

Based on the overall results, the data showed that ultrasonication treatment was effective in increasing the antibacterial activity of all test bacteria, this was due to the agglomeration state and variations in surface charge that could occur while in solution, with the help of the ultrasonication technique it was able to break up agglomerates [17]. This process was carried out in order to

Table 1. Minimum Inhibitory (MIC) and Minimum Bactericidal (MBC) Concentration of Synthesized Copper Nanoparticles (CuNPs) with ultrasonication and non-ultrasonication on different bacteria

Bacteria	Non-Ultrasonication (ppm or µg/mL)		Ultrasonication (ppm or µg/mL)	
	MIC	MBC	MIC	MBC
Escherichia coli FNCC 0091	1200	2400	600	1200
Salmonella typhimurium FNCC 0134	1200	2400	600	1200
Staphylococcus aureus FNCC 0047	75	150	37.5	75

obtain dissolved CuNPs with a maximum surface area and optimally dispersed. The mechanism is also related to the larger the surface area of CuNPs, the greater the interaction between the membranes of pathogenic microorganisms and CuNPs [25].

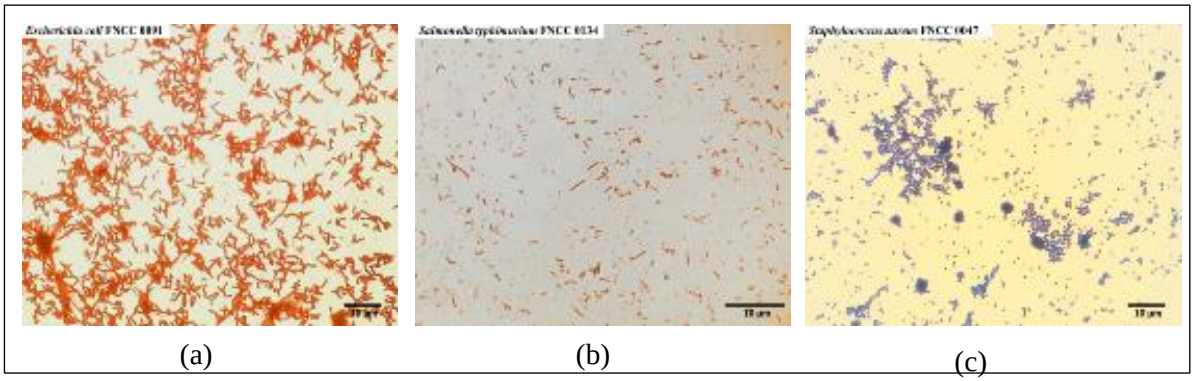


Figure 2. Bacteria visualization with Gram stain Using Binocular Light Microscope (400x magnification); **(a)** *Escherichia coli* FNCC 0091, **(b)** *Salmonella typhimurium* FNCC 0134, **(c)** *Staphylococcus aureus* FNCC 0047.

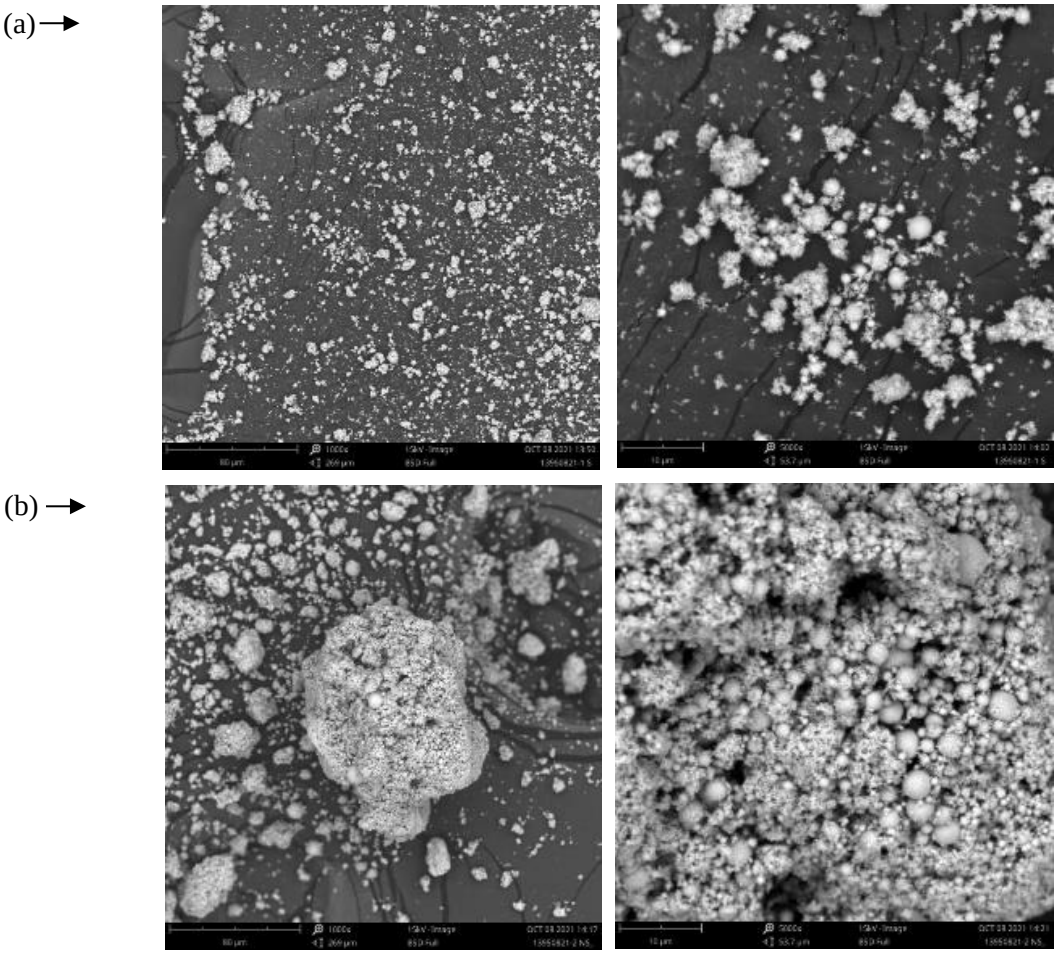


Figure 3. Visualization of Copper nanoparticles (CuNP) using a Scanning Electron Microscope (SEM). **(A)** CuNP preparation through Ultrasonic treatment for 15 minutes; **(B)** Non-ultrasonication.

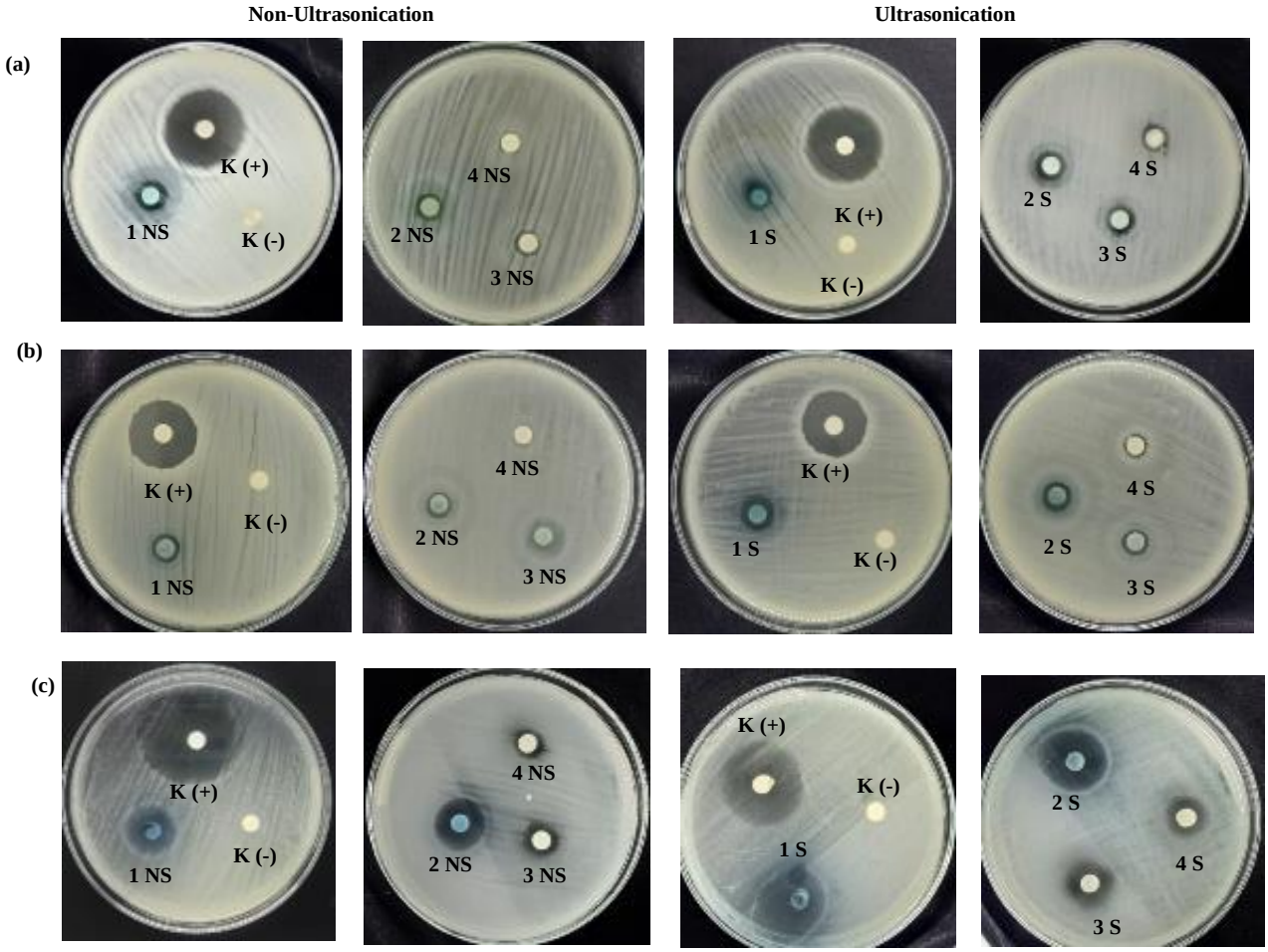


Figure 4. Inhibitory Zone Diameter of Synthesized Copper Nanoparticles (CuNPs) Antibacterial Activity Test. (A) *Escherichia coli* FNCC 0091; **(B)** *Salmonella typhimurium* FNCC 0134; **(C)** *Staphylococcus aureus* FNCC 0047. K+(Positive control); K- (Negative control); **1** (40000 ppm), **2** (20000 ppm), **3** (10000 ppm), and **4** (5000 ppm).

Furthermore, the results of the Disk Diffusion Test at the highest concentration of 40,000 ppm, showed that CuNPs treated with ultrasonication had the highest antibacterial activity on the pathogenic bacteria *Staphylococcus aureus* FNCC 0047 which had an inhibition zone diameter of 23.7 mm, then *Escherichia coli* FNCC 0091 had an inhibition zone diameter of 12.3mm, and the most resistant bacteria of the three bacteria, *Salmonella typhimurium* FNCC 0134, had an inhibition zone diameter of 11.7 mm (Supplementary Table 1).

This is consistent with the MIC and MBC tests, which revealed that CuNPs with ultrasonication treatment could inhibit the growth of *S. aureus* FNCC 0047 at the lowest concentration of 37.5 g/mL (Fig 4), whereas CuNPs could only inhibit the growth of *E. coli* FNCC 0091 and *S. typhimurium* FNCC 0134 at a concentration of 1200 g/mL. (Table 1). The absorbance value in MIC test's was also examined to see if the addition of CuNPs in various concentrations in MHB medium inhibited bacterial growth. The results show that at the MIC limit, the absorbance value drops dramatically, as shown in Table 2 and the MIC absorbance graph (Fig 5).

This is due to the fact that the cell membranes of gram-negative bacteria such as *E. coli* and *S. typhimurium* differ from the cell membranes of gram-positive bacteria such as *S. aureus* FNCC 0047. Gram-negative bacteria had a thin peptidoglycan cell wall but two membranes, one inner and one outer, both of which contain lipopolysaccharides, making them more resistant, whereas gram-positive bacteria do not had an outside membrane but are surrounded by a thick layer of peptidoglycan. [26].

This statement refers to the mechanism of cell destruction by CuNPs, which involves direct contact with

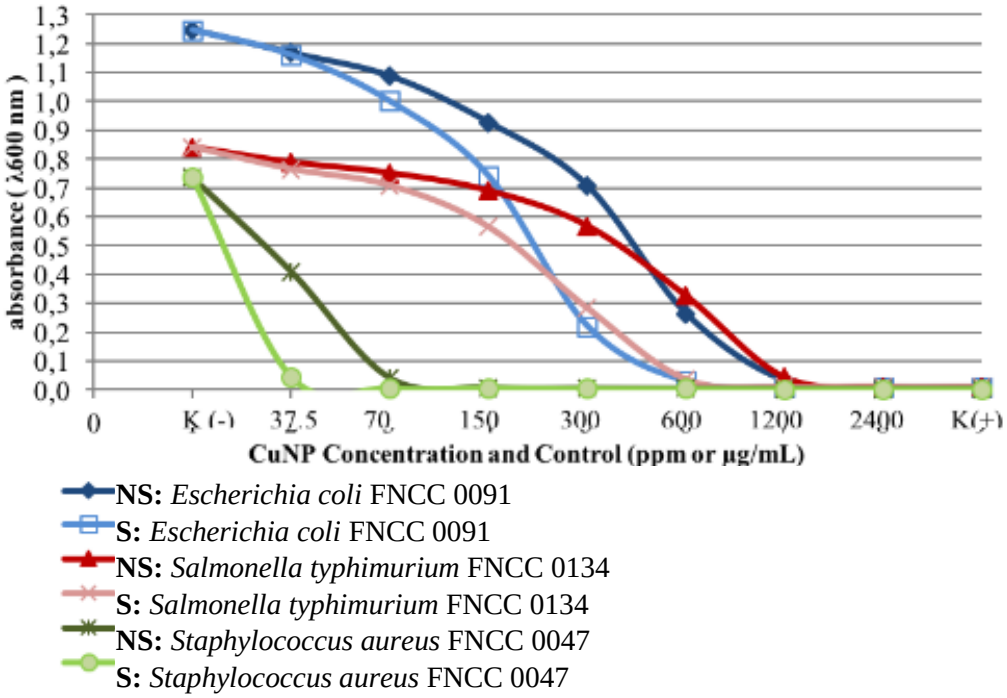


Figure 5. Absorbance of Minimum Inhibitory Concentration (MIC) with Ultrasonicated (S) and Non-Ultrasonicated (NS) treatment with various concentrations of CuNPs in different bacterial species.

the accumulation of CuNPs on the membrane surface, which then releases highly reactive and toxic metal ions to cells [11], so that if bacterial cells have two membranes lining them, CuNPs will have a more difficult time penetrating or damaging them.

Further testing using the Membrane Integrity Staining method using fluorescence microscope visualization and *Alkaline Phosphatase Assay* using ELISA Reader was carried out by treating the length of time CuNPs exposure to bacteria were 0, 4, and 24 hours, previously CuNP preparation was carried out by ultrasonication. In this test using 1 x MIC or a minimum concentration of CuNPs can inhibit the growth of each bacterium. The MIC used for *E. coli* FNCC 0091, and *S. typhimurium* FNCC 0134 was 1200 µg/ml, while *S. aureus* FNCC 0047 was 75 µg/ml. Alkaline phosphatase (ALP) has a physiological role in dephosphorylation/ inactivation of other enzymes and serves to help break down proteins [27]. The periplasmic region of gram-negative bacteria, including *E. coli*, contains alkaline phosphatase [28]. *Staphylococcus* also produces alkaline phosphatase, however only coagulase-positive staphylococci have this enzyme, whereas coagulase-negative staphylococci do not [29]. *S. aureus* is coagulase-positive resulting in two forms of coagulase: bound and free [30]. Based on this, the presence of alkaline phosphatase enzymes can be an indicator of bacterial cell damage.

The results of these two procedures support the findings of the disk diffusion, MIC, and MBC tests. The antibacterial ability of Synthetic Copper nanoparticles (CuNPs) can be seen using the Alkaline Phosphatase Assay test, because the higher the ALP enzyme identified, the greater the cell damage. CuNPs exhibited the maximum antibacterial effectiveness in *Staphylococcus aureus* FNCC 0047 with Alkaline Phosphatase (ALP) identified at 35.06 IU/L, ALP of *Escherichia coli* FNCC 0091 at 33.11 IU/L, and ALP of *Salmonella typhimurium* FNCC 0134 at 25.01 IU/L, according to this ALP Assay test (Table 3). The changes in ALP found were attributed not only to the influence of varied antibacterial activity, but also to the possibility that each bacterial species has a different total ALP enzyme.

The Alkaline Phosphatase Assay test, on the other hand, must pay attention to the optimum time of CuNPs exposure because the results of the Alkaline Phosphatase Assay performed in this study show a decrease in the alkaline phosphatase enzyme, which was detected at the CuNPs exposure time of 24 hours (Table 3) and the highest ALP was detected at the exposure time of 4 hours.

As a result, the optimum exposure time for CuNPs in this study ranges from 4 hours to no more than 24 hours, because CuNPs can cause DNA cation modification, denaturation, and inactivation of proteins in cells [10], and metal ions can bind with the enzyme's main functional

Table 2. Minimum Inhibitory Concentration (MIC) at various concentrations of Synthesized Copper Nanoparticles (CuNPs)

Bacteria	CuNPs Concentration (ppm or μg/mL)	Absorbance	
		Non-Ultrasonication	Ultrasonication
Escherichia coli FNCC 0091	MHB & Bacteria (-)	1.25±0.006^{h}	1.25±0.006^{g}
	CuNPs 37.5 ppm	1.17±0.005^{g}	1.16±0.006^{f}
	CuNPs 75 ppm	1.09±0.006^{f}	1±0.007^{e}
	CuNPs 150 ppm	0.926±0.005^{e}	0.740±0.005^{d}
	CuNPs 300 ppm	0.706±0.004^{d}	0.221±0.007^{c}
	CuNPs 600 ppm	0.263±0.006^{c}	0.030±0.004^{b}
	CuNPs 1200 ppm	0.031±0.002^{b}	0.010±0.001^{a}
	CuNPs 2400 ppm	0.009±0.0005^{a}	0.009±0.0006^{a}
	Chloramphenicol 150 ppm (+)	0.009±0.001^{a}	0.009±0.0006^{a}
Salmonella typhimurium FNCC 0134	MHB & Bacteria (-)	0.840±0.016^{h}	0.840±0.016^{g}
	CuNPs 37.5 ppm	0.788±0.004^{g}	0.764±0.006^{f}
	CuNPs 75 ppm	0.751±0.004^{f}	0.707±0.006^{e}
	CuNPs 150 ppm	0.689±0.003^{e}	0.564±0.006^{d}
	CuNPs 300 ppm	0.567±0.004^{d}	0.283±0.011^{c}
	CuNPs 600 ppm	0.325±0.005^{c}	0.037±0.004^{b}
	CuNPs 1200 ppm	0.043±0.005^{b}	0.011±0.003^{a}
	CuNPs 2400 ppm	0.012±0.002^{a}	0.011±0.001^{a}
	Chloramphenicol 150 ppm (+)	0.011±0.002^{a}	0.012±0.002^{a}
Staphylococcus aureus FNCC 0047	MHB & Bacteria(-)	0.735±0.005^{d}	0.735±0.003^{c}
	CuNPs 37.5 ppm	0.409±0.004^{c}	0.045±0.003^{b}
	CuNPs 75 ppm	0.039±0.003^{b}	0.007±0.0003^{a}
	CuNPs 150 ppm	0.007±0.002^{a}	0.007±0.001^{a}
	CuNPs 300 ppm	0.004±0.001^{a}	0.004±0.0003^{a}
	CuNPs 600 ppm	0.004±0.001^{a}	0.004±0.0003^{a}
	CuNPs 1200 ppm	0.003±0.001^{a}	0.003±0.001^{a}
	CuNPs 2400 ppm	0.003±0.001^{a}	0.003±0.001^{a}
	Chloramphenicol 150 ppm (+)	0.003±0.001^{a}	0.003±0.001^{a}

Note:* The mean ± standard error with 6 replications was used to indicate the value of antibacterial activity. Further analysis using Tukey's test, the difference between the superscript letters of the alphabet was significantly different at ($p<0.05$)

group, inactivating its function [15]. According to this remark, the prolonged CuNP exposure duration may diminish the activity of the identified Alkaline Phosphatase enzyme. As a result, the accuracy of this Alkaline Phosphatase Assay method may be affected by the increased exposure duration of CuNPs.

The Membrane Integrity Staining test was performed to support the findings of the Disk Diffusion Test, MIC, and MBC, as well as to clarify the conclusion that the longer the CuNPs were exposed, the more cell damage occurred (Fig 6).

The results of the Scanning Microscope Fluorescence (SEM) visualization show that the longer CuNPs are exposed, the more cell damage occurs. This is visible in the red fluorescence of bacterial cells, which increases after 24 hours of CuNP exposure.

CuNPs had the highest antibacterial activity on *Staphylococcus aureus* FNCC 0047 with an inhibition zone diameter of 23.7 mm and MIC at a concentration of 37.5 µg/ml. Further testing using the Membrane Integrity Staining and *Alkaline Phosphatase Assay* methods also showed the same results, namely CuNPs at the minimum concentration (MIC) had a greater inhibitory ability on *S. aureus* FNCC 0047 than *E. coli* FNCC 0091 and *S. typhimurium* FNCC 0134. Exposure time, the optimum CuNPs for *Alkaline Phosphatase Assay* testing is 4 to 24 hours, to minimize the effect of CuNPs in inactivating the Alkaline Phosphatase enzyme so that it does not affect the accuracy of the method.

Table 3. Alkaline Phosphatase (ALP) activity was detected in the bacteria tested.

Bacteria	CuNPs Exposure Time (Hours)	OD	ALP
Escherichia coli FNCC 0091	0	0.463	15.60±2.63[a]
	4	0.542	33.11±2.65[b]
	24	0.484	22.69±2.88[a]
Salmonella typhimurium FNCC 0134	0	0.399	13.43±3.93[a]
	4	0.516	25.01±1.31[b]
	24	0.483	23.74±3.19[b]
Staphylococcus aureus FNCC 0047	0	0.475	18.40±2.08[a]
	4	0.554	35.06±2.27[b]
	24	0.491	24.80±2.24[a]

Note: * OD values were obtained from 20 min incubation with ALP Assay-Kit. The mean ± standard error with three replications was used to indicate the value of Alkaline phosphatase activity. Further analysis using Tukey's test, the difference between the superscript letters of the alphabet was significantly different at ($p<0.05$)

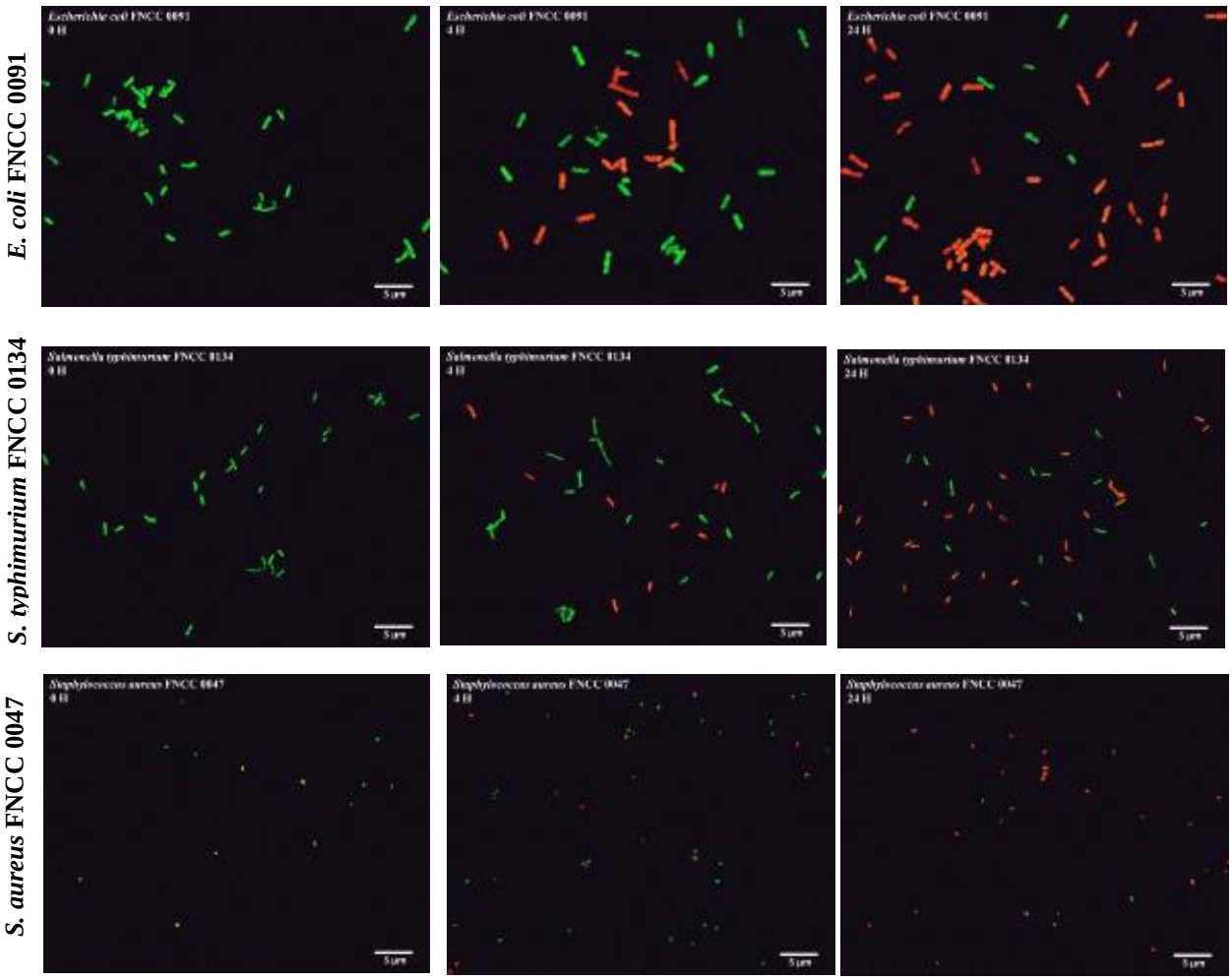


Figure 6. Visualization using a fluorescence microscope for bacteria *Escherichia coli* FNCC 0091, *Salmonella typhimurium* FNCC 0134, and *Staphylococcus aureus* FNCC 0047 with Synthesized Copper nanoparticle (CuNPs) exposure treatment for 0, 4, and 24 hours.

SUPPLEMENTARY DATA

See Supplementary Table 1 for the antibacterial activity of synthesized copper nanoparticles (CuNPs) with ultrasonication and non-ultrasonication based on disc diffusion method.

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SUPPLEMENTARY DATA

Supplementary table 1. Antibacterial activity of Synthesized Copper Nanoparticles (CuNPs) with Ultrasonication and non-ultrasonication based on disc diffusion method

Bacteria	CuNPs Concentration (ppm or μg/mL)	Inhibition Zone Diameter (mm)	
		Non-ultrasonication	Ultrasonication
Escherichia coli FNCC 0091	DI Water (-)	0±0.00^{a}	0±0.00^{a}
	Chloramphenicol 1000 ppm (+)	26±1.00^{d}	27.7±0.58^{e}
	CuNPs 5000 ppm	6.3±0.58^{b}	7.7±0.00^{b}
	CuNPs 10000 ppm	7.3±0.58^{b}	9.3±0.00^{c}
	CuNPs 20000 ppm	9±0.00^{c}	10,3±0.58^{c}
	CuNPs 40000 ppm	10.3±0.58^{c}	12.3±1.16^{d}
Salmonella typhimurium FNCC 0134	DI Water (-)	0±0.00^{a}	0±0.00^{a}
	Chloramphenicol 1000 ppm (+)	23.7±0.58^{e}	23.7±0.58^{e}
	CuNPs 5000 ppm	6±0.00^{b}	7±0.58^{b}
	CuNPs 10000 ppm	6.7±0.58^{b}	9±0.58^{c}
	CuNPs 20000 ppm	8.7±0.58^{c}	9.7±0.58^{c}
	CuNPs 40000 ppm	10.3±0.58^{d}	11.7±0.58^{d}
Staphylococcus aureus FNCC 0047	DI Water (-)	0±0.00^{a}	0±0.00^{a}
	Chloramphenicol 1000 ppm (+)	26±1.00^{f}	29.7±0.58^{f}
	CuNPs 5000 ppm	7.3±0.58^{b}	11.3±0.58^{b}
	CuNPs 10000 ppm	10±1.00^{c}	15±0.00^{c}
	CuNPs 20000 ppm	14.7±0.58^{d}	21±1.00^{d}
	CuNPs 40000 ppm	19.3±0.58^{e}	23.7±0.58^{e}

Note:* The mean ± standard error with three replications was used to indicate the value of antibacterial activity. Further analysis using Tukey's test, the difference between the superscript letters of the alphabet was significantly different at ($p<0.05$)