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Synthesis and Characterization of Cu(II)-EDTA Complexes: Antibacterial Studies (*Escherichia coli, Staphylococcus aureus*) and Inhibition of Dengue Virus Serotype 2 in Vero Cell

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Abstract

The Cu(II)-EDTA complex is known to have antibacterial and antiviral potential, but its effectiveness against pathogenic bacteria and dengue virus serotype 2 (DENV-2) still needs to be studied. This study synthesized and characterized the Cu(II)-EDTA complex of $CuSO_4$ precursors, and then tested the antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*, as well as the antiviral activity against DENV-2 in Vero cells. This study successfully synthesized and characterized the Cu(II)-EDTA complex using $CuSO_4$ as a precursor through the solvothermal method, producing blue crystals with a Cu ratio of 1:1. DSC analysis showed thermal stability up to 250°C with an endothermal peak at 270-300°C. The particles are 6.31 nm in size with a PDI of 0.076, indicating uniform distribution with nanoparticle size (<100 nm). FTIR confirms the formation of the complex through significant shifts in the O-H and C=O bands. SEM shows a layered morphology that can affect the solubility and release of substances. UV-Vis shows maximum absorbance peaks of EDTA at 244 nm and $CuSO_4$ at 740 nm. Antibacterial tests of Cu(II)-EDTA against *E. coli* and *S. aureus* showed that Cu(II)-EDTA had less activity than pure $CuSO_4$. For DENV-2, $CuSO_4$ was more effective with an EC_{50} value of 77.86 µg/mL, lower than Cu(II)-EDTA 356.13 µg/mL, indicating that $CuSO_4$ was better at inhibiting viral replication.

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INTRODUCTION

Transition metals and metalloids have interesting antimicrobial capabilities because they can target bacteria, viruses, and fungi broadly.[5] A prominent example of a transition metal with such properties is copper, which ranks as the third most abundant transition element in the human body. Copper is a bio-essential element used in DNA cleavage and has potential anti-HIV activity.[6] Copper (Cu) is essential for enzyme activity involved in hemoglobin formation and chemical redox reactions in the body. One copper compound, Copper(II) chloride, has the potential to inhibit DENV-2 with an IC_{50} of 0.13 μg/mL. However, excessive copper usage can cause toxicity in the liver, reproductive system, and neurons, as well as trigger stress in the endoplasmic reticulum, which may lead to apoptotic cell death.[7] In the study, the copper complex [Cu(2,4,5-triphenyl-1H-imidazol)$_2$(H_2O)$_2$].Cl_2 showed a decrease in the viability of Vero cells through mitochondria-related cell death, which is crucial in initiating apoptosis by releasing apoptosis-triggering factors and DNA fragmentation.[8]

Dengue fever is a serious global health problem spread by the *Aedes aegypti* and *Aedes albopictus* mosquitoes, especially affecting tropical and subtropical regions. Approximately 2.5 billion individuals are at risk of contracting the disease.[1] In 2023, over 80 countries reported close to five million dengue cases and more than 5000 related deaths.[2] The dengue virus is an RNA virus belonging to the genus Flavivirus in the Flaviviridae family, transmitted through mosquito bites, leading to Dengue Hemorrhagic Fever (DHF. It has four serotypes (DENV1 to DENV-4)[3], with serotype 2 (DENV-2) being a major contributor to fatalities caused by dengue fever.[4]

In addition, copper, historically utilized for its antimicrobial properties, has recently attracted renewed interest with the advancement of nanotechnology. Copper nanoparticles (CuNPs) have a larger surface area and higher toxicity than ordinary metals, making them a more effective choice for antimicrobial applications.[5]

Escherichia coli and *Staphylococcus aureus* are significant and pathogens responsible for a wide range of infections in both humans and animals. *E.coli* is particularly associated with skin and soft tissue infections, surgical wound infections, as well as bone and joint infections.[9] *Escherichia coli (E. coli)* is a facultative aerobic bacterium characterized by its Gram-negative staining and rod-like morphology. Although its presence in the colon is generally harmless as a commensal flora, certain strains of *E. coli* can be the main cause of diseases in humans and animals. Its impact ranges from commensal relationships to digestive tract diseases and extraintestinal pathologies such as diarrhea, urinary tract infections (UTIs), sepsis, pneumonia, and meningitis.[22,23] *Staphylococcus aureus (S. aureus)* is a Gram-positive, spherical-shaped bacterium capable of causing food poisoning, infections, and even death. This bacterium possesses various virulence components that play a role in its ability to cause disease. One of these is surface proteins that enable it to adhere to human tissues, leading to infection of the skin and soft tissue. Moreover, *S. aureus* is capable of producing multiple toxins that result in a range of clinical manifestations, from serious skin infections to food poisoning and staphylococcal scalded skin syndrome.[24,25]

Studies show that Cu^{2+} ions can inhibit *E. coli* and *S. aureus* and increase

when combined or chelated with molecules such as EDTA. EDTA is a commonly used chelation agent due to its ability to form stable complexes with metal ions, such as Cu-EDTA, which has applications in decontamination and treatment of metal poisoning.[10]

As a potent chelating agent, Ethylenediaminetetraacetic acid (EDTA) has been extensively utilized in medical practice, particularly for managing toxicity caused by heavy metals like mercury and lead. The antimicrobial effects of EDTA have been known for decades, effective against bacteria, yeast, and fungi. EDTA works by binding cations such as Mg^{2+} and Ca^{2+}, weakening the microbial cell wall and increasing the efficacy of other antimicrobials. Various EDTA salts, such as disodium, trisodium, and Ca-EDTA, exhibit antibiofilm and antimicrobial activity, with environmental pH being an important factor in their effectiveness. In Gram-negative bacteria, EDTA releases Mg^{2+} and Ca^{2+} ions from the outer cell wall, which increases sensitivity to additional antimicrobial agents.[11]

This study aims to synthesize and characterize Cu(II)-EDTA complexes based on $CuSO_4$, as well as test their antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*, along with an assessment of their cytotoxicity effects on dengue virus serotype 2 (DENV-2). The results of this research can contribute to the development of more effective antimicrobial and antiviral compounds, with the potential for wide applications in the medical and public health fields.

MATERIALS AND METHODS

Materials

The substances utilized in this study included Anhydrous $CuSO_4$ (Merck, Germany), Na-EDTA (Merck, Germany), sterile aquades, nutrient agar (Oxoid, UK), *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), Nalidixic acid (Oxoid, UK), NaCl (Merck, Germany), Minimum Essential Medium Eagle (Sigma-Aldrich, Germany), Fetal Bovine Serum (Sigma-Aldrich, Germany), Dimethyl sulfoxide (Merck, Germany), Viral ToxGloTM Assay reagent (Promega, USA), Vero Cell (ATCC CCL-81TM, USA), trypsin-EDTA (Thermo Fisher Scientific, USA), trypan blue (Thermo Fisher Scientific, USA), DENV-2 Surabaya isolate (Accession number: KT012509).

Synthesis Complex Cu(II)-EDTA

The synthesis of the compounds was carried out using the solvothermal method. That is, the Cu(II) compound used comes from anhydrous $CuSO_4$. The ratio of stoichiometry used (Cu: EDTA) is 1:1. The solution is mixed and then heated at 120°C for 24 hours. Then let it sit for 24 hours until the crystals slowly form[12] Cu(II)-EDTA crystals with blue color were obtained. Characterization of complex used UV-Vis Spectroscopy (Shimadzu UV-1650 PC), Fourier Transform Infra Red Spectroscopy (8400S Shimadzu), Differential Scanning Calorimetry (Perkin Elmer DSC 4000), and Scanning Electron Microscope (HITACHI FLEXSEM 100).

Antibacterial Activity Test

The antibacterial testing method used is the disc diffusion method, utilizing gram-negative (*E. coli*), which is frequently employed as the model bacterium to assess bactericidal efficacy, and gram-positive (*S. aureus*) bacteria, with slight modification[13], *E. coli* and *S. aureus* were subcultured in NA medium and incubated at 37°C for 24 hours. Furthermore, the bacteria were suspended in a 0.9% NaCl solution with a

concentration of 0.5 McFarland (1.5×10^8 CFU/mL). Six blank discs and one nalidixic acid disk (30 µg/disk) for positive control were placed on NA media. Test compound, containing EDTA, $CuSO_4$, Cu(II)-EDTA with concentrations of 250, 200, 150, 100, and 50 mg/mL, respectively. Sterile aquades was used for the negative control. Antibacterial effectiveness is assessed by measuring the inhibition zone that develops after 24 hours of incubation at a temperature of 37°C.

Antidengue Activity Test

The DENV-2 antiviral activity test was conducted based on the method by Sucipto et al (2019)[14] with slight modifications. Vero cell lines were employed for the assay. A confluent monolayer of Vero cells was added into 96 plate at a density of 5×10^4 cells/mL and incubated for 24 hours at 37°C with 5% CO_2. Then the medium was discarded and replace with 50 µl of MEM containing 10% FBS was added; 25 µl of samples (EDTA, and Cu(II)-EDTA) at concentrations of 31.25, 62.5, 125, 250, 500, and 1000 µg/ml; and 25 µl of DENV-2 stock at a concentration of 2 × 103 FFU/mL. Repeated 3 times. Next, the sample was incubated for 48 hours at 37°C with 5% CO_2. After incubation, 100 µl of Viral ToxGlo assay reagent was added and incubated at 37°C with 50% CO_2. The luminescence data obtained was then calculated as % of CPE (cytophatic effect) cells with the formula:

$$\%CPE = \frac{a-b}{c-b} x100\%$$

With a = luminescence of the treatment group; b = luminescence of the medium control; c = luminescence of the DENV-2 and cell control group. Based on the %CPE data, a linear regression graph is plotted with concentration (x) versus %CPE (y), using the equation y = ax + b to calculate the EC_{50} value.

Cytotoxicity Test

The cytotoxicity assessment was performed using the MTT assay method.[14] Vero cell lines were utilized for MTT assays, with confluent monolayers of Vero cells added to 96 plates (5×10^4 cells/mL) and incubated for 24 hours at 37°C with 50% CO_2. Then the medium was discarded and washed with PBS 1× 3 times. Then add 100 µl of MEM 10% FBS; 100 µl of samples (EDTA, and Cu(II)-EDTA) with concentrations of 31.25, 62.5, 125, 250, 500, and 1000 µg/ml; and incubated for 24 hours at 37 °C 5% CO_2. Repeated 3 times. At the end of incubation, the culture medium containing the sample is discarded, and washed with 100 µl of PBS, and 10 µl of MTT reagent is added. Incubate again for 4 hours and wash with PBS. Absorbance is read with a microplate reader at 595 nm. The data obtained is absorbance data, and the % of living cells is calculated by the formula:

$$\%\text{cell viability} = \frac{a-b}{c-b} x100\%$$

Where, a = absorbance with treatment; b = media control absorbance; c = absorbance of cell control. Next, a linear graph of concentration vs % of viability cells was created to determine CC_{50}.

RESULTS AND DISCUSSION

This study successfully synthesized and characterized the Cu(II)-EDTA complex using $CuSO_4$ as a precursor. The solvothermal method produced blue crystals Cu(II)-EDTA with a Cu stoichiometry ratio of 1:1. The results of the characterization were obtained by using Differential Scanning Calorimetry (DSC) to identify phase changes, such as

melting, crystallization, decomposition, or transitions in a material, in this case Cu(II)-EDTA (Figure 1).

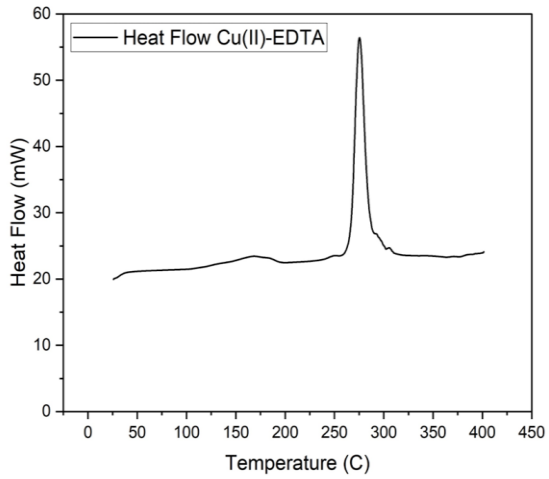

Figure 1. Thermal analysis measurement results of Cu(II)-EDTA at various heating rates using DSC.

The graph shows the thermal profile of Cu(II)-EDTA, with the x-axis indicating the temperature (in °C) and the y-axis indicating the heat flow (in mW). There is a significant endothermic peak around 270-300°C, which indicates a phase change or thermal decomposition process. Cu(II)-EDTA exhibits good thermal stability up to about 250°C, which means it is suitable for use in pharmaceutical formulations that require processing or storage at temperatures below that decomposition point. This knowledge of thermal stability is essential for the development of efficient and effective drug formulations, especially to ensure pharmaceutical effectiveness, safety, and stability of the final product.[15,16]

Particle size characterization is also necessary because the very small particle size facilitates penetration into the cell membrane, thereby increasing intracellular antibacterial activity and reactions. This is important because biofilm microorganisms are more resistant to antibacterial agents than planktonic pathogens, so effective treatment requires higher concentrations of agents.[17] The results obtained a particle size of 6.31 nm with a polydispersity index (PDI) of 0.076. This shows that the synthesized Cu(II)-EDTA is not yet qualified as a nanoparticle measuring <100 nm.[18] In addition, the PDI Cu(II)-EDTA value proves the uniformity of the particle size distribution because the closer the PDI value is to 0, the more uniform the sample size distribution is observed.[19]

In characterizing the functional groups contained in the synthesized molecules, the FTIR (Fourier Transform Infrared Spectroscopy) instrument is used. Here is a detailed analysis of the IR spectra of EDTA and Cu(II)-EDTA (Figure 2).

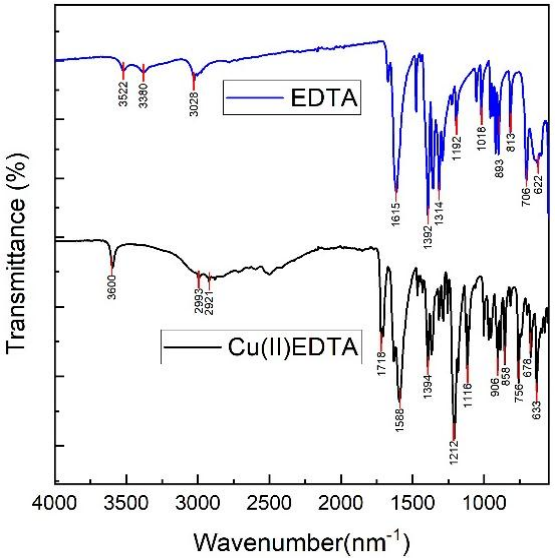

Figure 2. Results of measuring the spectra of EDTA and Cu(II)-EDTA chelates using FTIR

When EDTA is complexed with Cu^{2+} ions, there is a significant band shift. The O-H peak in the EDTA at 3522 cm^{-1} is lost or shifted, suggesting that the coordination of Cu^{2+} ions causes the -OH group to no longer be free. The C=O band at 1615 cm^{-1} in pure EDTA shifted to about 1588 cm^{-1}. This shift suggests that the carboxylate group ($-COO^-$) in EDTA participates in coordination with Cu^{2+} ions. The N-Cu band (stretching) at about 1212 cm^{-1} shows the interaction between the nitrogen atoms in EDTA and the Cu^{2+} ion. This indicates that nitrogen atoms are also involved in the

formation of complexes with Cu(II). This FTIR spectrum confirms that EDTA has successfully complexed with Cu(II) ions, which is indicated by a characteristic band shift in the C=O group, the loss of free O-H bands, and the emergence of new bands related to Cu-N and Cu-O interactions. This shift and change in IR peak intensity indicate a change in chemical structure, from free EDTA to Cu(II)-EDTA complex.

At 5000 magnifications (Figure 3A), the structure of the Cu(II)-EDTA particles is very clear. The particles exhibit an irregular and layered shape, suggesting that this complex has crystalline properties. At 2000 magnifications (Figure 3B), the layered structure and various particle sizes can be seen more clearly. The particles appear to be bound in a complex structure, suggesting that Cu(II)-EDTA is formed through a strong bond between Cu(II) and EDTA ions. At 1000 magnifications (Figure 3C), the overall structure of Cu(II)-EDTA can be clearly observed. The shape of the particles shows the morphology of large aggregates with a thick layer structure. The layered structure and the presence of agglomerations can affect the solubility and release properties of compounds in pharmaceutical applications. Morphology like this could mean that the Cu(II)-EDTA complex may have a slower release because the non-uniform and coated surface can slow diffusion in body fluids.

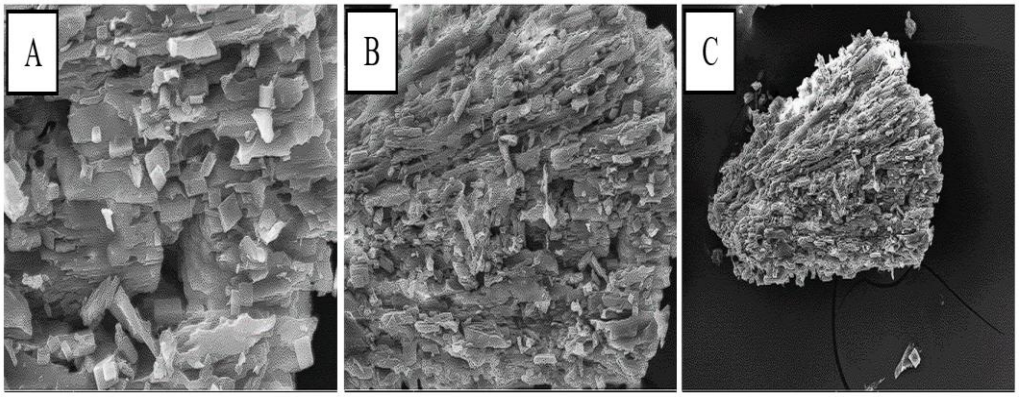

Figure 3. SEM images of Cu(II)-EDTA with magnification (A) 5000 times, (B) 2000 times, and (C) 1000 times

The results of the maximum wavelength measurement using the UV-Vis instrument (Figure 4) show that EDTA has a maximum wavelength in the UV region at about 244 nm, where EDTA absorbs strongly at 230 nm and flows to the absorbance range of 260 nm.[20] Meanwhile, the absorption of $CuSO_4$ is about 740 nm, where this result is similar to the wavelength reported by Hernández-López et al. (2021).[21]

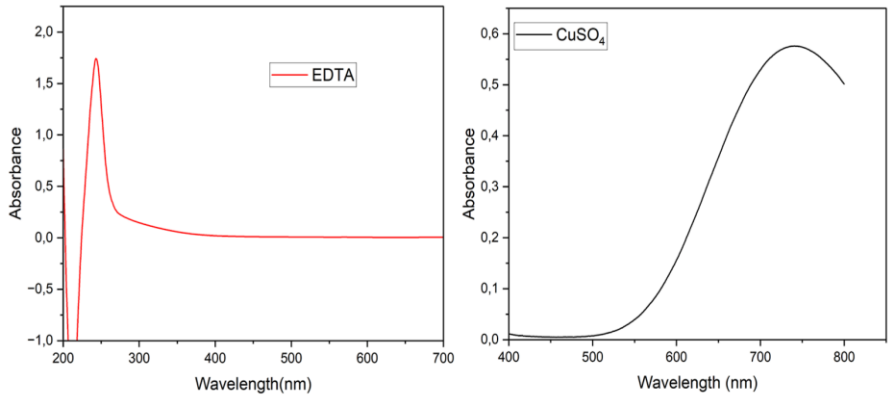

Figure 4. The maximum wavelength of EDTA and $CuSO_4$

An inverse relationship was observed between the concentration of the Cu(II)-EDTA complex and cell viability presented in the graph (Figure 5). The CC_{50} value of 415 µg/ml (Table 1) indicates that Cu(II)-EDTA exhibits a moderate to low level of cytotoxicity. This reduction in cell viability is dose-dependent, as evidenced by the strong correlation between increasing Cu(II)-EDTA concentration and its cytotoxic effects.

Table 1. CC_{50} values indicating cytotoxicity of the tested compounds

Compounds	CC_{50} (µg/ml)
Cu(II)-EDTA	415
$CuSO_4$	High Toxic
EDTA	High Toxic

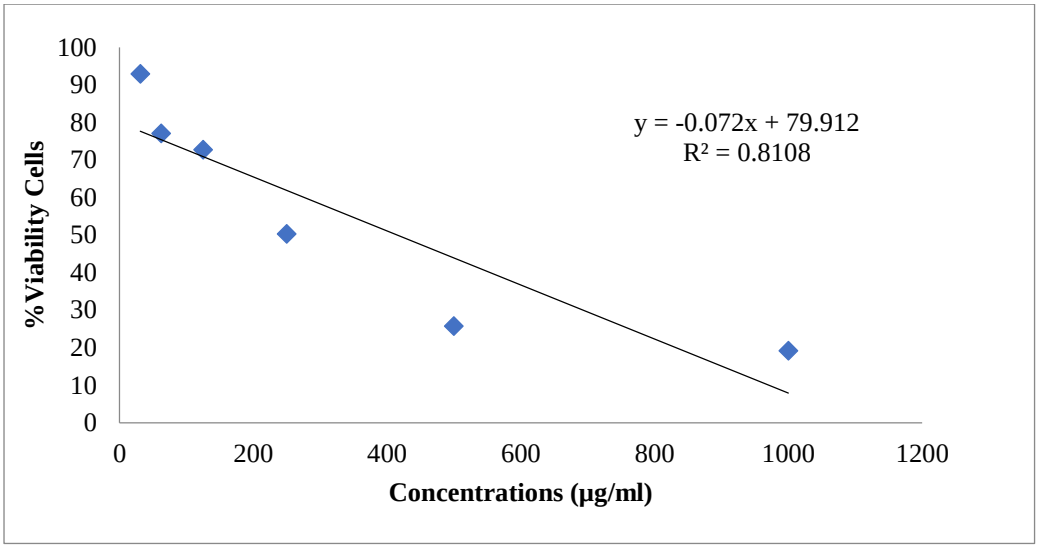

Figure 5. Cytotoxicity curve of Cu(II)-EDTA after treatment using the MTT assay.

The synthesized and characterized Cu(II)-EDTA that have been characterized are then evaluated for antibacterial activity against *E. coli* and *S. aureus*, with the results shown in Table 2 (*E. coli* antibacterial activity) and Table 3 (*S. aureus* antibacterial activity).

Table 2. *E.coli* antibacterial activity test results

Concentration (ppm)	EDTA	$CuSO_4$	Cu(II)-EDTA	K+
	Inhibition Zone ± SD	Inhibition Zone ± SD	Inhibition Zone ± SD	Inhibition Zone ± SD
250,000	24.05 ± 0.82	24.51 ±1.32	11.95 ± 0.15	31.32± 0.29
200,000	22.41 ± 0.30	23.17 ± 1.75	10,51 ± 0.06	
150,000	20.73 ± 0.60	22.17 ± 1.45	10.94 ± 0.83	
100,000	20.08 ± 1.60	20.61 ± 3.06	9.28 ± 1.32	
50,000	13.07 ± 1.99	15.24 ± 4.40	0.00 ± 0.00	

Table 3. *S. aureus* antibacterial activity test results

Concentration (ppm)	EDTA	$CuSO_4$	Cu(II)-EDTA	K+
	Inhibition Zone ± SD	Inhibition Zone ± SD	Inhibition Zone ± SD	Inhibition Zone ± SD
250,000	16.22 ± 2.14	26.35 ±1.09	11.03 ± 0.55	12.11 ± 0.74
200,000	16.04 ± 2.36	24.63 ± 2.10	8.99 ± 1.00	
150,000	15.75 ± 3.89	22.53 ± 0.32	9.40 ± 1.32	
100,000	15.84 ± 2.32	18.60 ± 2.43	7.95 ± 0.37	
50,000	0.00 ± 0.00	13.09 ± 0.63	7.60 ± 0.19	

The antibacterial activity of EDTA against *E. coli* showed the highest inhibitory zone of 24.05 mm at a concentration of 250,000 ppm and the lowest of 13.07 mm at 50,000 ppm, with increased concentrations resulting in a larger inhibitory zone. $CuSO_4$ produced the highest inhibition zone of 24.51 mm at 250,000 ppm and decreased to 15.24 mm at 50,000 ppm, demonstrating consistent antibacterial effects. In contrast, Cu(II)-EDTA showed the lowest activity with an inhibition zone of 11.95 mm at 250,000 ppm and 0 mm at 50,000 ppm, indicating significantly lower effectiveness than EDTA or $CuSO_4$ against *E. coli*.

EDTA showed the highest inhibition zone of 16.22 mm at 250,000 ppm against *S. aureus* (Table 3), but no inhibition zone (0 mm) at 50,000 ppm, indicating its effectiveness only at high concentrations. $CuSO_4$ has strong antibacterial activity with an inhibition zone of 26.35 mm at 250,000 ppm and decreases to 13.09 mm at 50,000 ppm. Cu(II)-EDTA showed a weaker effect, with an inhibition zone of 11.03 mm at 250,000 ppm and 7.60 mm at 50,000 ppm, much lower than $CuSO_4$.

EDTA has limited antibacterial activity due to its ability to chelate cations from the bacterial outer membrane. High concentrations of EDTA, such as 10%, can produce significant inhibitory zones, but lower concentrations indicate minimal effectiveness. EDTA activity remains as long as chelation has not formed a bond with metal ions.[19] In addition, Cu^{2+} ions from $CuSO_4$ produce Reactive Oxygen Species (ROS), such as O_2 and H_2O_2, which cause damage to *S. aureus* and *E. coli* bacterial cells. The reaction of Haber-Weiss and Fenton triggers the formation of hydroxyl radicals (•OH), which are highly reactive and damage the lipids, proteins, and DNA of bacteria. This damage interferes with the integrity of cell membranes, leading to the death of bacteria. This mechanism explains the powerful antibacterial effects of Cu^{2+}.[26] The Cu(II)-EDTA complex has lower antibacterial activity than pure $CuSO_4$ and EDTA because Cu^{2+} ions are tightly bound by EDTA, so the amount of free Cu^{2+} that can produce oxidative stress or attack bacteria is reduced. EDTA may bind Cu^{2+} too strongly, decreasing antibacterial effectiveness. However, at high concentrations, Cu(II)-EDTA still exhibits antibacterial activity, likely due to increased membrane permeability by EDTA and partial release of Cu^{2+} from the complex.

EDTA and $CuSO_4$ were also tested for antiviral activity against dengue virus using DENV-2 because it is more closely related to dengue hemorrhagic fever (DHF) cases, which are more severe compared to other serotypes. Research shows that DENV-2 is significantly more commonly associated with dengue cases than DENV-1. Moreover, DENV-2 and DENV-3 are reported to carry a twofold higher risk of triggering dengue infection compared to DENV-4 [21] The results of the antidengue assay indicated that the EDTA concentration needed to inhibit 50% of the replication of the DENV-2 virus was 356.13 µg/mL. This EC_{50} value indicates that EDTA has low potency as an antiviral agent against DENV-2, as the required concentration is quite high. In addition, $CuSO_4$ is more effective than EDTA in inhibiting DENV-2 replication, due to its lower EC_{50} value (77.86 µg/mL). This indicates that $CuSO_4$ exhibits greater antiviral efficacy against DENV-2 than EDTA.

Based on the slope of the two compounds (Figure 6), it can be seen that $CuSO_4$ has a greater inhibition effect at lower concentrations than EDTA. However, the effectiveness of $CuSO_4$ decreases drastically as its concentration

increases. EDTA showed a much slower decline in effectiveness. This could indicate that EDTA works more slowly or inefficiently in inhibiting DENV-2 replication than $CuSO_4$, but maintains a more stable inhibition at various concentrations.

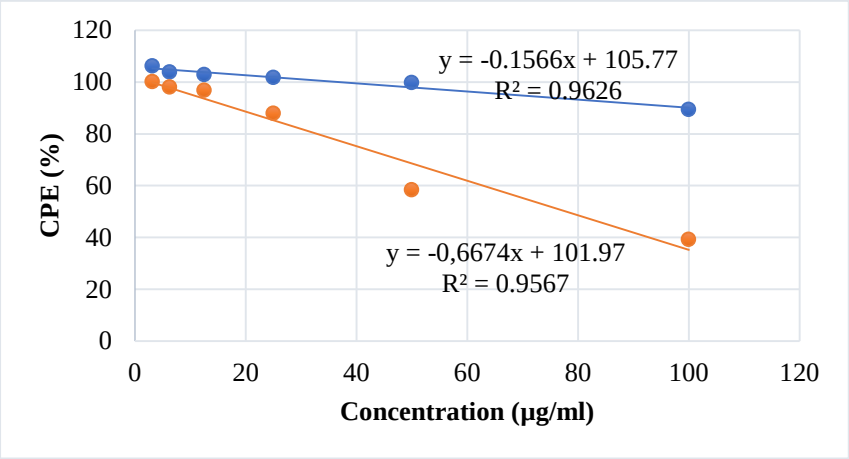

Figure 6. Regression curve of the inhibited DENV-2 after treatment with EDTA (blue line colour) and $CuSO_4$ (orange line colour)

STRENGTH AND LIMITATION

The strength and limitation of this study is there ae not many studies about antiviral from complex compound, especially DENV in Indonesia. The isolate we used for this study were native to Indonesia. This kind of research is certainly essential in Indonesia, as each isolate will have different effects on a drug candidate. Based on the results of this study, it can be used as a referenece for similar reasearch in the future.

CONCLUSIONS

This study succeeded in synthesizing the Cu(II)-EDTA complex using the solvothermal method. The complex has a small particle size and uniform size distribution, but the antibacterial and antiviral activity against DENV-2 is lower compared to $CuSO_4$. $CuSO_4$ was shown to be more effective in inhibiting DENV-2 replication, while the Cu(II)-EDTA complex showed potential as an antibacterial agent, although its effectiveness was lower.

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None to declare.

CONFLICT OF INTEREST

The authors state that there is no conflict of interest to disclose.

AUTHOR CONTRIBUTION

Conception, design, and material: BN and THS; supervision and resources: THS; data collection or processing and literature search: BN, THS, and TJK; analysis or interpretation: TJK and BN; writing manuscript: BN and TJK; and critical review: THS, TJK, HH, and SR.

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