UNIVERSITI PUTRA MALAYSIA

EVALUATION OF ANTIMICROBIAL EFFICACY OF PHOSPHANECOPPER (I) BENZOYLTHIOUREA SERIES ON GRAM-NEGATIVE BACTERIA ISOLATED FROM FELINE URINARY TRACT INFECTION

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CERTIFICATION

It is hereby certified that we have read this project paper entitled "Evaluation of antimicrobial efficacy of phosphanecopper (i) benzoylthiourea series on gram-negative bacteria isolated from feline urinary tract infection", by Saifuddin Rabbani Bin Adam Shah and in our opinion, it is satisfactory in terms of scope, quality, and presentation as partial fulfillment of the requirement for the course VPD 4999 – Project.

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TABLE OF CONTENTS

LIST OF THE TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

E. coli	=	*Escherichia coli*
K. pneumoniae	=	*Klebsiella pneumoniae*
P. aeruginosa	=	*Pseudomonas aeruginosa*
S. aureus	=	*Staphylococcus aureus*
M	=	Molar
G	=	gram
mg	=	miligram
mL	=	mililitre
µL	=	microlitre
CFU	=	colony forming unit
AST	=	antibiotic sensitivity test
MIC	=	minimum inhibitory concentration
MBC	=	minimum bactericidal concentration
UTI	=	urinary tract infections
ZOI	=	zone of inhibition

ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4901-Projek.

PENILAIAN KEBERKESANAN ANTIMIKROB SIRI PHOSPHANECOPPER (I) BENZOYLTHIOUREA KE ATAS BAKTERIA GRAM-NEGATIF DIASINGKAN DARI JANGKITAN SALURAN KENCING KUCING

Oleh

Saifuddin Rabbani Bin Adam Shah

2023

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Jangkitan saluran kencing (JSK) merupakan penyakit umum pada pesakit kucing, sering kali disebabkan oleh bakteria Gram-negatif. Kemunculan bakteria tahan antimikrob (AMR) dalam populasi kucing yang berkaitan dengan JSK telah menjadi kebimbangan yang meningkat di seluruh dunia. Oleh itu, pembangunan rawatan alternatif adalah penting untuk mengatasi masalah ini yang disebabkan oleh bakteria tahan pelbagai ubat (MDR). Oleh itu, kajian ini dijalankan untuk menentukan keberkesanan antimikrob sebatian Siri Phosphanecopper (I) Benzoylthiourea terhadap bakteria Gram-negatif yang diasingkan daripada jangkitan saluran kencing (JSK) kucing yang terdiri

daripada *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*), dan *Klebsiella pneumoniae* (*K. pneumoniae*). Tiga bakteria Gram-negatif yang disimpan di Makmal Bakteriologi Veterinar, Universiti Putra Malaysia, dikeluarkan dan disubkultur pada agar nutrien sebelum ujian kepekaan antimikrob (AST) menggunakan kaedah disk difusi Kirby-Bauer dan kaedah difusi sumur agar pada dulang agar Muller-Hinton (MH) bersama-sama dengan kepekatan inhibitori minimum (MIC) menggunakan kaedah pencairan kaldu dan kepekatan membunuh minimum (MBC) pada dulang MH. Sebatian terpilih daripada Siri Phosphanecopper (I) Benzoylthiourea diuji pada isolat klinik yang disimpan dengan kepekatan yang berbeza seperti Cu(I)ITri (3.125μM, 1.56μM, 0.78μM, 0.39μM), Cu(I)ITriDie (3.125μM, 1.56μM, 0.78μM, 0.39μM), Cu(I)ITriSar (6.25μM, 3.125μM, 1.56μM, 0.78μM), dan Cu(I)ITriGly (25μM, 12.5μM, 6.25μM, 3.125μM). Hasil kajian ini menunjukkan bahawa tiada zon perencatan yang jelas dapat diperhatikan menggunakan kaedah difusi cakera dan difusi sumur agar. Ujian kepekaan antimikrob (AST) menunjukkan bahawa ketiga-tiga isolat klinik tidak peka terhadap mana-mana sebatian yang dipilih pada kepekatan yang berbeza. Selain itu, hasil MIC dan MBC juga menunjukkan bahawa semua isolat klinik yang disimpan tidak peka terhadap semua sebatian terpilih kerana tiada sumuran yang jelas dapat diperhatikan dan hasil MBC terlalu banyak untuk dihitung (TNTC). Walau bagaimanapun, hasil MIC menunjukkan perbezaan kecil dalam bacaan kekeruhan optik pada panjang gelombang 600 nm untuk sumuran pertama dengan kepekatan 1.56μM (Cu(I)ITri), 1.56μM (Cu(I)ITriDie), 3.125μM (Cu(I)ITriSar), dan 12.5μM (Cu(I)ITriGly) terhadap E. coli, P. aeruginosa, dan

K. pneumoniae. Oleh itu, kompleks kuprum ini tidak menunjukkan aktiviti antimikrob yang signifikan terhadap isolat klinik yang disimpan, dan penyelidikan lanjut diperlukan dengan kemungkinan melibatkan kepekatan yang lebih tinggi dengan ujian tambahan.

Kata Kunci: Cu(I)ITri, Cu(I)ITriDie, Cu(I)ITriSar, Cu(I)ITriGly, kaedah penyebaran cakera, kaedah penyebaran sumur agar, MIC, MBC, ujian kepekaan antimikroba, isolat klinikal yang disimpan.

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfillment of the course VPD 4901 - Project.

EVALUATION OF ANTIMICROBIAL EFFICACY OF PHOSPHANECOPPER (I) BENZOYLTHIOUREA SERIES ON GRAM-NEGATIVE BACTERIA ISOLATED FROM FELINE URINARY TRACT INFECTION

by

Saifuddin Rabbani Bin Adam Shah

2023

Supervisor: Dr. Sharina Binti Omar

Co-supervisor: Dr. Nazzatush Shimar Binti Jamaludin

Urinary tract infections (UTIs) are a common ailment in feline patients, often caused by Gram-negative bacteria. The emergence of antimicrobial resistance (AMR) bacteria in feline populations associated with UTIs has been a growing concern worldwide. Thus, development of alternative treatments is crucial to overcome this problem caused by multi drug resistant (MDR) bacteria. Hence, this study was conducted to determine the antimicrobial efficacy of Phosphanecopper (I) Benzoylthiourea Series compound on gram-negative bacteria isolated from feline urinary tract infections (UTIs) comprising *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*), and

Klebsiella pneumoniae (*K. pneumoniae*). Three archived gram-negative bacteria were retrieved from Veterinary Bacteriology Laboratory, University Putra Malaysia and kept at room temperature and subcultured on nutrient agar prior to antimicrobial susceptibility test (AST) using Kirby-Bauer disk diffusion method and agar well diffusion method on Muller-Hinton (MH) agar plates along with minimum inhibitory concentration (MIC) using broth dilution method and minimum bactericidal concentration (MBC) on MH agar plates. Selected compounds from Phosphanecopper (I) Benzoylthiourea Series were tested on the archived clinical isolates with different concentrations such as Cu(I)ITri (3.125µM, 1.56µM, 0.78µM, 0.39µM), Cu(I)ITriDie (3.125µM, 1.56µM, 0.78µM, 0.39µM), Cu(I)ITriSar (6.25µM, 3.125µM, 1.56µM, 0.78µM) and Cu(I)ITriGly (25µM, 12.5µM, 6.25µM, 3.125µM). The findings of this study revealed that no distinct zone of inhibition can be observed using the disk diffusion and agar well diffusion methods, antimicrobial sensitivity testing (AST) indicated that the three clinical isolates were not susceptible to any of the selected compounds at varied concentrations. Moreover, results of MIC and MBC also showed that all the archived clinical isolates were not susceptible to all the selected compounds due to no clear well can be observed and the result for MBC is too numerous to count (TNTC). However, results of MIC showed slight difference in optical density (OD) reading at 600 nm wavelength (TECAN Sunrise) for the first wells with concentration of 1.56µM (Cu(I)ITri), 1.56µM (Cu(I)ITriDie), 3.125µM (Cu(I)ITriSar) and 12.5µM (Cu(I)ITriGly) against *E. coli*, *P. aeruginosa* and *K. pneumoniae*. Therefore, the copper complexes do not have significant antimicrobial activity against archived clinical isolates and further research is warranted potentially involving higher concentration with additional tests.

Keywords: Cu(I)ITri, Cu(I)ITriDie, Cu(I)ITriSar, Cu(I)ITriGly, disc diffusion method, agar well diffusion method, MIC, MBC, antimicrobial sensitivity testing, archived clinical isolates.

1.0 INTRODUCTION

1.1 Background

The emergence of antimicrobial resistant (AMR) bacteria in animal populations has been a growing concern worldwide. According to World Health Organization (2021) AMR bacteria occurs through genetic changes which are found in people, animals, food and the environment that can be transmitted from human to human or between human and animals, including from food of animal origin. Nowadays, AMR has a serious impact on society, both economically and in terms of healthcare. Multi drug resistant (MDR) bacteria have recently increased due to overuse and misuse of antibiotics to animals resulted in transmission of resistant bacteria (Caneschi et al., 2023).

Urinary tract infections (UTIs) are among the most common diseases reported in cats in various countries globally (Siti Aisyah Jeinie, 2017). According to Vercelli et al. (2021), urinary tract infections (UTIs) are frequently identified in small animal clinics without prior microbiological assessment, and they often receive inappropriate empirical antibiotic therapy. In the veterinary domain, bacterial infections causing UTIs have been a significant driver of the extensive utilization of antimicrobial medications. Consequently, the inappropriate administration of antibiotics in this context has contributed to growing concerns about antimicrobial resistance (Siti Noor Shuhada Iberahim, 2018). As a result, there is a need to develop alternative treatments to overcome this problem caused by MDR bacteria. Hence, studies have been done to find other alternatives of antimicrobial agents on metal compounds including copper metal.

Phosphanecopper (I) benzoylthiourea is a type of series that contains copper complexes bind together with ligands of amino acid and hydroxylamine that has been shown to possess antimicrobial properties. Rauf et al. (2009) has shown that thiourea ligands and their metal complexes exhibit antibacterial activity when they are complexed with copper. An in vivo study has demonstrated that biologically active compounds exhibit an increased bacteriostatic and carcinostatic effect when subjected to chelation, as reported by Chohan, Arif, Akhtar, and Supuran in 2006. Currently, there is ongoing research into the potential antibacterial properties of copper complexes. These complexes are meticulously designed to offer advantageous characteristics, particularly in medicinal applications. Chohan et al. (2006) have reported that, when combined with ligands such as amino acids, copper and various other metal ions enhance their inhibitory potency against gram-negative bacteria, including *E. coli*, and *P. aeruginosa*, as demonstrated in vitro.

In Malaysia, studies regarding the efficacy of copper complexes against bacteria of animal origin associated with UTIs in companion animals is scarce. Thus, this study provides valuable insights into the potential of copper-based compounds as an alternative treatment for gram negative bacterial infection and to contribute to the development of new and effective antimicrobial agents to combat bacterial infections in animals.

1.2 Objective

The objectives of this study are:

1. To identify antimicrobial effects of selected Phosphanecopper (I) Benzoylthiourea series [Cu(I)ITri], [Cu(I)ITriDie], [Cu(I)ITriGly] and [Cu(I)ITriSar] by using antibiotic sensitivity testing (AST) through disc diffusion and agar well diffusion methods.
2. To determine the effectiveness of selected Phosphanecopper (I) Benzoylthiourea series against selected archived Gram-negative bacteria isolated from feline UTIs as an alternative to antibiotic therapy using broth microdilution method to determine minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).

2.0 LITERATURE REVIEW

2.1 Common bacteria isolated in urinary tract infections (UTIs) in cats

Urinary tract infection in cats is a frequently encountered clinical condition in veterinary medicine which often treated with empirically prescribed antibiotics. As mentioned in the BSAVA Manual of Canine and Feline Nephrology and Urology, the origin and development of bacteria detected in the feline urinary tract may result from pathogens ascending from the lower urogenital tract to the initially sterile regions of the proximal urethra, bladder, and upper urinary tract. This process can consequently lead to the occurrence of infection (Bartges and Olin, 2017). Bacteria of urinary tract infection are divided into gram-positive and gram-negative bacteria. According to the study by Bartges and Olin (2017) and Litster *et al.* (2009), in their study of feline bacterial urinary tract infections, the most common isolated bacteria from feline UTIs consist of gram-positive *Enterococcus faecalis* (7%-21%) and gram-negative *E. coli* with the highest percentage (39%-59%) of positive cultures. Other bacteria that are commonly isolated are *Klebsiella* spp., *Pasteurella* spp., *Pseudomonas* spp., and *Corynebacterium* spp. (Bartges and Olin, 2017)

Several studies conducted in diverse demographic regions have yielded comparable results in identifying prevalent bacterial isolates in feline urinary tract infections (UTIs). For instance, research conducted in European countries like Norway, United Kingdom, Italy and Spain has indicated that *E. coli* is the most frequently isolated gram-negative bacterium, while *Enterococcus* spp, *Staphylococcus* spp, and *Streptococcus canis* are the most commonly isolated gram-positive bacteria (Eggertsdottir et al., 2007; Fonseca et al., 2021; Vercelli et al. 2021; Darwich et al. 2020). According to Chan et al. (2022) and Garces et al. (2022), these bacteria have also known to have already become multi drug resistance which means that the bacteria are resistant to atleast one agent in three or more class of antibiotic.

2.2 Antimicrobial properties of copper compound

Copper has been known for its antimicrobial activity where it can rapidly kill bacteria. Numerous studies have investigated the efficacy of copper compounds in inhibiting the growth and proliferation of bacteria. According to Benhalima et al. (2019) the antibacterial mechanisms of copper are still being studied, but it is known to produce inactivation of enzymatic pathways, formation of reactive oxygen species, precipitation of bacterial proteins, modification of their cell wall and destruction or alteration of the synthesis of nucleic acids, without being mutagenic. Copper nanoparticles (CuNPs) have the ability to infiltrate the bacterial cell wall, leading to damage at the cellular level. Within the cell, these nanoparticles indirectly influence DNA or protein synthesis, deactivate enzymes, and stimulate the production of hydrogen peroxide (Beatriz et al., 2021). Copper has also been used as materials in touch surfaces such as door handles, bed rails and places which highly contaminated with microbes commonly in hospitals for its antimicrobial ability in contact killing (Grass et al., 2010). According to few other studies done by Applerot et al. (2012) and Cronholm et al. (2011) stated that copper ions and CuNPs act as redox-active species, inducing oxidative stress by generating reactive oxygen species (ROS) that damage cellular components, potentially leading to apoptosis. Therefore, ongoing studies on the antimicrobial effect of copper compounds are being done to create an alternative for antibiotic therapy.

2.3 Amino acid, diethanolamine and thiourea ligands complexed copper

Amino acids play a crucial role in various biochemical processes, serving as the building blocks of proteins that form the structural foundation for all living organisms. Due to their ability to coordinate transition metals through amino or carboxylic groups, amino acids are recognized as effective chelating agents, and the interaction of these complexes has been extensively studied. Broeren et al. (2013) highlight the involvement of amino acid interactions in enzymatic activity and the stability of protein structures, making them valuable model systems for understanding metal-protein interactions in biological systems. Additionally, Moore et al. (2019) note that metal-amino acid chelates can facilitate the transport of metal ions across membranes, forming through the reaction of metal salts with amino acids.

Diethanolamine-based amino acid derivatives have potential antibacterial activity which the molecule has amphiphilic nature, small size, one or more positive charges, required degree of hydrophobicity and hydrophilic-lipophilic balance value to overcome resistance of pathogenic bacterial strains (Korotkin et al., 2022). Rauf et al. (2009) stated that thiourea ligands and their metal complexes exhibit antibacterial activity when complexed with copper. Copper and various other metal ions enhance their inhibitory potency against gram-negative bacteria including *E. coli* and *P.*

aeruginosa, when combined with ligands such as amino acids (Chohan et al., 2006). An in vitro study by Arslan et al. (2009) reported that all the Cu complexes with thiourea derivative ligand possess inhibitory effect on the growth of gram-negative bacteria including *E. coli* (ATCC 25922) and *P. aeruginosa* (ATCC 27853).

2.4 Antibiotic sensitivity test

According to Balouiri et al. (2016) agar disk-diffusion test by using Kirby-Bauer disk diffusion method is commonly used in many microbiology laboratories for routine antimicrobial susceptibility testing providing qualitative results categorizing bacteria as susceptible, intermediate, or resistant. The author also mentioned that disk-diffusion method is not appropriate to determine the minimum inhibitory concentration (MIC) as it is impossible to quantify the amount of antimicrobial agent diffused into agar medium and the advantage is its low cost, simplicity, and the ease to test large quantity of bacteria and antimicrobial agent. The agar well diffusion method is similar to disk-diffusion method except it is widely used to evaluate the antimicrobial activity of plants, microbial extract and metal compounds with better efficacy (Balouiri et al., 2016). The assessment of MIC and MBC plays a vital role in the evaluation of new compounds tested on microorganisms, providing insights into their effectiveness against microorganisms. Dilution methods consisting of broth micro or macro dilution is the most common basic AST method to estimate the concentration of the tested antimicrobial agent quantitatively against bacteria (Schwarz et al., 2010)

3.0 METHODOLOGY

3.1 Compound preparation

The copper compounds were retrieved from Bangunan Makmal Kimia Universiti Malaya, designed by Dr. Nazzatush Shimar a biochemist in University of Malaya. The copper compound precursor was mixed with the ligands to produce copper complexes by specific chemical procedures. This newly design copper complexes has been tested for its concentration on fish embryo acute toxicity (FET) test based on OECD guidelines (2013) to produced desired concentration within the range of lethal concentration (LC) 100 and LC 50 where 100% and 50% of the fish embryo population died after 96hr of exposure to the copper compound. This concentration is stable, avoids precipitation and suitable for use in experimental study. The compounds are Copper (I) Iodide Triphenylphosphine [Cu(I)ITri], Copper (I) Iodide Triphenylphosphine + N-[bis(2-hydroxyethyl)amino]thioxomethyl]-benzamide [Cu(I)ITriDie], Copper (I) Iodide Triphenylphosphine + N-[(benzoylamino)thioxomethyl]-glycine [Cu(I)ITriGly], Copper (I) Iodide Triphenylphosphine + N-[(benzoylamino)thioxomethyl]-methyl-glycine [Cu(I)ITriSar]. These compounds were then weighed for stock concentration of 6.25μM, 12.5μM and 50μM to match LC100. The compounds were dissolved in dimethyl sulfoxide (DMSO) and further diluted with sterile distilled water until reached 0.5% DMSO. Two-fold serial dilution was done to make four different concentrations to be used in AST procedure.

3.2 Bacterial sample collection

The bacteria sample used consist of Gram-negative bacteria of *Escherichia coli* (691), *Pseudomonas aeruginosa* (505), *Klebsiella pneumoniae* (071), *S. aureus* ATCC 25923 were retrieved from archived clinical isolates in Veterinary Bacteriology Laboratory, Veterinary Laboratory Services Unit (VLSU), Universiti Putra Malaysia (UPM). These archived clinical isolates were taken from urine samples of feline species that were sent to the laboratory for bacterial isolation and identification between the period of 1st January 2021 and 30th September 2022. The archived isolates used were from feline species with tentative diagnosis of bacterial cystitis, feline lower urinary tract disease (FLUTD), feline idiopathic cystitis (FIC) and other correlation with UTIs diseases.

3.3 Bacteria subculture from archived samples

The archived bacterial isolates were kept in agar slant vial at room temperature. Nutrient agar media were prepared prior to bacteria culture which was kept in a chiller at 4°C. The isolates were then cultured onto a nutrient agar in order to reactivate the bacteria. The samples were incubated in an incubator at 37°C for 18-24 hours. The isolates were subculture again to obtain a higher number of pure colonies from a single culture plate. Screening using Gram staining was done once to identify and confirm the samples are gram-negative based on the labelling on the stock samples. New subculture will be done every time prior to AST procedures.

3.4 Antimicrobial susceptibility test

Antimicrobial susceptibility testing (AST) procedures in this study include Kirby-Bauer Disk Diffusion Susceptibility Test, Agar Well Diffusion test and Broth Microdilution method to determine MIC and MBC. The procedure was done based on the laboratory Manual of Antimicrobial Susceptibility Testing Techniques 2023 Department of Microbiology, FBSB, UPM following the guidelines of CLSI 2020 30th Edition M100 Performance Standard for Antimicrobial Susceptibility Testing. Bacterial suspension was adjusted to match turbidity of 0.5 McFarland which was equivalent to 1-2 x 10^8 cfu/ml and the bacteria was lawned onto Mueller-Hinton (MH) agar. 30µL of each copper compound was dispensed onto blank disk together with Ampicillin 10µg/ml as negative control and DMSO 0.5% as positive control. The disk was placed on the MH agar and incubated at 37°C for 24 hours and the zone of inhibition (ZOI) was measured after the incubation. As for the agar well diffusion method, similar to disk diffusion method except wells was bored on MH agar using micropipette tip and 100µL of each compound was dispensed into each well together with Ampicillin and DMSO in the other respective well.

Next, broth microdilution method was used to determine the MIC and MBC value. Mueller-Hinton (MH) broth and 96 well plates were used in this method. Bacterial suspension was prepared, and turbidity was adjusted to 0.5 McFarland which was equivalent to 1-2 x 10^8 cfu/ml. About 150µL of MH broth was dispensed into all wells from column 1-12 from row A-H and then 150µL of each compound with different concentration was dispensed into well 1 from row A-H. Two-fold dilution was done from column 1-10 while well 12 was left as blank well and well 11 served as control without any antibiotic or compound and one plate was made with ampicillin added in each well to act as control measures. Then, 50µL of the bacterial suspension was added into all wells and the optical density (OD) reading at 600nm wavelength using Tecan Sunrise was measured before incubating the plates at 37°C for 18-20 hours. After the incubation, the OD reading was measured once again to aid our eye in evaluating the difference in turbidity level before and after incubation. The lowest concentration of compound at which the bacterial growth is completely inhibited is identified as the MIC value. Furthermore, after the MIC was determined 100µL was drawn and dispensed onto MH agar from the 1xMIC, 2xMIC and 4xMIC wells which the inhibition of growth occurs. The mixture from the wells was spread using a sterile spreader and the MH plates were incubated at 37°C for 24 hours. After the incubation, plates were inspected, and visible colonies were counted and the lowest concentration showing no bacterial growth is reported as the MBC value.

4.0 RESULTS

Based on test done on disk diffusion method and agar well diffusion method, it was observed that there was no zone of inhibition (ZOI) around the disk containing the tested copper compound observed on the agar lawned with *E. coli*, *P. aeruginosa* and *K. pneumoniae* against Cu(I)Tri and Cu(I)TriDie at concentrations of 3.125 µM, 1.5625 µM, 0.785 µM, and 0.39 µM, Cu(I)TriGly at concentrations of 25 µM, 12.5 µM, 6.25 µM, and 3.125 µM, and Cu(I)TriSar at concentrations of 6.25 µM, 3.125 µM, 1.5625 µM, and 0.785 µM. As for the positive and negative control for antimicrobial agent using ampicillin 10µg/ml and 0.5% DMSO, both controls showed no zone of inhibition on *E. coli*, *P. aeruginosa* and *K. pneumoniae.* However, only for plates lawned with *Staphylococcus aureus (S. aureus)* ATCC 25923 which act as our quality control bacteria for this experiment, does have zone of inhibition ranging from 27mm to 35mm in both disk diffusion and agar well diffusion methods showing that it is susceptible to ampicillin 10µg/ml. The zone of inhibition was manually measured using a caliper at its breakpoint using AST standards provided by the Clinical and Laboratory Standards Institute (CLSI), M100, 30th Edition, 2020.

The minimum inhibitory concentration (MIC) was determined based on the turbidity of the well plate, where a clear well indicates complete inhibition of bacterial growth while a cloudy or turbid well indicates growth of bacteria. The turbidity was observed using naked eye aided with Tecan Sunrise optical density (OD) reader at 600nm wavelength. In this experiment, a clear well was not observed for either *E. coli*, *P. aeruginosa* and *K. pneumoniae* against the control antimicrobial agent Ampicillin and the tested compounds Cu(I)Tri, Cu(I)TriDieth, Cu(I)TriGly, and Cu(I)TriSar. However, only on the first well which is the highest concentration of the copper compound, it appears less cloudy, and the OD reading is slightly lower compared to all other wells for *E. coli*, *P. aeruginosa* and *S. aureus* ATCC 25923. On the other hand, only *S. aureus* ATCC 25923 exhibited a clear well at 0.156µg/ml for the positive control antimicrobial agent ampicillin indicating the minimum inhibitory concentration for ampicillin against *S. aureus* ATCC 25923. However, no clear well was observed for *S. aureus* ATCC 25923 against any of the tested copper compounds.

Hence, the investigation proceeded with a modified minimum bactericidal concentration (MBC) to assess the antibacterial effectiveness of the initial three wells and well 10, with the goal of detecting any nuanced antibacterial effects at varying compound concentrations. Nevertheless, all examined compounds displayed *E. coli*, *P. aeruginosa* and *K. pneumoniae* colonies that were excessively too numerous to count (TNTC) on the MH agar, even at the maximum concentration of each compound.

Figure 1 shows the result of disk diffusion method, the diameter of the ZOI on *S. aureus* ATCC 25923 for amoxicillin (Ab) which serves as positive control (d=27mm). There are no ZOI observed for DMSO (negative symbol) represents the negative control and c1, c2, c3 and c4 which represents different concentration for Cu(I)Tri, Cu(I)TriGly, Cu(I)TriSar, and Cu(I)TriDie.

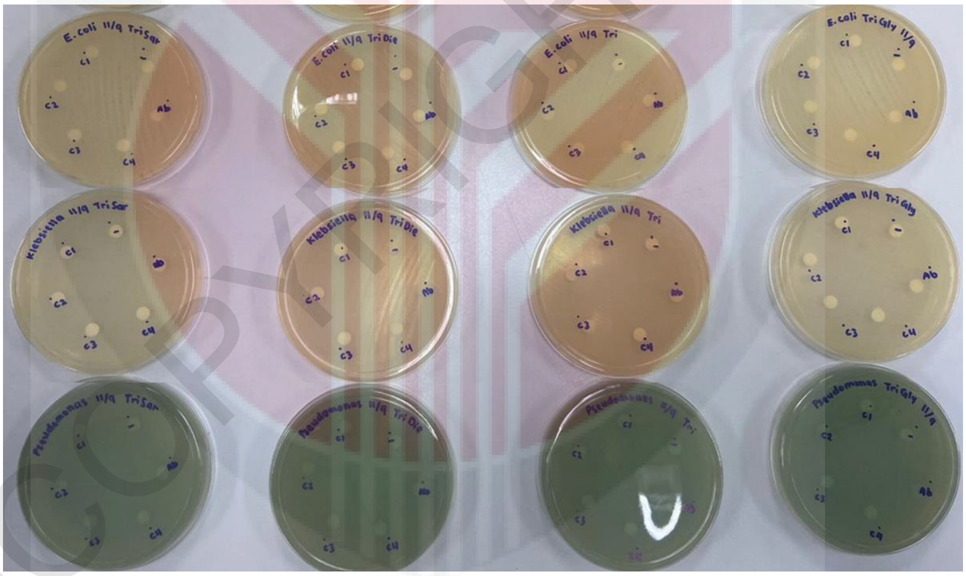

Figure 2 shows the result of disk diffusion method for *E. coli*, *P. aeruginosa* and *K. pneumoniae*, there are no ZOI observed for Ampicillin (Ab) represents positive control, DMSO (negative symbol) represents negative control and c1, c2, c3 and c4 which represents different concentration for Cu(I)Tri, Cu(I)TriGly, Cu(I)TriSar, and Cu(I)TriDie.

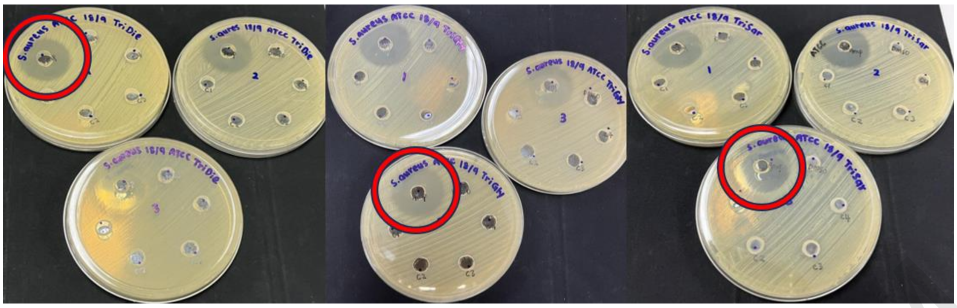

Figure 3 shows the result of agar well diffusion method for *S. aureus* ATCC 25923, the diameter of ZOI observed for Ampicillin (Ab) which represents positive control (d=35mm). There are no ZOI observed for DMSO (negative symbol) represents negative control and c1, c2, c3 and c4 which represents different concentration for Cu(I)Tri, Cu(I)TriGly, Cu(I)TriSar, and Cu(I)TriDie.

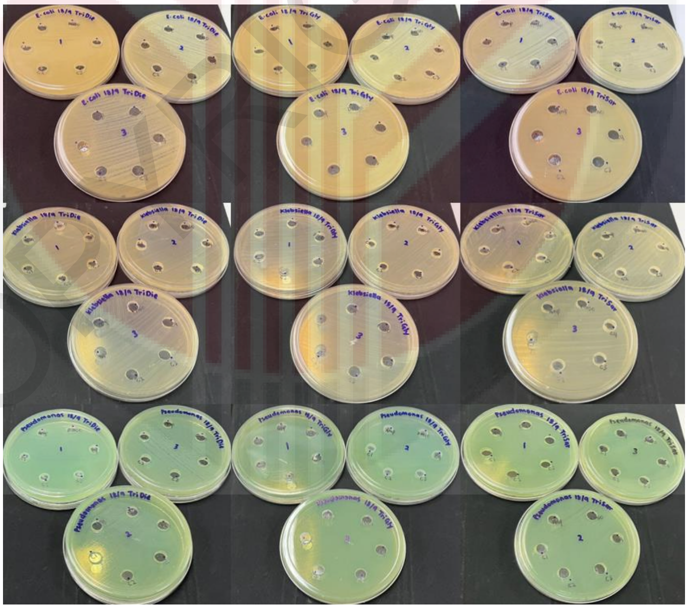

Figure 4 shows the result of disk diffusion method for *E. coli*, *P. aeruginosa* and *K. pneumoniae*, there are no ZOI observed for ampicillin (Ab) represents positive control, DMSO (negative symbol) represents negative control and c1, c2, c3 and c4 which represents different concentration for Cu(I)Tri, Cu(I)TriGly, Cu(I)TriSar, and Cu(I)TriDie.

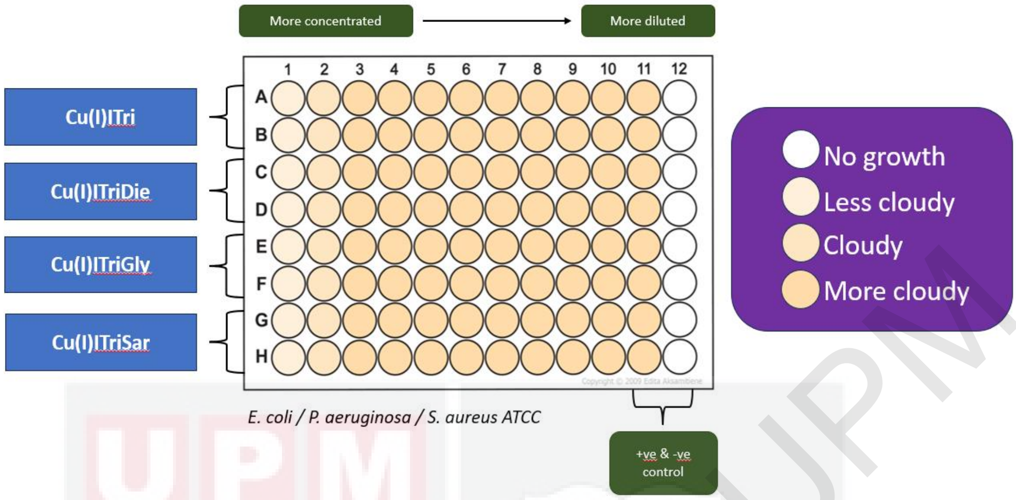

Figure 5 shows the illustrations of MIC results for *E. coli*, *P. aeruginosa*, *K. pneumoniae* and *S. aureus* ATCC 25923 against all tested compounds, in this experiment, there are no clear well observed except for well 1 which showed less cloudy appearance.

Compound	Optical Density Reading at 600nm Wavelength (TECAN Sunrise)			
	E. coli	*P. aeruginosa*	*K. pneumoniae*	*S. aureus* ATCC
	1st well	1st well	1st well	1st well
Cu(I)ITri	0.067	0.061	0.124	0.061
Cu(I)ITriDieth	0.053	0.041	0.121	0.061
Cu(I)ITriGly	0.049	0.052	0.125	0.067
Cu(I)ITriSar	0.047	0.045	0.126	0.069
Control (11th well)	0.112	0.105	0.029	0.075

Table 1 shows the OD reading at 600nm wavelength, the reading on the first well which is the highest concentration of the compound is lower compared to the control well for *E. coli*, *P. aeruginosa and S. aureus* ATCC 25923.

Staphylococcus Aureus ATCC									
Well/ compound	**1**	**2**	**3**	**4**	**5**	**6 - 9**	**10**	**11**	**12**
Cu(I)Tri	TNTC	TNTC	TNTC				TNTC		
Cu(I)TriGly	TNTC	TNTC	TNTC				TNTC		
Cu(I)TriDieth	TNTC	TNTC	TNTC				TNTC		
Cu(I)TriSar	TNTC	TNTC	TNTC				TNTC		
Ampicillin	0	0	0	6	24				

Table 2 shows MBC results for *S. aureus* ATCC 25923. Only ampicillin has MBC value at 1.25μg/mL and the rest are too numerous to count (TNTC)

E. coli*, *P. aeruginosa*, *K. pneumoniae							
Well/ compound	**1**	**2**	**3**	**4 - 9**	**10**	**11**	**12**
Cu(I)Tri	TNTC	TNTC	TNTC		TNTC		
Cu(I)TriGly	TNTC	TNTC	TNTC		TNTC		
Cu(I)TriDieth	TNTC	TNTC	TNTC		TNTC		
Cu(I)TriSar	TNTC	TNTC	TNTC		TNTC		
Ampicillin	TNTC	TNTC	TNTC				

Table 3 shows MBC results for *E. coli, P. aeruginosa* and *K. pneumoniae*. All tested copper compounds including positive control ampicillin is too numerous to count (TNTC)

5.0 DISCUSSIONS

In this study, three Gram-negative bacteria consisting of *E. coli, P. aeruginosa and K. pneumoniae* were used as sample of microorganism to be tested against Cu(I)ITri, Cu(I)ITriDie, Cu(I)ITriSar and Cu(I)ITriGly. These bacteria are among the most common bacteria to be found in UTIs cases in feline, being *E. coli* with the highest percentage (Bartges and Olin, 2017). According to Chan et al. (2022) and Garces et al. (2022), these bacteria are also known to have already become multi drug resistance which means that the bacteria are resistant to at least one agent in three or more classes of antibiotic. Thus, this study is to evaluate the antimicrobial efficacy of the tested copper compound complexes.

Metal complexes were employed to evaluate their antimicrobial characteristics instead of utilizing metal ions alone. This choice was made because the liposolubility of metal complexes increases as the polarity of metal ions diminishes, a result of the positive charge of metal ions being shared with ligands. Consequently, the enhanced solubility promotes increased penetration of the complexes into the lipid membrane of bacteria (Abebe and Hailemariam, 2016). Other studies also show that metal complexes exhibit more antimicrobial effect compared to the ligands and metals alone (Popova et.al, 2012). However, in this study, the copper complexes does not exhibit antimicrobial activity against the clinical isolates. This disparity may be due to the copper compound used in the experiment which are a newly designed and has not been tested for its compatibility against microorganisms.

Furthermore, an *in vitro* study done by Arslan et al. (2009) reported that the testes copper complexes with thiourea and amino acids derivatives ligand possess inhibitory effect on the growth of gram-negative bacteria including *E. coli* (ATCC 25922) and *P. aeruginosa* (ATCC 27853). Ironically, in this study, the copper complexes reveal no effect on the clinical isolates on all antimicrobial testing methods including disk diffusion, agar well diffusion and broth microdilution to determine MIC and MBC. The variation observed could be ascribed to the utilization of different amino acids and ligands in this study, namely glycine, methyl-glycine, diethanolamine, and benzoylthiourea ligands. The different degrees of lipophilicity and hydrophilicity exhibited by these ligands might lead to distinct antimicrobial activity outcomes.

Additionally, in this study, ampicillin was used alternatively to amoxicillin as positive control. However, no ZOI was observed on all the AST procedures done for ampicillin against *E. coli, P. aeruginosa and K. pneumoniae.* The absence of antibacterial activity of ampicillin against these bacteria could be attributed to the fact that the samples were derived from archived clinical isolates, which may have developed resistance over time. On the other hand, according to Santo et al. (2010) mentioned that potential challenge when applying copper might be the probable emergence and spread of resistant bacteria. Eventhough copper seems to be a relatively low risk of resistance emerging among bacteria, many studies have reported the potential challenge of copper resistance towards bacteria including gram-negative bacteria (Lemire et al., 2013). An *in vitro* study done by Benhalima et al. (2019) comparing the MICs of copper compound, resistance was found among

43.75% on gram-negative bacteria of tested *Enterobacteriaceae* and one isolates of *P. aeruginosa.* According to Vincent et al. (2017) Gram-negative bacteria activate copper resistance mechanisms through 'activator-type' systems, such as CueR-like and CusRS-like which respond to increased cytoplasmic and periplasmic copper concentrations, regulating gene transcription, and exporting copper to the extracellular space. He also mentioned that Multicopper oxidases (MCO) are synthesized to convert Cu(I) to less toxic Cu(II), while resistance nodulation cell division (RND)-type copper efflux systems export copper to the periplasmic space. This might elucidate the absence of Zones of Inhibition (ZOI) for all compounds against the clinical isolates, indicating that the compounds may be ineffective in inhibiting bacterial growth due to the aforementioned mechanism that confers resistance against copper compounds.

Moreover, smaller particles of copper in nano (CuNPs) form have better antimicrobial activity due to their greater capacity to penetrate cells and dissolve faster in nanoparticle form by releasing ion to the surroundings (Applerot et al., 2012). An *in vitro* study done by Ermini and Voliani (2021) compares the action of copper ions released by copper sulfate and CuNPs with ciprofloxacin in a “zone of inhibition” test. Interestingly, the copper ion sample does not create any growth inhibition against *K. pneumoniae* and *S. aureus* while CuNPs evidenced a strong antibacterial activity. However, in this study, the copper complexes particles are larger and are not in form of nanoparticles which evidently provide better effect against bacteria. Therefore, this justifies why there is no ZOI in all AST methods.

Lastly, at high concentrations, copper exhibits better bacteriostatic properties, inhibiting bacterial growth, and exerts a toxic impact on the majority of microorganisms (Lemia et al., 2019). An *in vitro* study by Benhalima et al. (2019) showed that a concentration as low as 800μg/ml of copper inhibited bacterial growth in 80% of the isolates. In addition, a higher copper concentration of 1600μg/ml ensures inactivation, meaning it prevents the multiplication of all the isolates. However, in this study, the compound used has been tested for its concentrations on fish embryo acute toxicity (FET) test based on OECD guidelines (2013) to produced desired concentration within the range of Lethal concentration 100 and LC 50 where 100% and 50% of the fish embryo population died after 96hr of exposure to the copper compound. Hence, this compound is only safe to be used in experimental studies using the concentration below than LC100 and cannot adjust to higher concentration because it will affect the stability and cause precipitation which may not be dissolve accurately.

6.0 CONCLUSION

Based on the study, the tested Phosphanecopper (I) Benzoylthiourea Series consisting of Cu(I)ITri, Cu(I)ITriDie, Cu(I)ITriGly, Cu(I)ITriSar does not have significant antibacterial activity against the archived clinical isolates of gram-negative bacteria isolated from feline UTIs specifically *E. coli*, *P. aeruginosa* and *K. pneumonia.* There are no ZOI observed on agar well diffusion and disk diffusion method for the copper complexes against the archived clinical isolates. There is no clear wells observed for broth microdilution method to determine MIC and the result of MBC is TNTC. Even though there is a slight difference in turbidity and OD reading of the MIC plates which appeared less cloudy on the first well which is the highest concentration tested, however, it is not significant as it does not reduce the bacterial growth thus not having evidence of bactericidal or bacteriostatic effect.

7.0 RECOMMENDATION

The findings of this research suggest testing metal complexes on different bacterial strains, emphasizing the varied effects of different metal compounds on distinct bacteria. Further investigations are recommended, possibly involving modifying the molecular structure of the ligands by adjusting their lipophilicity and hydrophilicity and can be used with higher concentration that is suitable for experimental studies. Exploring alternative copper application methods, like usage of nanoparticles form of copper, is advised, as the study indicates that smaller copper particles exhibit better antimicrobial activity, attributed to their enhanced cell-penetration capabilities. Lastly, additional test involving time-kill assay is required for extra evaluation.

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APPENDICES

Table 4: Concentration of Phosphanecopper (I) Benzoylthiourea series used in experiment.

Metal	Concentration of Phosphanecopper (I) Benzoylthiourea series (Molar)				DMSO (negative control)	Ampicillin (positive control)
	C1	C2	C3	C4		
Cu(I)Tri	3.125μM	1.56μM	0.78μM	0.39μM		
Cu(I)TriSar	6.25μM	3.125μM	1.5625μM	0.78μM	0.5%	10μg/ml
Cu(I)TriGly	50μM	25μM	12.5μM	6.25μM		
Cu(I)TriDieth	3.125μM	1.5625μM	0.78μM	0.39μM		

Table 5: Lethal concentration of Phosphanecopper (I) Benzoylthiourea series.

LC^{50} and LC^{100}

Compounds	LC^{50}	LC^{100}
B.Dieth	>1600 μM	>1600 μM
B.Gly	200 μM	400 μM
B.Sar	400 μM	800 μM
Cu(I)ITri	3.125 μM	6.25 μM
Cu(I)ITriDieth	3.125 μM	6.25 μM
Cu(I)ITriGly	50 μM	100 μM
Cu(I)ITriSar	6.25 μM	12.5 μM

Table 6: Phosphanecopper (I) Benzoylthiourea series characteristics

Compound	Chemical Formula	Experimental Melting Point (°C)	Molecular Weight (g/mol)	Colour	Percentage Yield (%)
B.Die	$C_{12}H_{16}N_2O_3S$	112	268.34	White	55.68
B.Gly	$C_{10}H_{10}N_2O_3S$	200	238.27	Light yellow	66.65
B.Sar	$C_{11}H_{12}N_2O_3S$	132	252.29	Off white	54.26
Cu(I)ITri	$C_{36}H_{30}CuI_2P_2$	260	841.24	White	70.11
Cu(I)ITriDie	$C_{48}H_{46}CuIN_2O_3P_2S$	182	983.36	Light yellow	61.70
Cu(I)ITriGly	$C_{52}H_{45}CuIN_2O_3P_2S$	192	953.29	Yellow	61.27
Cu(I)ITriSar	$C_{47}H_{42}CuIN_2O_3P_2S$	166	967.32	Yellow	81.81

Figure 6: Molecular structure of copper compound