UNIVERSITI PUTRA MALAYSIA

EVALUATION OF PHOSPHANECOPPER(I) BENZOYLTHIOUREA SERIES AS AN ALTERNATIVE ANTIMICROBIAL TREATMENT FOR GRAM-POSITIVE BACTERIA ISOLATED FROM FELINE URINARY TRACT INFECTION

FARRA IZWANIE BINTI MOHD YUSOF

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In partial fulfillment of the requirement for the

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CERTIFICATION

It is hereby certified that we have read this project paper entitled "Evaluation of phosphanecopper-(I) benzoylthiourea series as an alternative antimicrobial treatment for gram-positive bacteria isolated from feline urinary tract infection", by Farra Izwanie binti Mohd Yusof and in our opinion, it is satisfactory in terms of scope, quality, and presentation as partial fulfillment of the requirement for the course VPD 4901 - Project.

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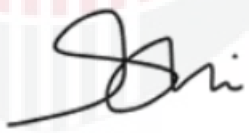

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Lots of love, Farra.

TABLE OF CONTENTS

LIST OF THE TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

S. aureus	=	*Staphylococcus aureus*
E. faecalis	=	*Enterococcus faecalis*
S. pseudintermedius	=	*Staphylococcus pseudintermedius*
M	=	Molar
g	=	gram
mg	=	miligram
L	=	litre
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
OD	=	optical density

ABSTRAK

Abstrak bagi kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar sebagai sebahagian daripada pemenuhan separuh jalan kursus VPD 4901 - Projek.

PENILAIAN SIRI PHOSPHANECOPPER (I) BENZOYLTHIOUREA SEBAGAI RAWATAN ANTIMIKROB ALTERNATIF UNTUK BAKTERIA GRAM-POSITIF YANG DIISOLASI DARIPADA JANGKITAN SALURAN KENCING KUCING

oleh

Farra Izwanie Bt. Mohd Yusof

2023

Penyelia: Dr. Sharina Omar

Ketahanan antimikrob (AMR) tanpa ragu merupakan salah satu cabaran terbesar dalam perubatan veterinar. AMR meningkat disebabkan penggunaan meluas ubat antimikrob dalam perubatan veterinar. Selain itu, interaksi rapat antara haiwan kesayangan dan manusia membolehkan penularan bakteria AMR, seterusnya menyumbang kepada isu ini. Dalam amalan haiwan kecil, jangkitan saluran kencing (UTI) sering didiagnos, dan seringkali memerlukan penggunaan antibiotik empirik sebagai pendekatan rawatan. Oleh itu, tujuan kajian ini adalah untuk menilai kesan antimikrob siri fosfanakuprum(I) benzoilthiourea sebagai rawatan antimikrob alternatif untuk bakteria gram-positif, khususnya *Staphylococcus pseudintermedius* (*S. pseudintermedius*) dan *Enterococcus faecalis* (*E. faecalis*). Dua bakteria gram-positif yang diarkibkan diisolasi dari spesies kucing yang dijangkiti UTI dari Makmal Bakteriologi Veterinar, Universiti Putra Malaysia. Isolat dikumpulkan antara 1 Januari 2021 hingga 30 September 2022 dari spesies kucing dari kedua-dua jantina dan

pelbagai umur dengan latar belakang yang berbeza. Isolat yang diarkibkan disimpan pada suhu bilik dan dikekalkan di atas agar nutrien sebelum menjalankan ujian kerentanan antimikrob (AST), kepekatan minimum perencatan (MIC), dan kepekatan minimum membunuh bakteria (MBC). AST dilakukan menggunakan kaedah penyebaran cakera Kirby-Bauer dan kaedah penyebaran sumur agar pada plat Muller-Hinton (MH). Selain itu, MIC ditentukan menggunakan kaedah pencairan calit, dan MBC ditentukan pada plat MH. Sebatian terpilih dari siri fosfanakuprum(I) benzoilthiourea diuji pada pelbagai kepekatan 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). AST menunjukkan kedua-dua isolat tidak memberi kesan terhadap mana-mana sebatian tembaga terpilih pada pelbagai kepekatan, kerana tiada zon penahanan yang jelas diperhatikan dalam kedua-dua kaedah penyebaran cakera dan penyebaran sumur agar. Selain itu, keputusan MIC dan MBC juga menunjukkan bahawa tiada sebatian tembaga terpilih yang berkesan terhadap kedua-dua isolat, kerana tiada sumuran yang jelas dapat dilihat, dan keputusan MBC terlalu banyak untuk dihitung (TNTC). Oleh itu, Cu(I)ITri, Cu(I)ITriDie, Cu(I)ITriSar, dan Cu(I)ITriGly dari Siri Phosphanecopper (I) Benzoylthiourea tidak mempunyai aktiviti antibakteria yang signifikan terhadap bakteria gram-positif yang diarkibkan, khususnya *S. pseudintermedius* dan *E. faecalis*, yang diisolasi dari spesies kucing yang dijangkiti UTI. Kajian lanjut diperlukan, yang mungkin melibatkan penyesuaian struktur ligand yang digunakan dalam sebatian tembaga.

Kata kunci: kucing, AST, MIC, MBC, antibakteria

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 PHOSPHANECOPPER (I) BENZOYLTHIOUREA SERIES AS AN ALTERNATIVE ANTIMICROBIAL TREATMENT FOR GRAM-POSITIVE BACTERIA ISOLATED FROM FELINE URINARY TRACT INFECTION

by

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2023

Supervisor: Dr. Sharina Omar

Antimicrobial resistance (AMR) is undoubtedly one of the greatest challenges of veterinary medicine. AMR incidence is increasing because of the extensive use of antimicrobial drugs in veterinary medicine. In addition, the close interaction between pet animals and humans enables the transmission of AMR bacteria, further contributing to this issue. In small animal practice, urinary tract infections (UTIs) are frequently diagnosed, and often necessitate the use of empirical antibiotics as a treatment approach. Hence, the purpose of this study is to evaluate the antimicrobial effect of phosphanecopper-(I) benzoylthiourea series as an alternative antimicrobial treatment for gram-positive bacteria, specifically *Staphylococcus pseudintermedius* (*S. pseudintermedius*) and *Enterococcus faecalis* (*E. faecalis*). Two archived gram-positive bacteria were isolated from feline species infected with UTIs from Veterinary Bacteriology Laboratory, University Putra Malaysia. The isolates were collected

between 1st January 2021 until 30th September 2022 from feline species of both genders at various ages with different backgrounds. The archived isolates were subcultured on nutrient agar prior to conducting antimicrobial susceptibility tests (AST), minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). AST was performed using Kirby-Bauer disk diffusion method and agar well diffusion method on Muller-Hinton (MH) agar plates. Additionally, MIC was determined using broth dilution method, and the MBC was determined on MH agar plates. Selected compounds from phosphanecopper(I) benzoylthiourea series were tested at various 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). AST results revealed that both isolates were not susceptible to any of the selected copper compounds at various tested concentrations, as no clear zone of inhibition was observed in both disk diffusion and agar well diffusion methods. Moreover, the MIC and MBC results also demonstrated that neither of the selected copper compounds was effective against both isolates, as no clear well was visible, and the MBC result was too numerous to count (TNTC). Therefore, Cu(I)ITri, Cu(I)ITriDie, Cu(I)ITriSar and Cu(I)ITriGly from the Phosphanecopper (I) Benzoylthiourea series do not have significant antibacterial activity against archived gram-positive bacteria, specifically *S. pseudintermedius* and *E. faecalis*, which were isolated from feline species infected with UTIs. Additional research is necessary, potentially involving a redesign of the ligand utilised in the copper compound.

Keywords: feline, AST, MIC, MBC, antibacterial

1.0 INTRODUCTION

Antimicrobial resistance (AMR) is undoubtedly one of the greatest challenges of veterinary medicine. AMR has seen a recent surge in cases due to the imprudent of antibiotics. Furthermore, the administration of antibiotics to animals, both those intended for food production and companion animals, has led to the emergence and spread of antibiotic-resistant bacteria (Caneschi et al., 2023). In addition, close interaction between pet animals and humans enables the transmission of AMR bacteria, potentially elevates the likelihood of genetic transfer between microorganisms, contributing to the spread of antibiotic resistance (Warnes et al., 2011). In small animal practice, urinary tract infections (UTIs) are frequently diagnosed, and often necessitate the use of empirical antibiotics as a treatment approach (Fonseca et al., 2021). According to Pomba et al. (2010), *Staphylococcus pseudintermedius* and *Enterococci faecalis* are common causes of urinary tract infections in cats. Therefore, the need for the discovery of innovative antimicrobials is necessary, and this includes evaluating the potential use of copper metal as an alternative antimicrobial agent.

According to Benhalima et al. (2019), at high concentrations, copper exhibits bacteriostatic properties, inhibiting bacterial growth, and exerts a toxic impact on the majority of microorganisms. In that study, a copper (II) sulfate pentahydrate ($CuSO_4.5H_2O$) with a stock solution concentration of 294.3966×10^3 µM exhibited bactericidal efficacy against 9 out of 25 tested bacteria in the MIC test. In addition, a few *in vivo* studies have shown that copper has demonstrated effectiveness against antibiotic-resistant bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococci* (VRE) (Warnes et al., 2011; Noyce et

al., 2006). Other than that, Chohan et al., (2006) reported that biologically active compounds become more bacteriostatic and carcinostatic upon chelation.

In addition, thiourea ligands are a useful chelating agent towards metal complexes. An *in vivo* study by Rauf et al. (2009) has shown that thiourea ligands and their metal complexes exhibit a wide range of biological activity including antibacterial. Additionally, Siew et al. (2014) said that Benzoyl thioureas ligands are considered useful chelating agents because of their aptly positioned C=O and C=S functional groups, which enable them to effectively encapsulate metal ions within their coordinating structure, yielding to a stable metal complex.

Over the past few years, numerous organic copper complexes have undergone testing for their antibacterial properties and their mode of action likely involves binding to amino acids and metal ions, which may potentially free up atoms and enhance their biological activity (DeAlba et al., 2017; Fernanda et al 2009). Moreover, copper-amino acid chelate shows better antimicrobial effects. Based on DeAlba et al. (2017) study, serial dilutions of nanoparticles were introduced to *Escherichia coli*, *Staphylococcus aureus*, and *Enterococcus faecalis*, revealing that the antimicrobial effect of the copper-amino acids chelate better than copper nanoparticles. Furthermore, the addition of amino acids, particularly glycine, enhances the solubility of poorly soluble copper (II) salts leading to an increase in the bioavailability of the metal ion (Sabrina et al., 2010). Other than that, Kim et al. (2020) proposes that copper-based combined microbicides such as hydroxylamine (HA) can effectively inactivate different bacterial and viral species in water.

Therefore, this has led to the objectives of this study are to identify antimicrobial effects of phosphanecopper-(I) benzoylthiourea series against selected gram-positive bacteria isolated from feline urinary tract infection by using Kirby-Bauer

disk diffusion method and agar well diffusion method and to determine minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of phosphanecopper-(I) benzoylthiourea series against selected gram-positive bacteria isolated from feline urinary tract infection.

2.0 LITERATURE REVIEW

2.1 Antimicrobial properties of copper compound

Copper and its compounds have been shown to possess antimicrobial properties against a wide range of microorganisms, including bacteria, viruses, and fungi (Grass et al., 2011). Copper compounds possess the capability to exhibit bactericidal activity through a mechanism involving the generation of reactive oxygen species (ROS), which leads to irreversible damage to membranes (Salah et al., 2021). Several studies have suggested a correlation between the antibacterial properties of copper and its capacity to release copper ions, causing damage to cell membranes. In addition, numerous recent studies on new copper-based material confirm this theory (Zhang et al., 2013; Liu et al., 2014; Mathews et al., 2015; Emam et al., 2017). The antimicrobial efficacy of copper exhibits a proportional increase with its concentration (Salah et al., 2021).

2.2 Amino acids

Amino acids are organic molecules found in all living organisms. Amino acids can chelate metal ions and play essential roles in various biological processes. The chelation of amino acids with metal compounds often results in an enhancement of the solubility of the metal complex. This increased solubility can have significant implications for a wide range of chemical and biological processes. Sabrina et al. (2010) established that addition of amino acids particularly glycine, alanine, and serine enhances the solubility of poorly soluble copper (II) salts leading to an increase in the bioavailability of the metal ion.

2.3 Antimicrobial properties of Benzoylthiourea ligand

The antimicrobial potential of benzoylthiourea ligands with copper complexes has been studied by a few researchers. Rauf et al. (2009) demonstrated that thiourea ligands and their metal complexes exhibit diverse biological activities, including antibacterial (Rauf et al., 2009). Furthermore, Binzet et al. provided additional evidence supporting that thiourea derivatives exhibit greater efficacy in terms of antibacterial activity compared to their antifungal effects (Binzet et al., 2007).

2.4 Bacteria isolation from small animal

Based on the research carried out by Pomba et al. (2010), the common causes of urinary tract infections in cats are *Staphylococcus pseudintermedius* and *Enterococcus faecalis*. Additionally, as indicated by Jantom et al. (2021), there has been documented antimicrobial resistance in *S. pseudintermedius*, with an increasing prevalence of methicillin-resistant strains (MRSP). The antimicrobial resistance profile of *S. pseudintermedius* has been a subject of concern in human and veterinary medicine due to the potential of zoonotic transmission of resistant strains. Numerous studies documented that *S. pseudintermedius* have elevated resistance levels to various antimicrobial classes including methicillin-resistant strains (MRSP). The resistance patterns identified in *S. pseudintermedius* includes antibiotic groups of β-lactam, tetracyclines, macrolides, lincosamides, chloramphenicol, aminoglycosides, trimethoprim, fluoroquinolones, rifampicin, and fusidic acid. This extensive resistance poses challenges in effectively treating infections caused by *S. pseudintermedius*, restricting the choices for treatment available.

Enterococcus species are a group of bacteria that can be found in various environments, including soil, water, and the gastrointestinal tract of animals (Boehm et al.,2014). Kristich et al. (2014) reported that *Enterococcus* species, particularly *Enterococcus faecalis* and *Enterococcus faecium*, exhibit intrinsic resistance, acquired resistance, and tolerance to various antimicrobial agents. For example, penicillin, ampicillin, the majority of cephalosporins, and all semi-synthetic penicillins due to presence of low-affinity penicillin-binding proteins (Mohamed et al., 2018)

2.5 Antibiotic sensitivity testing

The minimum inhibitory concentration (MIC) and the zone of inhibition are two significant measures used in microbiology to evaluate the efficacy of antimicrobial agents, but they represent distinct aspects of antimicrobial activity. The Kirby-Bauer disk diffusion method and agar well diffusion method are qualitative measures to determine the sensitivity or resistance of pathogenic aerobic and facultative anaerobic bacteria to various antimicrobial comp (Hudzicki, 2009). The size of the zone of inhibition is directly proportional to the sensitivity of the organism to the antibiotic, with larger zones indicating higher susceptibility (Thompson, 1995).

Moreover, MIC is a quantitative measure that determines the susceptibility or resistance of specific bacterial strains against antimicrobial agents. It represents the lowest concentration of an antimicrobial agent that inhibits the visible growth of a microorganism (Kowalska-Krochmal & Dudek-Wicher, 2021).

3.0 MATERIALS AND METHODS

3.1 Preparation of molar concentration of Phosphanecopper (I) Benzoylthiourea series

Stock solution with 6.25µM was prepared by diluting 1mg of Cu(I)Tri in 190.19 ml of DMSO and distilled water and 1mg of Cu(I)TriDieth in 162.72ml of DMSO and distilled water. Other than that, stock solution with 50µM was prepared by diluting 0.5mg of Cu(I)TriGly in 10.49ml of DMSO and distilled water. Furthermore, stock solution with 12.5µM was prepared by diluting 1mg of Cu(I)TriSar in 82.72ml of DMSO and distilled water. Then, four different concentrations were prepared from the stock solution in order to be tested (Table 1).

3.2 Bacteria subculture from archived samples

The archived isolates of gram-positive bacteria namely *Staphylococcus pseudintermedius* and *Enterococcus faecalis* were collected from Bacteriology Lab, Veterinary Laboratories Services Unit (VLSU) of Universiti Putra Malaysia. Nutrient agar media were prepared prior to bacteria culture which was kept in a chiller at 4^{o}C. The archived bacteria were revived onto nutrient agar and incubated at 37^{o}C for 24 hours in order to reactivate the bacteria. The isolates were subculture again to obtain a higher number of pure colonies from a single culture plate.

3.3 Inoculum suspension

The colonies from pure culture were transferred aseptically into 2ml sterile distilled water in a test tube and mixed thoroughly. The turbidity of the inoculum was compared against 0.5 McFarland standard (Aryal, 2021). The result of suspension contains 1.5 X 10^8 colony forming unit per ml (CFU/mL) solution. Suspensions were prepared for *Staphylococcus pseudintermedius* and *Enterococcus faecalis.*

3.4 Agar well diffusion method

Mueller Hinton (MH) agar (Oxoid, UK) was bored using the back of sterile 1mL pipette tip to create evenly spaced wells. The inoculum of *S. pseudintermedius* and *E. faecalis* were streaked over the entire MH agar surface by using a sterile cotton swab to create a lawn of bacteria. The plate was left to dry for few minutes. One hundred (100 µL) of 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 0.78 µM were dispensed in each well. About 100 µL of 10µg ampicillin was used as a positive control as recommended by Clinical and Laboratory Standards Institutes (CLSI) in M100 - Performance Standards for Antimicrobial Susceptibility Testing, 30th Edition (2020). The culture plates were then incubated in an incubator at 37°C for 24 hours. To ensure reproducibility, the whole procedure was repeated thrice. Antimicrobial activity was evaluated by measuring the diameter of zone inhibition (ZOI).

3.5 Disc diffusion method

Antimicrobial susceptibility testing (AST) procedure in this study was done according to the Kirby-Bauer Disk Diffusion Susceptibility Test Protocol by American Society of Microbiology standard procedures (Hudzicki, 2009). The inoculum of *S. pseudintermedius* and *E. faecalis* were lawned uniformly using sterile swabs over the entire Mueller Hinton agar. Ten microliters of of 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 0.78 µM were dropped onto sterile empty discs (Oxoid, UK) using micropipette (Eppendorf, Germany) . The sterile forceps were used to adhere the discs onto the agar aseptically. Ampicillin 10µg of disk content was used as control. The culture plates were incubated at 37°C for 24 hours. To ensure reproducibility, the whole procedure was repeated thrice. Antimicrobial activity was evaluated by measuring the diameter of zone inhibition (ZOI).

3.6 Minimum inhibitory concentration (MIC)

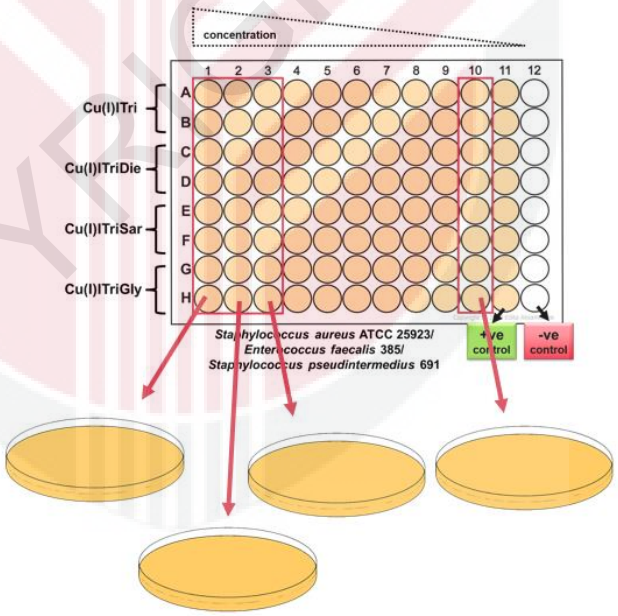
The methodology of this section was adopted from Clinical and Laboratory Standards Institutes (CLSI) in M07-A10 - Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically, 10th Edition (2015). Based on the result of agar well diffusion method, only the highest concentration of each compound will be tested for the determination of minimum inhibitory concentration (MIC). Bacterial suspension was prepared by transferring a small amount of *S. pseudintermedius* and *E. faecalis* from the overnight bacterial culture into a sterile tube containing sterile saline water. The turbidity of bacterial suspension was adjusted to achieve a

turbidity equivalent to that of a 0.5 McFarland standard. This results in a suspension containing approximately 1.5 X 10^8 colony forming unit per ml (CFU/mL).

Then, a bacterial suspension of optical density (OD) (machine turbidity reader, TECAN Sunrise, Swiss) 0.01 in 20mL of saline was prepared. The final OD of bacterial suspension in each well was determined to be between 0.001 and 0.005. About 50µL of bacterial suspension were added into each well in 96-well microtiter plate. Next, 150µL of MH broth were added into all wells from column 1 - 12, row ABCDEF. After that, 150µL of Cu(I)Tri, Cu(I)TriDieth, Cu(I)TriGly and Cu(I)TriSar of the highest concentration were added into well no. 1. A two fold dilutions were made from column 1 to 10 and the excess 150µL were discarded. The content was gently mixed by pipetting up and down. The steps were repeated for ampicillin of highest concentration. Column 11 served as broth control without any antibiotic or compound and column 12 served as negative control that had broth only. The OD readings at 600nm were measured prior to incubation at 37°C for 24 hours and post incubation period. The lowest concentration of the compounds and ampicillin at which bacterial growth is completely inhibited is identified as MIC value.

3.7 Modified minimum bactericidal concentration (MBC)

Minimum bactericidal concentration (MBC) was determined by plating the first three wells (row A-H, column 1-3) and well 10 of each compound. About 100µL of each compound in selected wells were inoculated onto MH agar (Oxoid, UK) to compare the bacterial growth. Comparison was made to determine whether the compound has visible antibacterial activity from the culture plate. The plates were incubated at 37°C for 24 hours. After incubation, the plates were subjected for enumeration of bacterial growth on the plate. Difference in number of bacterial growths from first three wells and well 10 indicates that the compound has slight antibacterial activity.



4.0 RESULTS

Based on the agar well diffusion and disc diffusion methods it was observed that there was no zone of inhibition (ZOI) observed on the MH agar lawned with *Staphylococcus pseudintermedius* and *Enterococcus faecalis* 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 0.78 µM. However, for both the disk diffusion method and agar well diffusion method, zone of inhibition (ZOI) was observed only on the agar lawned with *S. pseudintermedius* against ampicillin, which acted as a control for this experiment, with an average ranging from 27mm to 35mm. No ZOI was observed on control of ampicillin against *E. faecalis* for both disk diffusion method and agar well diffusion method. The zone of inhibition was manually checked 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. In this experiment, a clear well was not observed for either *S. pseudintermedius, E. faecalis*, or *Staphylococcus aureus* ATCC 25923 against the control antimicrobial agent ampicillin, tested compounds Cu(I)Tri, Cu(I)TriDieth, Cu(I)TriGly, and Cu(I)TriSar. Conversely, only *S. aureus* ATCC 25923 exhibited a clear well at 0.156µg/ml for the positive control antimicrobial agent, ampicillin. However, no clear well was observed for *S. aureus* ATCC 25923 against any of the compounds.

Therefore, the experiment proceeded with modified minimum bactericidal concentration (MBC) to compare the antibacterial activity of the first three wells and

well 10, aiming to identify any subtle antibacterial effects at different compound concentrations. Despite this, all tested compounds exhibited colonies of *S. pseudintermedius* and *E. faecalis* that were too numerous to count (TNTC) on the MH agar, even at the highest concentration of each compound.

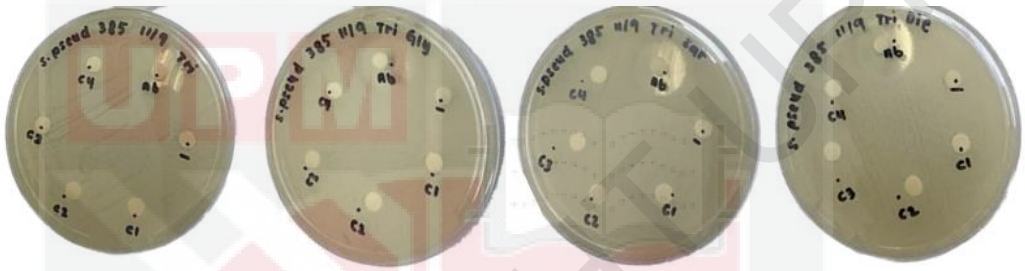


Figure 1 shows the result of the disk diffusion method, the diameter of the zone of inhibition (ZOI) on *S. pseudintermedius* for ampicillin (ab), which serves as a positive control (d = 27mm). The negative symbol represents the negative control. For Cu(I)Tri, Cu(I)TriGly, Cu(I)TriSar, and Cu(I)TriDie, no zone of inhibition was observed.

Figure 2 shows the result of the disk diffusion method, no diameter of the zone of inhibition (ZOI) on *E. faecalis* for Cu(I)Tri, Cu(I)TriGly, Cu(I)TriSar, and Cu(I)TriDie, ampicillin (ab), which serves as a positive control and negative control (-).

Figure 3 shows the result of the agar well diffusion method, the diameter of the zone of inhibition (ZOI) on *S. pseudintermedius* for ampicillin (ab), which serves as a positive control (d = 35mm). The negative symbol represents the negative control. For Cu(I)TriGly, Cu(I)TriSar, and Cu(I)TriDie, no zone of inhibition was observed.

Figure 4 shows the result of the agar well diffusion method, no diameter of the zone of inhibition (ZOI) on *E. faecalis* for Cu(I)TriGly, Cu(I)TriSar, and Cu(I)TriDie, ampicillin (ab), which serves as a positive control and negative control (-).

Table 2 shows MBC results for *Staphylococcus aureus* ATCC in which ampicillin has MBC shown at 1.25 µg/mL.

***Staphylococcus aureus* ATCC 25923**												
Well/ compound	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**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	2	4						

Table 3 shows MBC results for *Staphylococcus pseudintermedius*. where ampicillin shown MBC at 0.078 µg/mL.

Staphylococcus pseudintermedius												
Well/ compound	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**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	9				

Table 4 shows MBC results for *Enterococcus faecalis*. All tested compounds including positive control of ampicillin showing TNTC on MH agar.

Enterococcus faecalis												
Well/ compound	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**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								TNTC	

5.0 DISCUSSIONS

In this study, metal complexes with benzoylthiourea, diethanolamine and amino acids (glycine and sarcosine) were used in order to determine its antimicrobial properties instead of metal ions alone. This is because Rauf et al. (2009) demonstrated that thiourea ligands and their metal complexes display diverse biological activities, encompassing antibacterial effects. Additionally, Sabrina et al. (2010) found that the incorporation of amino acids, specifically glycine, alanine, and serine, improves the solubility of copper(II) salts with low solubility, consequently elevating the bioavailability of the metal ion.

Moreover, copper-amino acid chelate shows better antimicrobial effects. In a study conducted by DeAlba et al. (2017), serial dilutions of nanoparticles and copper-EDTA chelate were introduced to *Escherichia coli, Staphylococcus aureus*, and *Enterococcus faecalis.* The findings indicated that the copper-amino acids chelate displayed superior antimicrobial efficacy compared to copper nanoparticles. However, in this study the copper-amino acids chelate did not effectively inhibit bacterial growth on all antimicrobial susceptibility testing methods, including the disk diffusion method, agar well diffusion method, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC). This discrepancy may be attributed to the use of different amino acids and ligands in this research, specifically glycine, methyl-glycine, diethanolamine, and benzoylthiourea ligands. The diverse lipophilicity and hydrophilicity of these ligands may result in distinct antimicrobial effects.

Furthermore, ampicillin served as a positive control in this study because it is commonly employed in susceptibility tests to assess the activity of amoxicillin as recommended by Clinical and Laboratory Standards Institutes (CLSI) in M100 - Performance Standards for Antimicrobial Susceptibility Testing, 30th Edition (2020).

In this study, ampicillin was utilized to gauge susceptibilities to amoxicillin. Amoxicillin is the commonly used treatment option in UTIs due to its superior oral bioavailability. However, no ZOI observed on disk diffusion method and agar well diffusion method of ampicillin against *E. faecalis*. This lack of antibacterial activity of ampicillin against *E. faecalis* may be attributed to the fact that the samples were obtained from archived clinical isolates, which could have potentially developed resistance over time.

According to Vincent et al. (2018), gram-positive bacteria have the potential to develop resistance to copper through a repressor regulator released from DNA. Furthermore, a study by Hasman et al. (2005) revealed that the tcrYAZB operon imparts copper resistance in *E. faecium, E. faecalis*, and other enterococcal species. This observation might elucidate the absence of zones of Inhibition (ZOI) for all compounds against *E. faecalis* in disk diffusion and agar well diffusion methods, indicating that the compounds may be ineffective in inhibiting bacterial growth due to the presence of a repressor regulator that confers resistance against copper compounds.

In addition, *Staphylococcus pseudintermedius* has potential to develop resistance to copper. A study conducted by Worthing et al. (2018) which focused on characterizing *Staphylococcal* Cassette Chromosome mec (SCCmec) elements derived from methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) infections in Australian animals, has demonstrated that *S. pseudintermedius* exhibits resistance to copper. The study revealed that these isolates carried the copA gene, a genetic element known to confer resistance to copper (Worthing et al., 2018). This discovery highlights the potential adaptability of *Staphylococcus pseudintermedius* to copper-based antimicrobial agents, thereby justifying the absence of ZOI in disk

diffusion and agar well diffusion methods of *S. pseudintermedius* against all tested copper complexes.

While Applerot et al 2012 reported that smaller particles have a better antimicrobial activity due to their greater capacity to penetrate cells, this study demonstrates a contrasting outcome suggesting that the copper compound that was used was not nanoparticle. In this study copper ion chelate with amino acids were used against *Staphylococcus pseudintermedius* and *Enterococcus faecalis*. This could justify why there was no effective inhibition of bacterial growth in all antimicrobial susceptibility testing (AST) methods of all tested copper complexes against both of the isolates.

Lastly, at high concentrations, copper exhibits bacteriostatic properties, inhibiting bacterial growth, and exerts a toxic impact on the majority of microorganisms (Benhalima et al., 2019). The primary mechanisms of copper's antimicrobial activity include generation of reactive oxygen species (ROS), as copper ions released from surfaces it will induce hydroxyl radical production, causing irreversible damage to bacterial membranes (Salah et al., 2021). According to an *in vivo* study by Benhalima et al. (2019), a copper (II) sulfate pentahydrate ($CuSO_4.5H_2O$) with stock solution's concentration of 294.3966×10^3 µM was utilized in MIC and MBC methods against 25 isolates. The findings revealed that an MIC concentration of 800 µg/ml effectively eliminated 36% (9/25) of the isolated strains, while the minimum bactericidal concentration (MBC) at 1600 µg/ml exhibited optimal bactericidal efficacy against the tested bacteria (Benhalima et al., 2019). However, the highest concentration used in this study was determined to be the last concentration safest for zebrafish, as tested by the chemists from the University of Malaya. This could justify why the compound does not show any antimicrobial effect,

as the concentration employed in the study is not as high, following LD50 and LD100 considerations.

6.0 CONCLUSION

Based on the results, the null hypothesis was accepted, indicating that Cu(I)ITri, Cu(I)ITriDie, Cu(I)ITriSar, and Cu(I)ITriGly from the phosphanecopper(I) benzoylthiourea Series do not exhibit antibacterial activity against archived Gram-positive bacteria, specifically *S. pseudintermedius* and *E. faecalis*. These bacteria were isolated from feline species infected with Urinary Tract Infections (UTI), as evidenced by the absence of zones of Inhibition (ZOI) in the disk diffusion test, the agar well diffusion test yielding no clear wells observed on the minimum inhibitory concentration (MIC) test. Additionally, the minimum bactericidal concentration (MBC) test showed too numerous to count (TNTC) colonies, further supporting the lack of antibacterial efficacy.

7.0 RECOMMENDATIONS

Based on the results of this study, it is recommended that metal complexes be tested on other types of bacteria, as it suggests that different types of metal compounds exhibit varying effects on distinct bacterial strains. Additionally, further research is deemed necessary, potentially involving a modified molecular structure of the ligand by altering its lipophilicity and hydrophilicity. Lastly, it is advisable to explore different methods of applying copper, such as copper coatings or copper nanoparticles, as the study suggests that smaller-sized copper particles demonstrate enhanced antimicrobial activity due to their greater capacity to penetrate cells.

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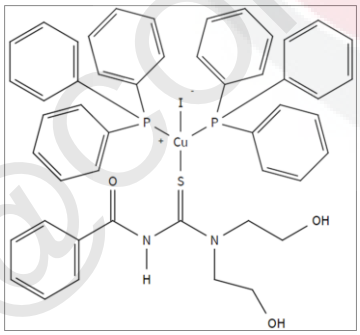
APPENDICES

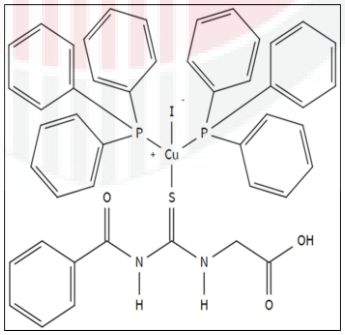
Table 1: Concentration of Phosphanecopper (I) Benzoylthiourea series

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

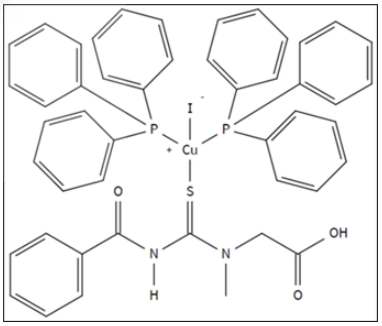
Figure 5: Molecular structure of compound



Cu(I)TriDieth



Cu(I)TriGly



Cu(I)TriSar