ANTIBACTERIAL ACTIVITY OF 5-CHLORO SUBSTITUTED PHENYL *N*-ACYLHYDRAZONE DERIVATIVES WITH AROMATIC SUBSTITUTION AT META- AND PARA- DIRECTORS AS POTENTIAL ADJUVANTS

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By

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ABSTRACT

ANTIBACTERIAL ACTIVITY OF 5-CHLORO SUBSTITUTED PHENYL *N*-ACYLHYDRAZONE DERIVATIVES WITH AROMATIC SUBSTITUTION AT META- AND PARA- DIRECTORS AS POTENTIAL ADJUVANTS

Long Yi Xian

The rise of antibiotic-resistant bacterial strains has imposed the exploration for the compounds that is new in order to enhance the antibacterial efficacy for food safety, animal health as well as human clinical settings. This research investigates the antibacterial properties of 5-chloro substituted phenyl *N*-acylhydrazone (NAH) derivatives, with a focus on derivatives featuring aromatic substitutions at meta- and para-positions as potential adjuvants. A total of 9 NAH derivative compounds were tested against a range of bacteria individually as well as in combination with ciprofloxacin, streptomycin and chloramphenicol as adjuvants, including *Bacillus cereus* (ATCC 13061), *Bacillus subtilis* subsp. spizizenni *(*ATCC 6633), *Staphylococcus. aureus* (ATCC 6538), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella* Typhimurium (ATCC 14028), Methicillin-resistance *Staphylococcus aureus* (ATCC 33591) and Methicillin-resistance *Staphylococcus aureus* (ATCC 43300), to assess their effectiveness.

Both minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were identified through a broth microdilution assay to determine antibacterial activity. Compound 1 (3,4-Cl$_2$) obtained MIC values with the range of 3.91–31.25 µg/mL which demonstrated a broad spectrum of antibacterial activity. In contrast, NAH derivative compounds with the substitution of CH$_3$, F, OCH$_3$ and H demonstrated specific-species antibacterial activity against *S. aureus* (ATCC 6538), characterized by MIC value of 62.50 µg/mL. When combined with standard antibiotics, particularly ciprofloxacin, the NAH derivatives exhibited synergistic effects, significantly enhancing bactericidal activity against *B. cereus* (ATCC 13061), *B. subtilis* subsp. *spizizenni(*ATCC 6633) and Methicillin-resistant *S. aureus* (ATCC 33591). However, the combination of NAH derivatives with chloramphenicol did not produce a bactericidal effect, as indicated by the lack of a minimum bactericidal concentration (MBC) value. This suggested a predominantly inhibitory rather than lethal interaction. Furthermore, the fractional inhibitory concentration (FIC) index calculations showed that streptomycin-adjuvant combinations considered did not exhibit significant interaction, whereas combinations including adjuvant compound 2 and 9 displayed antagonism against *B. subtilis* subsp. *spizizenni*(ATCC 6633). Overall, most of the antibiotic-adjuvants combinations showed indifference in their interaction with the selected bacteria. Hence, it is not recommended for further study of these antibiotic-adjuvant combinations.

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DECLARATION

I hereby declare that this final year project report is based on original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

Long Yi Xian

APPROVAL SHEET

This final year project entitled "**ANTIBACTERIAL ACTIVITY OF 5-CHLORO SUBSTITUTED PHENYL *N*-ACYLHYDRAZONE DERIVATIVES WITH AROMATIC SUBSTITUTION AT META- AND PARA- DIRECTORS AS POTENTIAL ADJUVANTS**" was prepared by LONG YI XIAN and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (HONOURS) Food Science at University Tunku Abdul Rahman.

Approved by:

KahCheng

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Dr. Teo Kah Cheng   Date: 27/8/2024

Supervisor

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UNIVERSITY TUNKU ABDUL RAHMAN

FACULTY OF SCIENCE

Date: 30/8/2024

SUBMISSION OF FINAL YEAR PROJECT

I, **LONG YI XIAN** (ID No: **20ADB02199**) hereby certify that I have completed this final year project titled "ANTIBACTERIAL ACTIVITY OF 5-CHLORO SUBSTITUTED PHENYL N-ACYLHYDRAZONE DERIVATIVES WITH AROMATIC SUBSTITUTION AT META- AND PARA- DIRECTORS AS POTENTIAL ADJUVANTS" under supervision of Dr. Teo Kah Cheng from the Department of Agriculture and Food Science, Faculty of Science.

I understand that the University may upload the softcopy for my final year project report in pdf format to the UTAR Institutional Repository, which may be made accessible to the UTAR community and public.

Yours truly,

(Long Yi Xian)

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ABC	ATP binding cassette
ATCC	American Type Culture Collection
B. cereus	*Bacillus cereus*
B. subtilis subsp. *spizizenni*	*Bacillus subtilis* subsp. spizizenni
Br	Bromo
CHL	Chloramphenicol
CIP	Ciprofloxacin
Cl	Chloro
DMSO	Dimethyl Sulfoxide
E. coli	*Escherichia coli*
ESKAPE	*Enterococcus faecium,* S*taphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanni, Pseudomonas aeruginosa* and *Enterobacter* species
F	Fluoro
FIC	Fractional Inhibitory Concentration
H	Hydrogen

INT	Iodonitrotetrazolium
K. pneumoniae	*Klebsiella pneumoniae*
MATE	Multidrug and toxin extrusion
MBC	Minimum Bactericidal Concentration
MFS	Major facilitator superfamily
MH	Mueller-Hinton
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
NAH	*N*-acylhydrazone
NO_2	Nitro
OCH3	Methoxy
OD	Optical density
OM	Outer membrane
PACE	Proteobacterial antimicrobial compound efflux
P. aeruginosa	*Pseudomonas aeruginosa*
P. fluorescens	*Pseudomonas fluorescens*
P. putida	*Pseudomonas putida*

PAβN	Phenylalanyl arginyl β-napthylamide
PBPs	Penicillin-binding proteins
RND	Resistance nodulation, cell division
S. aureus	*Staphylococcus aureus*
SCC*mec*	staphylococcal cassette chromosome *mec*
SMR	Small multidrug resistance
S. Typhimurium	*Salmonella* Typhimurium
STR	Streptomycin

CHAPTER 1

INTRODUCTION

1.1 Background of the Antibiotic

The term 'antibiotic' is referred to the medical used and it is commonly applied for eliminate infection. The development of antibiotics in the 1940s was considered as a major breakthrough in medicine due to their effectiveness in treating bacterial infections. Many believed that these drugs would eliminate infectious diseases altogether from human populations (Aminov, 2009). It contributed to a 23-year increase in the average human lifespan. In accordance with Nagarajan (1993), one example of antibiotic is Vancomycin, which is produced by *Amycolatopsis orientalist*. It is an antibiotic used to treat serious Gram-positive infections. However, there has been a declining trend in antibiotic research and development, along with a rise in antibiotic resistance in various human infections, which leading to significant antimicrobial resistance issues (Hutchings, Truman and Wilkinson, 2019).

1.2 Antimicrobial resistance

Generally, the rise of antimicrobial resistance presents a significant challenge, particularly in the case of multidrug-resistant Gram-positive and Gram-negative

bacteria. In accordance with Neu (1992), antibiotics can effectively hinder bacterial protein and cell wall synthesis, as well as DNA replication. One of the ways that form antibiotics resistance in bacteria is through chromosomal mutation or exchanging of genetic material via the processes. For example, transformation through DNA exchange, transduction such as bacteriophage, or conjugation by plasmids. A further instance for this is the difficulties formed during the treatment of *P. aeruginosa* biofilms with standard antibiotic therapy due to the development of multidrug-resistant *P. aeruginosa* infections (Frieri, Kumar and Boutin, 2017). On top of that, several authors have revealed that the occurrence of antibiotic resistance poses a major risk to the safety and effectiveness in surgical procedures as well as immunosuppressive chemotherapy. This statement can be supported by the research from Friedman, Temkin and Carmeli (2016). In USA, around 38.7% to 50.9% of bacteria rendering illness and infections during surgery. While 26.8% of bacteria is resistance to standard antibiotics triggering infections after chemotherapy. According to Levy (1998), this resistance is driven by most resistance genes as well as the overuse of antibiotics. If a community's bacterial lacks antibiotic resistance gene, the antibiotic can effectively treat infections which caused by any bacterial species.

1.3 Antibiotic Adjuvants

Adjuvants are nonantibiotic substances that assists antibiotics function better by either preventing resistance or enhancing the body's response to infection, as

acknowledge by the findings from Wright (2016). They are typically used in clinical settings to target specific compounds that block resistance to certain antibiotics, such as beta-lactamases that cause bacteria resistant to beta-lactam antibiotics. As stated by Douafer, et al. (2019), the concept of adjuvants focuses on how to improve the antibiotics' effectiveness against resistant bacteria. Adjuvants work in synergy with antibiotics, meaning their combined effect is stronger than their individual effects, leading to increased bacterial elimination and reduced resistance development. According to Kumar, et al. (2013), utilizing adjuvants with antibiotics provide numerous benefits. One of the advantages is they can restore the existing bacteria effectiveness by inhibiting resistance mechanisms, causing pathogens susceptible to antibiotics. Additionally, adjuvants can improve antibiotic activity by few ways including enhancing penetration into bacterial cells, improve the stability as well as hindering efflux pumps that remove antibiotics from bacterial cells.

1.4 *N*-acylhydrazones and Acylhydrazones Derivatives

N-acylhydrazones are a type of organic compounds that consist of a hydrazone functional group (-N-N=CH-CO-). When a 5-chloro substituted phenyl group is substituted, they form a specific category of molecules that possess potential antibacterial properties. Congiu and Onis (2013) mentioned that there is a series of *N*-acylarylhydrazone derived from natural safrole, which exhibited anti-inflammatory properties more powerful than dipyrone and indomethacine. According to Socea, et al. (2022), the structures of acylhydrazones

group(−CONHN=) featuring an electrophilic carbon atom (CH=N), a nucleophilic imine nitrogen atom (CH=N:), as well as an amino nitrogen atom (–NH–). Consequently, acylhydrazone are electrophilic and nucleophilic molecules. They have been extensively explored across various research fields due to their diverse pharmacological characteristics. According to Congiu and Onnis (2013), several acylhydrazones have been evaluated for their antitumor effects. For example, a sequence of indole-2-carboxylic acid benzylidene hydrazides were found to render apoptosis in T47D cells, possibly through caspase activation and inhibition of tubulin polymerization. Additionally, in accordance with the findings from Cui, et al. (2022), a range of compounds containing the acylhydrazone functional group have demonstrated effective bactericidal, herbicidal, or insecticidal activities. For instance, benquinox, saijunmao, metaflumizone, and diflufenzopyr.

The objectives of this study are:

1. To study the *in vitro* antibacterial effect of *N*-acylhydrazone (NAH) in combination with ciprofloxacin, streptomycin and chloramphenicol against selected Gram-positive, Gram-negative bacteria and Methicillin-resistant strains.

2. To determine the antibacterial activity of *N*-acylhydrazones against selected Gram-positive, Gram-negative bacteria and Methicillin-resistant strains using minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).

CHAPTER 2

LITERATURE REVIEW

2.1 *N*-acylhydrazones (NAH)

In accordance with Rollas and Küçükgüzel (2007), *N*-acylhydrazones (NAH) are represented by the chemical formula R1–NHN $=$ CH–R2, with R1 and R2 representing distinct functional groups. Fraga and Barreiro (2006) have proposed that NAH is prevalent in therapeutic chemistry. The synthesis of NAH typically involves the condensation of aldehydes or ketones with hydrazides (Figure 2.1).
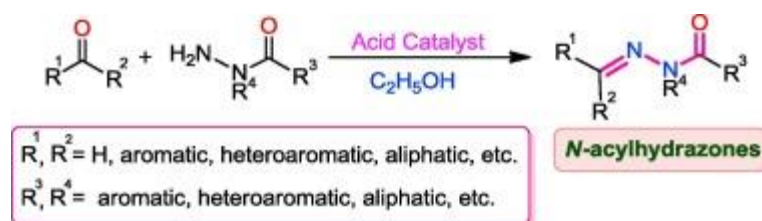
Figure 2.1: Synthesis of *N*-acylhydrazones (Frago, et al., 2006).

2.1.1 Synthesis of *N*-acylhydrazones (NAH)

In accordance with Hincapié-Otero, et al. (2021), NAH is characterized by the CHNNHC(O)– linkage. Generally, they are synthesized by condensing a hydrazide and an aldehyde as well as ketone using a small amount catalyst such as glacial acetic acid. Generally, the traditional method of synthesizing

hydrazides involved hydrazination of methyl or ethyl esters of carboxylic acids. However, it was found that ethyl dehydroabietate does not easily convert to the hydrazide. This may be due to the steric hindrance from adjacent moieties in the molecule as well as low reactivity of ester. Nevertheless, the hydrazide can obtain a good yield from dehydroabietate chloride, which exhibits better reactivity. Subsequently, the hydrazide can undergo reflux in ethanol to condense with various substituted aromatic aldehydes which yield the NAH more effectively (Gu, et al., 2012). One of the examples from Morjan, et al. (2022) stated that NAH derivatives compounds 11–17 synthesized through the condensation of 2-hydroxy-3,5-dinitrobenzohydrazide using aldehydes or ketones. Subsequently, the resulting mixtures were subjected to reflux in ethanol, leading to 75% to 85% yield of the targeted compounds (Figure 2.2). In fact, it is important to identify NAH for food safety and meet the regulatory from FDA to ensure the safety and high quality of this chemical entity.

4) $R_1 = H$, $R_2 = Ph$
5) $R_1 = H$, $R_2 = 4$-ClPh
6) $R_1 = H$, $R_2 = 4$-OMePh
7) $R_1 = CH_3$, $R_2 = Ph$
8) $R_1 = R_2 = CH_3$
9) $R_1 + R_2 = C_5H_{10}$
10) $R_1 + R_2 = C_7H_{14}$

11) $R_1 = H$, $R_2 = Ph$
12) $R_1 = H$, $R_2 = 4$-ClPh
13) $R_1 = H$, $R_2 = 4$-OMePh
14) $R_1 = CH_3$, $R_2 = Ph$
15) $R_1 = R_2 = CH_3$
16) $R_1 + R_2 = C_5H_{10}$
17) $R_1 + R_2 = C_7H_{14}$
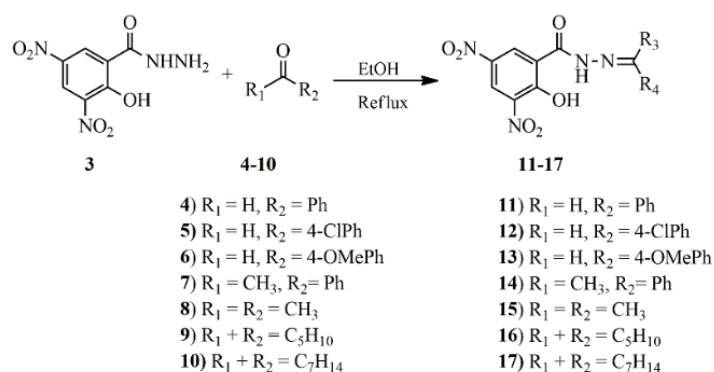
Figure 2.2: Synthesis of *N*-acylhydrazones by condensation of 2-hydroxy-3,5-dinitrobenzohydrazide (Morjan, et al., 2022).

2.1.2 Biological activity of *N*-acylhydrazones

Research supports that NAH exhibits pharmacological characteristics that lead to the development of new biologically active drugs. According to Nikolova-Mladenova et al. (2017), NAH demonstrates significant anticancer properties relevant to cancer treatment. For instance, Kassab et al. (2018) revealed that the anticancer properties of NAH derivatives of benzotriazole counter to ovarian (OVCAR-3) as well as colon (HCT-15) cancer cells, resulting in increasing of inhibition (71.17% and 70.14%). Moreover, NAH demonstrates wide spectrum biological actions, encompassing antibacterial, antiviral, anticancer, anti-inflammatory, antitubercular as well as antifungal characteristics as illustrated in Figure 2.3 (Biliz et al., 2023).
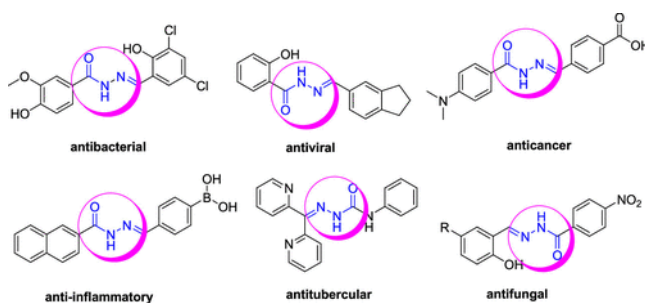
Figure 2.3: Biological activity of NAH (Biliz, et al., 2023).

2.1.3 *N*-acylhydrazones Antibacterial Activity

The compound NAH demonstrates strong antibacterial activity due to its flexible nature and the presence of hydrogen bond donors and acceptors, as indicated by Kasab and Kadewy (2018). In accordance with the study from Oliveira, et al. (2012), NAH derivative compounds (4a–4e) exhibited a potent antibacterial

activity compared to chloramphenicol, which act as a standard drug (Figure 2.4). Compounds 4a–4e showed an active antibacterial activity against the *S. aureus* (4–32 µg/mL), where Compounds 4a, 4b and 4d exhibited most active antibacterial activity against effluxing strains of *S. aureus* including SA-1199B, RN-4220, IS-58. Regarding the MRSA strains which are 007 as well as 05H, the highest active compounds are 4b, 4d, 4e. Besides, chloramphenicol showed moderately antibacterial activity with MIC and MBC, which is 64 µg/mL (Oliveira, et al., 2012). Generally, lipophilicity was found to influence the antibacterial activity in which compounds with the greater lipophilic compounds, 4b and 4d showing the highest effectiveness. Lipophilicity plays a crucial role, emphasized by Brown et al. (2021), affects the compounds' interaction with cell membranes and their permeability. Wu, et al. (2013) also mentioned that lipophilicity is crucial in interaction between site of action inside the cell with cell membrane to contact with the target. The permeability of small molecules is directly associated with their solubility in nonpolar solvents and aqueous environments (Orbach and Finkelstein, 1980).
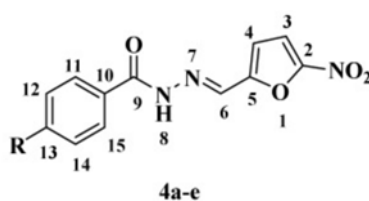
Figure 2.4: NAH derivative compounds of 4a–4e (Oliveira, et al., 2012).

Other findings from Aarjane, et al. (2020) mentioned that NAH derivatives Compounds 3a–3h (Figure 2.5) which were synthesized from acridone showed the antibacterial activity towards Gram-positive bacteria (*S. aureus*) and Gram-

negative bacteria (*P. putida, Klebsiella pneumonia, E. coli*). Compounds 3a, 3b, and 3e revealed moderately antimicrobial activity compared with tested compounds while the highest active compound is 3a, characterized by MIC value of 19.61 µg/mL. Furthermore, compound 3f exhibited high antibacterial activity towards *P. putida* with MIC value, 38.46 µg/mL. This result similar to the standard antibiotics, chloramphenicol (37.03 µg/mL). Regarding *E. coli*, all the tested compounds show moderately antibacterial activity, where MIC values ranging from 38.46 to 74.0 µg/mL. In contrast, an inactive antibacterial activity was detected in *Klebsiella pneumonia*.
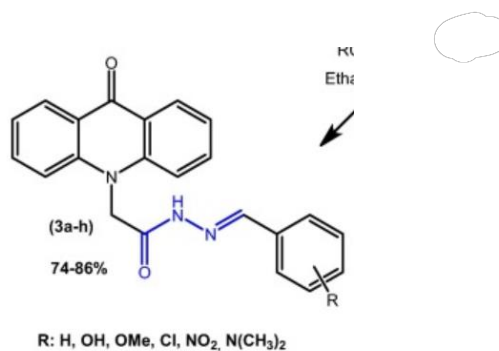
Figure 2.5: NAH derivative compounds of 3a–3h (Aarjane, et al., 2020)

Moving on to another findings from Gu, et al. (2012), NAH derivative compounds 4a–q which are synthesized from dehydroabietic acid (Figure 2.6) exhibited antibacterial activity towards Gram-positive bacteria (*S. aureus* and *B. subtilis*) and Gram-negative bacteria (*E. coli* and *P. fluorescens*). There are several compounds, including 4c, 4d, 4l–n and 4p demonstrated active antibacterial activity with MIC value 1.9–7.8 µg/mL against Gram-positive bacteria. Compound 4p exhibited particularly strong activity against *S. aureus* and *B.* (1.9 µg/mL) compared to the positive control, amikacin. In addition,

Compounds 4l, 4n, and 4p also displayed potent inhibition (3.9–7.8 µg/mL) against the Gram-negative bacteria. Moreover, Compounds 3, 4a, 4e, 4g, 4h, and 4k demonstrated intermediate antibacterial activity towards some bacteria strains, characterized by the MIC values ranging from 15.6–31.2 µg/mL. In contrast, Compounds 4b, 4f, 4i, 4j, 4o, and 4q revealed inactive antibacterial activity towards the four bacteria strains. The obtained results demonstrated the halogen group F and Cl atoms, were found to enhance the antibacterial activity when substituted to a compound, as suggested by Plech, et al. (2011). Besides, the presence of NO_2 atoms crucial for antibacterial activity since Compounds 4l, 4m, and 4p revealed high antibacterial activity towards selected bacteria.
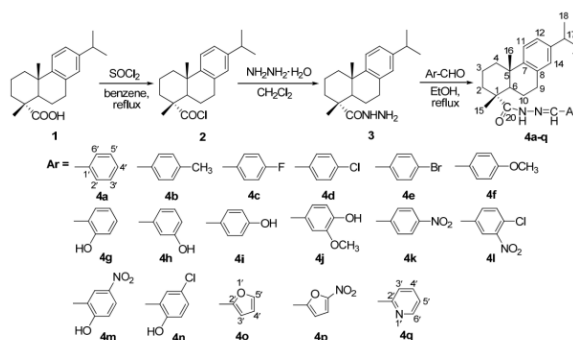
Figure 2.6: Structures and synthesis of *N*-acylhydrazones from dehydroabietic acid (Gu, et al., 2012).

2.2 Antibiotics Classification

According to Russel, et al. (2004), antibiotics is known as the organic compounds that produced by microorganisms which harmful to other organisms. One of the key targets of antibiotics in bacteria is ribosome. Mitcheltree, et al. (2021) mentioned that lacosamide is a class of antibiotics that target ribosome.

While the founding of the class lincomycin (Figure 2.7), was isolated in 1963 from a streptomycete in Nebraska and was utilized in streptococcal, pneumococcal and staphylococcal infections treatment (Lin et al., 2018).
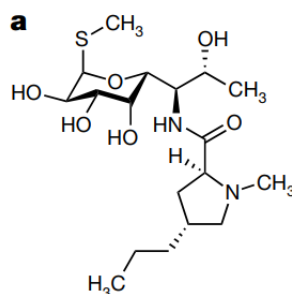
Figure 2.7: Structure of lincomycin (Mitcheltree, et al., 2021).

Etebu and Arikekpar (2016) proposed several methods to categorize antibiotics including structures of the molecules, mode of action, as well as range of activity. van Hook, et al. (2012) suggested that antibiotics are being classified according to either chemical or molecular structure including beta-lactams, macrolides, tetracyclines, quinolones, aminoglycosides, sulphonamides, glycopeptides, and oxazolidinones. Antibiotics in the similar structural category will exhibit similar effectiveness, poisonousness as well as potential side effects.

2.2.1 Beta-lactam

According to Ghuysen (1991) beta-lactam consist of three carbon and one nitrogen ring which is greatly reactive (Figure 2.8). Due to beta-lactam antibiotics have the ability of inhibit the bacterial transpeptidases functions, they are also known as penicillin-binding proteins (PBPs). Typically, 4 PBPs existed in most of the bacteria species, where the PBPs inhibitory of beta-lactam

antibiotics is due to the structure, geometric and stereochemical similarities of PBPs within the amide bonds and enzyme substrate (Cochrane and Lohans, 2021). It can be classified in several ways, such as the physiological impact of beta-lactam on bacterial cell growth, sustainability, shape and integrity.
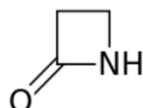
Figure 2.8: Structure of beta-lactam (Etebu and Arikekpar, 2016).

2.2.2 Quinolones

Moving on to quinolones, Gregory (2015) posited that quinolones establish a class of antibiotics characterized by a core structure that related to 4-quinolone (Figure 2.9). These antibiotics have been approved for its effectiveness due to their potent and broad-spectrum antimicrobial activity (Andersson and MacGowan, 2003). Furthermore, Sissi and Palumbo (2010) suggested that quinolone antibiotics function to prevent two type IIA bacterial topoisomerases, specifically DNA gyrase as well as topoisomerase IV. Whereby the quinolone genesis development can be traced back to the first-generation quinolone, which exhibited restriction against only Gram-negative bacteria, apart from *Pseudomonas* spp. (Blondeau, 2004). Subsequently, Sharma et al. (2009) proposed the beginning of the second-generation quinolone which known as flumequine, exemplifying that the addition of one fluorine (F) atom at the R6 position could enhance the activity spectrum. This modification has enhanced the quinolone action.

Furthermore, the second-generation fluoroquinolones such as enoxacin, norfloxacin, and ciprofloxacin were able to hinder the antibacterial activity of all Gram-negative organisms, including *Pseudomonas* species (Ruiz, 2003). Van Caekenberghe et al. (1984) suggested that these drugs were further enhanced by the adding of a cyclopropyl group to R1 position as well as a piperazine ring to the R7 position. The cyclopropyl group improved the overall antibacterial properties, whereas R7 piperazine ring enhanced the strength against Gram-negative bacteria (Peterson, 2001). This combination rendered ciprofloxacin the most potent compound amongst the early second-generation compounds, hence it acts as the preferred choice for *P. aeruginosa* inhibition. In fact, fluoroquinolones aids in prevention of severe diseases including renal, respiratory, as well as sexually spread bacterial infections, as mentioned by Van Bambeke, et al. (2005). In third-generation quinolones, Gram-negative and Gram-positive coverage are improved. In contrast, fourth generation fluoroquinolones can treat anaerobic, Gram-positive and Gram-negative bacteria (Ambrose, 1997).

Figure 2.9: Structure of 4-quinolone (Pham, Ziora and Blaskovish, 2019).
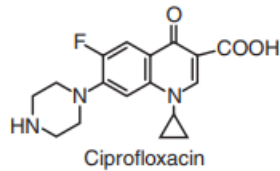
Figure 2.10: Structure of ciprofloxacin (Heeb, et al., 2010).

2.2.3 Aminoglycoside

Aminoglycosides have a wide range of antibacterial activity which preventing bacteria from binding to ribosomal subunits and hinder protein synthesis as well (Peterson and Kaur, 2008). They are efficient at inhibiting aerobic Gram-negative and Gram-positive bacteria. According to Beeker and Cooper (2013), aminoglycosides are composed of a 2-deoxystreptamine (2-DOS) ring bound with two or more amino-modified sugars through glycosidic bonds (Figure 2.11). Vaara (1992) has suggested that aminoglycosides also attach to the bacterial ribosome and hinder the production of bacterial protein. The transportation of these polar molecules through the Gram-negative bacteria's outer membrane is facilitated by molecules themselves. It involves drug-induced interference with connections of Mg^{2+} linking neighbouring lipopolysaccharide molecules (Hancock, Farmer, Li and Poole, 1991).

Aminoglycosides can be categorized according to both chemical structure as well as biosynthesis. Streptomycin (Figure 2.12), as mentioned by Schatz et al. (2005), is the earliest identified aminoglycoside. It is commonly used in the treatment of bubonic plague, tularaemia, and tuberculosis. Additionally, Cunha (2006) suggested that some aminoglycosides derived from paromamine contain

2-DOS as a fundamental structure. For example, kanamycin, neomycin and gentamicin (Figure 2.13). Research has shown that kanamycin comprises 4,6-substituted 2-DOS derivatives, with 2-amino- or 2,6-diamino-glucose acting as ring B and 3-aminoglucose as ring C (Yokoyama et al., 2007). On the other hand, neomycin consists of 1 or 2 hexoses and a furanose bound to position 4 and 5 of 2-DOS. Furthermore, gentamicin is also a type of aminoglycoside derived from paromamin. Hong et al. (2009) proposed that the structure of gentamicin involves a 4,6-substituted 2-DOS and 2 hexoses which consist of either extra carbon side chains or an unsaturated B ring.
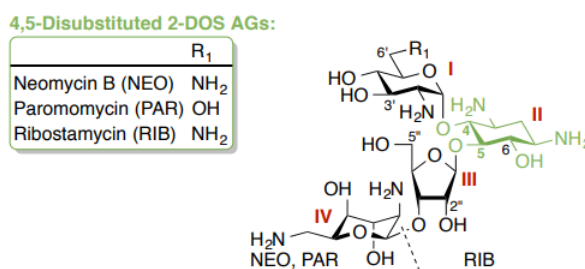
Figure 2.11: Structure of aminoglycosides (Garneau-Tsodikovaa and Labby, 2016).
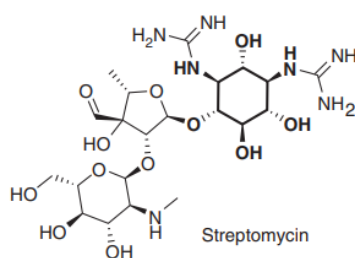
Figure 2.12: Structure of streptomycin (Krause, Serio, Kane and Connolly, 2016).
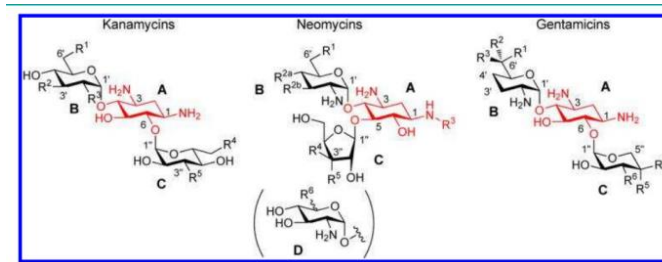
Figure 2.13: Structure of Kanamycins, Neomycins and Gentamicins (Becker and Cooper, 2013).

2.3. Type of Antibiotic Resistance

Boucher, et al. (2009) mentioned that antibiotic resistance among bacteria become one of most critical challenges against human health nowadays, especially the ESKAPE pathogens including *Enterococcus faecium, S. aureus, Klebsiella pneumoniae, Acinetobacter baumannii, P. aeruginosa,* and *Enterobacter* species. Based on the research from MacGowan (2008), The resistance of pathogenic bacteria to antibiotics can be characterized either through microbiological or clinical methods. The resistance of microbiological usually relates to the existence of a genetically specified resistance mechanism, which either acquired or mutated. This classification categorized the bacteria as resistance or susceptible based on specific threshold tests carried out in a phenotypic laboratory (MacGowan and Macnaughton, 2017).

Acquired resistance is derived from the main chromosome or additional chromosome structures including plasmids and transposons (Aljanaby and Aljanaby, 2018). The examples related to inherent antibiotic resistance including all Gram-positive bacteria resistance towards colistin, *Enterobacteriaceae*

resistance against glycopeptides and linezolid, as well as the intrinsic resistance of *P. aeruginosa* against broad spectrum of antibiotics.

Moving on to cross resistance, several studies have demonstrated that cross resistance refers to the phenomenon wherein specific microorganisms exhibit resistance to a particular antibiotic and concurrently develop resistance to other antibiotics that operate via identical or related mechanisms (Etebu and Arikekpar, 2016). Moreover, Jahne, et al. (2015) mentioned that this occurrence is commonly observed in cases where the antibiotics share structural similarities, such as erythromycin, neomycin and kanamycin resistant, as well as resistance to cephalosporins and penicillins. Notably, the study of Szybalsky and Bryson (1952) suggested that if there is a strain produced resistant to one antibiotic, exhibits a significantly greater resistance to another, this may indicate the presence of biological activity as well as potentially chemical similarity within the two antibiotics. In fact, cross resistance has been continually declared to appear among chemically similar antibiotics and other chemotherapeutic agents.

The concept of multi-drug resistance, as elucidated by Alanis (2005), pertains to pathogens exhibiting resistance to multiple antibiotics, rendering them resistance to therapy with single drug interventions. This commonly involves the acquisition of distinct drug-resistant genetic elements, typically occurred in R-plasmids (Tóth, et al., 2020). Subsequently, the resistance in multidrug also could arise by enhanced gene expression including efflux pumps, enzymatic antibiotic inactivation as well as target structure transformations (Salloum, Michel and Teyyara, 2020). Notably, methicillin-resistant *S. aureus* (MRSA)

serves as a prominent example, demonstrating resistance not only to methicillin but also to other antibiotics including aminoglycosides, macrolides, tetracycline, chloramphenicol and lacosamide which cause main problems within healthcare (Nikaido, 2009; de Lencastre, Oliveira and Tomasz, 2007). For instance, MRSA is a significant contributor to bacteraemia, causing 12% of endocarditis cases. Generally, the typical infection sources are central venous catheters and pneumonia (Montazeri, et al., 2015). Generally, research indicates that MRSA affects more than 150,000 patients yearly in European Union (Kanerva, et al., 2007; Köck, et al., 2010).

2.4 Antibiotics Resistance Mechanisms

2.4.1 Modification of Antibiotics Molecules

Prashanth et al. (2012) proposed that modifications occurring in drug-related receptors and the target regions' sites are different, affecting complex enzymes and ribosomes. Macrolide antibiotics is the prevalent resistance to variant ribosomal targets (Shaikh et al., 2007). According to the findings from Kumar et al. (2023), ribosomal mutation in aminoglycosides and oxazolidinones or ribosomal protection in tetracyclines are the examples of drugs resistance to targeting ribosomal subunits. These mechanisms disrupt the drug's capacity to form attachment with ribosome (Roberts, 2004). Regarding drugs such as fluoroquinolones which target the synthesis of nucleic acid, resistance is mediated by alterations in DNA gyrase in Gram-negative bacteria such as gyrA and topoisomerase IV in Gram-positive bacteria such as grlA (Hawkey, 2003). Moreover, Redgrave et al. (2014) have mentioned that these mutations lead to

structural modifications in gyrase and topoisomerase, thereby reducing as well as eliminating the drug binding ability to the compounds.

2.4.2 Antibiotic Penetration and Efflux

Generally, most bacteria have various types of efflux pumps. Blair, et al. (2015) mentioned that bacterial efflux pumps are important in Gram-negative bacterial pathogens formed inherent resistance to numerous drugs. Typically, it is used to treat Gram-positive bacterial infections. Research has revealed that Gram-positive efflux pumps consist of chromosomes that may confer intrinsic resistance (Piddock, 2006), and some are also carried on plasmids (Costa, et al., 2013). According to Blair et al. (2014), these pumps are categorized as 5 primary groups based on their configuration as well as energy source. For instance, ATP-binding cassette family, multidrug and toxic compound extrusion family, small multidrug resistance family, major facilitator superfamily as well as resistance-nodulation-cell division family. As mentioned by Breidenstein, de la Fuente-Núñez, and Hancock (2011), resistance commonly evolves via active pump systems in the antibiotics that consist of tetracycline group. Li, et al. (2020) found that tetracyclines are expelled from the cell by energy-dependent active pumping systems, leading to resistance control in plasmids and chromosomes. Additionally, active pumping systems have been found to effectively resist quinolones, 14-membered macrolides, chloramphenicol and beta-lactams (Guo, et al., 2020).

2.4.3 Permeability of Inner and Outer Membrane

Santajit and Indrawattana (2016) have mentioned that alterations in the inner and outer membranes permeability can result in reduced drug absorption by the cell or instantaneous removal of drugs by pump systems. According to the findings of Dugassa and Shukuri (2017), it has been observed that Gram-negative bacteria possess an outer cell membrane, that imposes drug pass through specific channels within the membrane for cellular material entry or exit. The drugs must successfully pass through these channels to attach with the cell wall or enter the cell. Any genetic mutation affecting these channels can hinder the entry of antibiotics into the cell, despite the antibiotics retaining their functional activity. This is due to the alteration of the electrical charge as well as the physical arrangement at the membrane may hinder the antibiotics from reaching their intended target site (Galdiero et al., 2012). Notably, a reduction in outer membrane permeability can significantly contribute to quinolone and aminoglycoside resistance (Li et al., 2012).
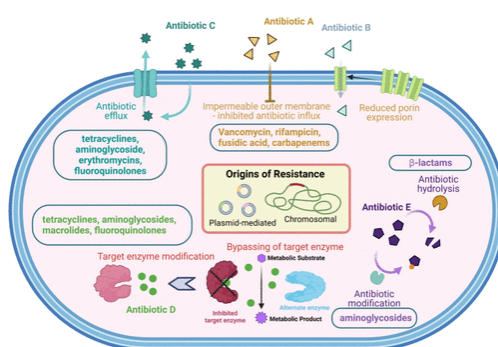
Figure 2.14: Mechanisms of Antibiotic Resistance (Dhanda, Acharya and Haldar, 2023).

2.5 Classification of Antibiotic Adjuvants

Antibiotic adjuvants can boost antibiotic efficacy rather than directly kill bacteria. They operate through mechanisms such as restricting resistance, improving intracellular antibiotic accumulation, complementing bactericidal actions, hinder the signalling and regulatory pathways as well as improving the host reaction against the infection of bacteria (Liu, et al., 2019). These antibiotic adjuvants are co-administered with antibiotics, resulting in a combination drug. The concept of antibiotic adjuvants draws from the successful use of antibiotic combinations in clinical practice which is combination of Antibiotic A and Antibiotic B (Eliopoulous and Eliopoulous, 1988; Drusano, et al., 2015). Regarding to the bacterial target and the corresponding mechanism, antibiotic adjuvants (AA) can be broadly subdivided into Class I and Class II categories (Wright, 2016). Class I AA works via targeting active as well as passive resistance mechanisms in bacteria, while Class II AA enhances the antibiotic efficacy within the host (De Oliveira et al., 2020). The subcategories within Class I adjuvants are further delineated based on their respective mechanisms.

2.5.1 Class IA Adjuvants

As mentioned by Gill, Franco, and Hancock (2015), Class IA adjuvant which known as "inhibitors of active resistance," directly hinder the antibiotic resistance by targeting inactivating enzymes, efflux pump systems as well as alternate targets. One of the examples is beta-lactamase. Generally, it is the only adjuvant currently used in clinical practice. When beta-lactam antibiotics are

enzymatically degraded by beta-lactamase enzymes, they lose their efficacy. These enzymes are commonly grouped into two categories including serine beta-lactamases which characterized by the presence of a residue of serine for hydrolysis, and metallo beta-lactamases, whose hydrolytic activity is facilitated by a metal ion such as Zn^{2+} (Bush and Bradford, 2019). A prevalent scheme for categorization, founded on protein sequence similarities, identifies four principal of beta-lactamase classes including A, B, C and D (Drawz and Bonomo, 2010). Bush (2013) postulates that classes A, C, and D considered as serine- beta-lactamases, since they aids in catalytic serine residue to form the reactive nucleophile. On the other hand, the enzymes TEM, SHV, CTX-M, and KPC categorized as Class A, while AmpC as well as plasmid-encoded CMY-type cephalosporinases considered Class C. Lastly, the OXA enzymes such as oxacillinases classified as Class D (Smet et al., 2008; Poirel Naas and Nordmann, 2010; Philippon et al., 2019). Conversely, class B enzymes represent zinc-dependent hydrolases and showed resistance to a most of beta-lactam antibiotics such as penicillins, cephalosporins as well as carbapenems (Linciano et al., 2019).
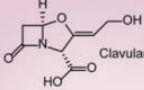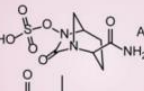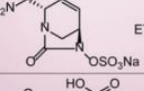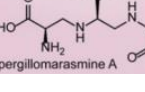
Figure 2.15: Examples of beta lactamase as Class IA Adjuvants (Liu, et al., 2019).

2.5.2 Class IB Adjuvants

Additionally, Khalan and Wright (2011) postulated that Class IB adjuvants boost antibiotic effectiveness by evading intrinsic resistance mechanisms, including metabolic pathways as well as physiological methods, rather than directly inhibiting specific resistance components. Consequently, Class IB adjuvants known as "inhibitors of passive resistance" (Wright, 2016; Sheard et al., 2019). One of the examples of Class IB adjuvant is efflux pump inhibitors. Generally, there will be a higher activity and presence of efflux pump in Gram-negative bacteria which contribute to both intrinsic and acquired resistance against antibiotics (Yoon, et al., 2015). These pathogens possess various types of efflux pumps, including ATP binding cassette (ABC), small multidrug resistance (SMR) family, major facilitator superfamily (MFS), multidrug and toxin extrusion (MATE) family, resistance nodulation, cell division (RND) family, and proteobacterial antimicrobial compound efflux (PACE) superfamily (Huang et al., 2022). The RND superfamily is particularly considerable as a main group of efflux pump in Gram-negative bacteria. However, the findings from Lamers, Cavallari and Burrows (2013) mentioned that phenylalanine-arginine beta-naphthylamide (PAbN) has been observed to hinder the activity of numerous RND family pumps, consequently lowering intrinsic resistance as well as eliminate the resistance against various classes of antibiotics. For instance, beta-lactams and quinolones. On the other hand, MFS, ABC, MATE, and SMR efflux pumps are present in both Gram-positive and Gram-negative bacteria (Blanco, et al., 2016; Auda, Ali salmon and Odah, 2020). In accordance with Abdel-Karim, et al. (2022), Class Ib adjuvants including efflux pump inhibitors (EPIs), have been recognized for their capability to hinder these efflux pumps, thus

averting antibiotic displacement, and encouraging the accumulation of greater antibiotic concentrations within bacterial cells, ultimately boosting their effectiveness.

2.5.3 Class II Adjuvants

Class II adjuvants, also known as "host modulating adjuvants," function by targeting the cellular processes to increase the antibiotics effectiveness of host. This can be achieved by either initiating an immune response or increasing phagocytosis (Wright, 2016; Dhanda et al., 2023). Antibiotic adjunct therapies focusing on "host defence-targeted" mechanisms show a less likely of inducing microbial resistance compared to direct pathogen targeting. For instance, certain immunomodulatory peptides (LL-37), have demonstrated ability to boost the intrinsic immune system's antibacterial activity by upregulating the response of neutrophil antimicrobial and downregulating pro-inflammatory cytokines, as well as IFN-c (Mansour, Pena and Hancock, 2014). Additionally, Bai et al. (2013) mentioned that compounds such as BAY 11-7082, which known as the inhibitor of IkBα kinase, have shown potential in hindering activation of nuclear factor-kappa B (NF-kB). Thereby enhancing macrophage apoptosis and autophagy which crucial in the intracellular *Mycobacterium tuberculosis* (MTB). Furthermore, targeting host defence mechanisms, such as regulating pattern recognition receptor (PRR) signalling pathways, has demonstrated effectiveness. This is exemplified by the 4C-Staph/T7-alum vaccines with the use of a TLR7-agonist (SMIP.7-10) and T7-alum adjuvant in efficiently treating staphylococcal strain-induced peritonitis in mice (Mancini et al., 2016). Guchhait et al. (2015).

proposed that utilize of non-peptide amphiphilic tobramycin analogues that is similar to host defence peptides is able to enhance the innate resistant by particular immune cells such as neutrophils that is crucial in eliminating bacterial pathogens.

2.6 Implications for Animal Health and Food Safety Consideration

2.6.1 Potential Applications of Antibiotics

In line with the findings of You and Silbergeld (2014), the application of antibiotics is not only confined to clinical settings for treating human ailments, it also extends to agricultural and veterinary contexts. In agricultural practices, antibiotics are utilized for treating animals, enhancing efficiency of feed modification, ensuring food safety, and preventing disease outbreaks. There is a notable concern regarding the similarity between the antibiotics used in agricultural and veterinary settings and those prescribed to humans, encompassing their uses, types and mechanisms of action (Hong et al., 2013).

Antibiotics including aminoglycosides, β-lactams, chloramphenicol, fluoroquinolones, glycolipids, ionophores, macrolides, quinolones, streptogramins, sulfonamides, and tetracyclines are commonly applied in both cattle and dairy productions, as mentioned by Kim and Ahn (2022). Zaher, et al. (2013) suggested that tetracyclines are frequently administered to handle the diseases of cattle, including infection of respiratory, gastrointestinal as well as integumentary systems. Especially *Pasteurella multocida*, and *Streptococcus*

spp., act as the primary causes of infectious disease among cattle. Additionally, antibiotics are applied in poultry farming to support the well-being and expedited growth of chickens. Among the antibiotics that frequently used in poultry, virginiamycin, bacitracin, salinomycin and tilmicosin are prescribed for treating infectious diseases triggered by Avian Pathogenic *E. coli* (APEC) and *S. pullorum* (Kim and Ahn, 2022). Therefore, antibiotics play a crucial role in treating disease outbreak amongst the animal.

2.6.2 Safety and Consideration

According to Billah et al. (2015), antibiotics is valuable in promoting human health, supporting veterinary medicine and sustaining agriculture, all of which are crucial for safeguarding populations and upholding food security when administered in controlled doses. However, it entails potential direct and indirect effects for human health. Direct effect is the exposure to antibiotic-resistant bacteria originating from food animals, while indirect effect arise from the contact with resistant organisms within the ecosystem, such as water and soil, due to the usage of antibiotics to food animals (Landers, et al., 2012). A relevant example is the application of tylosin in poultry, swine and cattle, distributed through medicated feed, drinking water as well as injection for disease prevention as well as prevent the growth of pathogen (Hurd, et al., 2004).

Furthermore, the overuse of antibiotics in animals caused emergence of antimicrobial resistance (AMR), a critical public health issue. Research indicates that the utilization of antibiotics in food producing has significantly become the

main cause of selection and transmission of resistant bacteria (Von Wintersdorff, et al., 2016). The World Health Organization (WHO) has estimated AMR will become top ten in global public health threats by 2050, it could result in over 10 million annual fatalities. Notably, certain antibiotics, such as tetracyclines and ampicillin, exhibit low oral bioavailability in pigs and poultry, impacting both the animals and the environment (Bibbal, et al., 2007) In addition, Hansen, Aarestrup and Sørensen (2002) mentioned that the unabsorbed antibiotics modify the intestinal microbiota as well as retain microbiologically active in the excreted animals' faeces which impact the environment organisms as well.

CHAPTER 3

METHODOLOGY

3.1 Experimental Flowchart
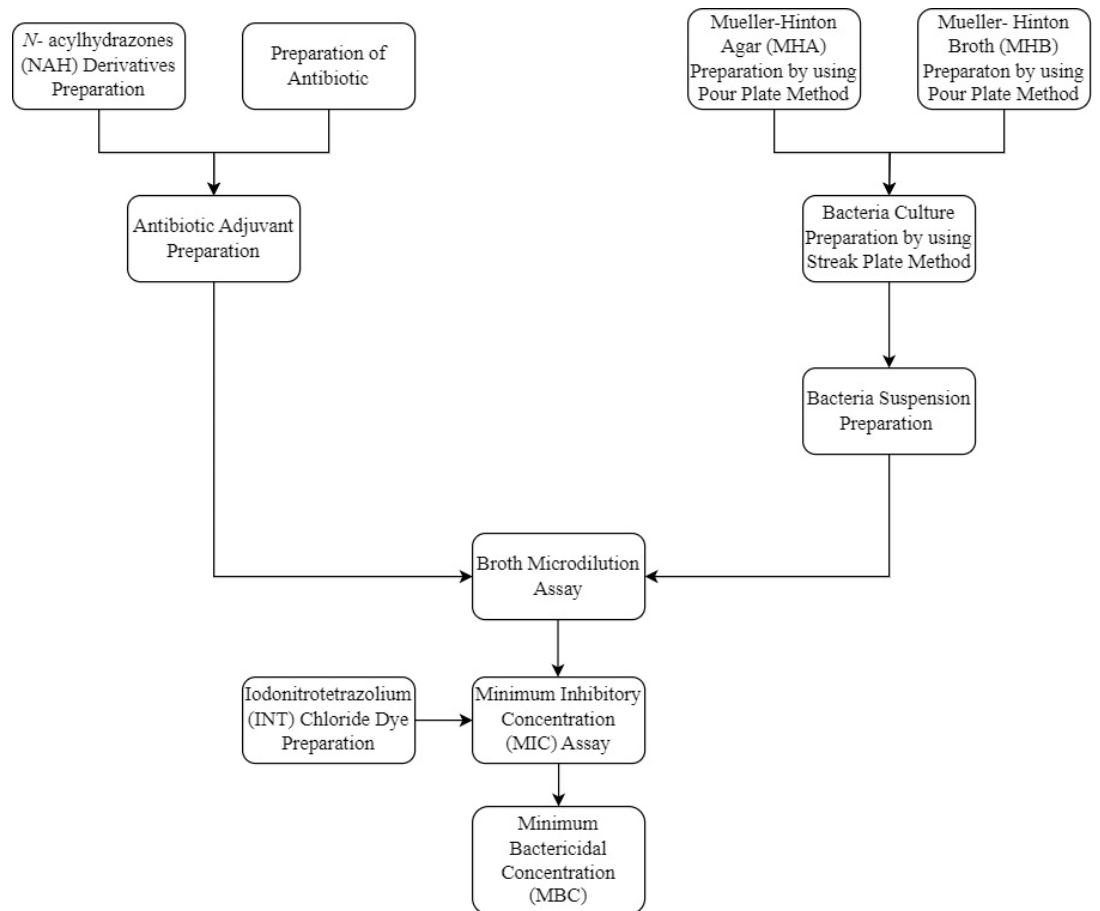
Figure 3.1: Overview of experiment.

3.2 Materials and Apparatus

3.2.1 *N*-acylhydrazones Derivative Compounds

Nine *N*-acylhydrazones (NAH) derivatives compounds of different aromatic meta- and para- substitutions were provided by Dr. Teo Kah Cheng who, an assistant professor from the Department of Agricultural and Food Science and Dr. Sim Kooi Mow, associate professor from the Department of Chemical Science in the Faculty of Science at University Tunku Abdul Rahman in Kampar. The compounds were authenticated through NMR spectroscopic analysis. Table 3.1 and Figure 3.2 displayed the nine NAH derivatives with different aromatic substitutions.
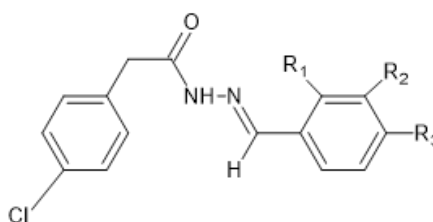
Figure 3.2: Core chemical structure of 5-chloro substituted phenyl *N*-acylhydrazones with aromatic substitution at meta and para directors as potent adjuvants.

Table 3.1: Nine NAH derivative compound with their substituent groups and different functional R substituents respectively.

NAH Derivative Compunds	Substituent Groups	R Substituents		
1	3,4-Cl$_2$	H	Cl	Cl
2	3,4-OCH$_3$	H	OCH$_3$	OCH$_3$
3	4Br	H	H	Br
4	4CH$_3$	H	H	CH$_3$

Table 3.1 Continued: Nine NAH derivative compound with their substituent groups and different functional R substituents respectively.

5	**4Cl**	**H**	**H**	**Cl**
6	4F	H	H	F
7	$4NO_2$	H	H	NO_2
8	$4OCH_3$	H	H	OCH_3
9	H	H	H	H

3.2.2 Bacteria Strains

In this project, the bacteria used were as follows: Gram-positive bacteria, including *Bacillus cereus* (ATCC 13061), *Bacillus subtilis* subsp. *spizizenni*(ATCC 6633) and *Staphylococcus. aureus* (ATCC 6538). The Gram-negative bacteria included *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) and *Salmonella* Typhimurium (ATCC 14028). Additionally, two strains of Methicillin-resistance *Staphylococcus aureus* with ATCC 33591 and ATCC 43300.

3.2.3 Apparatus, Consumable and Glasswares

Table 3.2 showed the apparatus, consumable and glasswares used in this project with their manufacturer and country origin.

Table 3.2: List of apparatus, consumable and glasswares.

Apparatus/ Consumable and glassware	Manufacturer	Country of Origin
96- well plates	Premier Diagnostic	Malaysia
Aluminium foil	MyChef	Malaysia
Beaker (250 ml, 500 ml, 1000 ml)	Duran	Germany
Bunsen burner gas cartridge	HmbG	Malaysia
Centrifuge tubes (15 ml)	Fisher Scientific	China
Cotton Swab	Biomedia	Malaysia
Cuvette (1.5 ml)	-	-
Glass sample vial tubes (5 ml)	HmbG	Malaysia
Gloves	IRONSkin	Malaysia
Inoculating loop	-	-
Laboratory spatula	-	-
Measuring cylinder (100 ml)	Glassco	Scotland
Micro spatula	-	-
Micropipette tips (0.5 – 10 μl)	Axygen Scienfic	United States
Parafilm	Fisher Scientific	United States
Petri dish	NEST	China
Portable Bunsen burner	HmbG	Malaysia
Schott bottles (250 ml, 500 ml, 1000 ml)	Duran	Germany
Spark lighter	Spark-L	Japan
Weighing boat	-	-

3.2.4 Chemical Reagents and Media

Table 3.3 presented the chemical reagents and media used in this project with their manufacturer and country origin.

Table 3.3: List of chemical reagents and media.

Chemical Reagents/ Media	Manufacturer	Country of Origin
95% ethanol	Systerm Chemicals	Malaysia
absolute ethanol	Chemical Industries (Malaya)	Malaysia
Chloramphenicol	Bio Basic Inc.	Canda
Ciprofloxacin	Bio Basic Inc.	Canada
Dimethyl Sulfoxide (DMSO)	Synerlab	France
Distilled water	Faculty of Science, UTAR Kampar	Malaysia
Iodonitrotetrazolium (INT) chloride powder	Sigma-Aldrich	United States
Mueller-Hinton (MH) agar	Titan Biotech	India
Mueller-Hinton (MH) broth	Condalab	Spain
Streptomycin sulfate, *Streptomyces* sp.	Merck	China

3.2.5 Instruments

Table 3.4 presented the instruments used in this project with their manufacturer and country origin.

Table 3.4: List of instruments.

Instruments	Manufacturer	Country of Origin
Analytical balance	Mettler- Toledo	United States
Autoclave machine	Hirayama	Japan
Drying oven	Binder	Germany
Freezer (-20˚C)	Liebherr	United States
Incubator (37˚C)	Memmert	Germany
Laminar Air Flow Cabinet	Esco	Singapore
Microbalance	Mettler- Toledo	United States
Micropipette (100-1000 µL)	Eppendorf	Germany
Micropipette (20-200 µL)	Eppendorf	Germany
Refrigerator (4˚C)	KIM	Malaysia
UV- Vis Spectrophotometer	Thermo Fischer Scientific	USA
Vortex mixer	Scientific Industries	United States

3.3 Methodology

3.3.1 Preparation of NAH Derivatives Compounds

A quantity of 3 mg of the initial NAH derivative compound was measured using a microbalance and placed into a glass vial. Subsequently, a total volume of 3 mL Dimethyl sulfoxide added to the glass vial in a 1:1 ratio, resulting in a final concentration of 1000 mg/mL. These steps were repeated for the remaining 8 NAH derivative compounds. After being vortexed, the NAH derivative compounds were stored at room temperature.

3.3.2 Antibiotic Preparation (Ciprofloxacin)

An amount of 0.625 mL of 100 µg/mL ciprofloxacin solution was transferred to a sterile centrifuge tube using micropipette and diluted with 9.375 mL sterile distilled water. This resulted in achieving a 10 mL ciprofloxacin solution with final concentration of 6.25 µg/mL. Subsequently, the centrifuge tube was being vortexed, labelled, covered with aluminium foil and kept in a refrigerator at 4°C.

3.3.3 Antibiotic Preparation (Streptomycin)

One mg of streptomycin powder was weighed by using an analytical balance then transferred to a sterile centrifuge tube. Subsequently, 10 mL of sterile distilled water were added into streptomycin powder, yielding a 100 µg/mL final concentration. The centrifuge tube was then vortex using a vortex mixer, labelled, covered with aluminium foil as well as kept in a refrigerator at 4˚C.

3.3.4 Antibiotic Preparation (Chloramphenicol)

First, 1 mg of chloramphenicol powder was weighed using an analytical balance and added to a sterile centrifuge tube. Then, a 10mL sterile distilled water was mixed with chloramphenicol powder and vortexed to create a 100 µg/mL concentration. Next, 2.5 mL of the 100 µg/mL chloramphenicol solution was moved to another sterile centrifuge tube, followed by the addition of 7.5 mL of sterile distilled water, resulting in a 25 µg/mL final concentration. The tube was then vortexed, labelled, covered by using aluminium foil, and kept in the refrigerator at 4˚C.

3.3.5 Preparation of Adjuvants

A volume of 1 μg of 9 NAH derivative compound was pipetted into different sterile glass vial and labelled correspondingly. Subsequently, 1 μg of the prepared ciprofloxacin solution was introduced into the initial glass vial containing the NAH derivative compound. This step was repeated for the remaining 8 NAH derivative compound. The adjuvant solutions were stored in refrigerator at 4˚C. The entire process was repeated for the streptomycin-adjuvant and chloramphenicol-adjuvant solutions.

3.3.6 Preparation of Iodonitrotetrazolium (INT) Chloride Dye

A volume of 4 mg of INT chloride powder was weighed using an analytical balance and then transferred to a sterile centrifuge tube. Subsequently, 3 drops of absolute ethanol (99%) were added to the centrifuge tube to facilitate the dissolution of the INT chloride powder. Then, a volume of 10 mL sterile distilled water was introduced into the centrifuge tube to obtain a 0.4 mg/mL final concentration. Next, the centrifuge tube was vortexed, labelled, wrapped with aluminium foil and stored in refrigerator at 4˚C.

3.3.7 Mueller-Hinton (MH) Agar Preparation

A quantity of 15.2 g MH agar powder was measured using an analytical balance and subsequently transferred into a 500 mL Schott bottle. Then, 400 mL of distilled water was poured into the Schott bottle and shaken gently to dissolve the MH agar powder. Subsequently, the Schott bottle was labelled and subjected

to autoclave. The sterile MH agar solution was poured into petri dishes and allowed to cool in a laminar flow cabinet at room temperature for approximately 45 min for solidification. Once solidified, the MH agar plates were sealed with parafilm and kept in the refrigerator at 4˚C.

3.3.8 Mueller-Hinton (MH) Broth Preparation

An amount of 8.4 g of MH broth powder was measured by using an analytical balance then transferred into a 500 mL Schott bottle. Following this step, 400 mL of distilled water was poured into the Schott bottle and swirled to enable the dissolution of the MH broth powder. The Schott bottle was then labelled and subjected to autoclaving. The sterile MH broth solution was sealed with parafilm and kept in a refrigerator at 4˚C.

3.3.9 Preparation of Bacteria Suspension

An amount of 5 mL and 4.95 mL of sterile MH broth were pipetted to labelled sterile centrifuge tubes. Bacteria colonies from the prepared culture plate were inoculated into the centrifuge tubes containing 5 mL of MH broth using an inoculating loop. The bacteria suspension was then vortexed. Subsequently, 1 mL of bacteria suspension and 1 mL of sterile MH broth were pipetted into different cuvettes, and absorbance was identified by using a UV-Vis Spectrophotometer at 625 nm. The optical density (OD) reading ranged from 0.08 to 0.10A, indicating 1×10^8 CFU/mL bacterial concentration. Once the desired OD achieved, 50 µg of the bacteria suspension was pipetted into the centrifuge tube containing 4.95 mL of sterile MH broth, formed a final bacterial

suspension concentration of 1×10^6 CFU/mL (Clinical and Laboratory Standard, 2017).

3.3.10 Broth Microdilution Assay

Figure 3.3 represents the setup of 96 well plates for *N*-acylhydrazones (NAH) derivative compounds and adjuvants (Clinical and Laboratory Standard, 2017). A quantity of 100 µL of sterile MH broth was pipetted to four corners of the wells that labelled as "S" represented sterility control while 50 µL of sterile MH broth was added to the remaining wells, except for the empty ones labelled as blank. DMSO control was prepared by pipetting 50 µL of Dimethyl sulfoxide (DMSO) solution to the selected well (12B) and serial dilution was conducted by transferring 50 µL of mixture to the subsequent wells (12B). This step was repeated for wells 12C, 12D, and 12E. 50 µL of well 12E was discarded to achieve a final concentration of 6.25% of DMSO. For negative control, 50 µL of dissolved NAH derivative compounds were pipetted correspondingly into the wells labelled as "N" that represented negative control. Then, a volume of 50 µL of the first dissolved NAH derivative compounds was pipetted into the designated well (2A) and pipette mixed 10 times. Serial dilution was then conducted by transferring 50 µL of the mixture to the subsequent wells from row A to row H. For instance, 2A to 2B, 2B to 2C, and the final 50 µL in 2H was discarded. The mixture was pipette mixed before each time of transferring. The 96-well- plate was then labelled and sealed by using parafilm and incubated for 24 h at 35˚C (Serafilm, et al., 2019). All steps were repeated for adjuvants and positive controls (CIP, STR, and CHL). Table 3.2 and Table 3.3 represented the

concentration of the NAH derivative compounds, positive controls, and antibiotic-adjuvant combinations in respective wells of the 96-well plate.
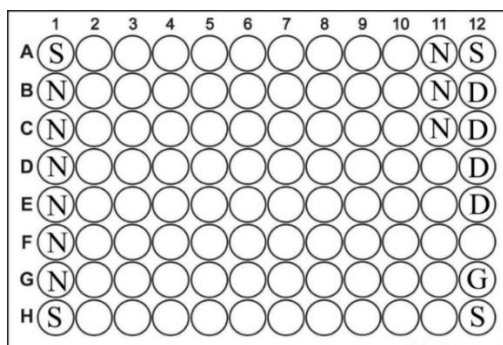
S   : Sterility Control   N   : Negative Control

D   : DMSO Control   G   : Growth Control

Empty   : Blank

Column 2 : Compound 1 (3,4-Cl$_2$)

Column 3 : Compound 2 (3,4-OCH$_3$)

Column 4 : Compound 3 (4Br)

Column 5 : Compound 4 (4CH$_3$)

Column 6 : Compound 5 (4Cl)

Column 7 : Compound 6 (4F)

Column 8 : Compound 7 (4NO$_2$)

Column 9 : Compound 8 (4OCH$_3$)

Column 10: Compound 9 (H)

Figure 3.3: Arrangement of 96 well plates for NAH derivative compounds and adjuvants.
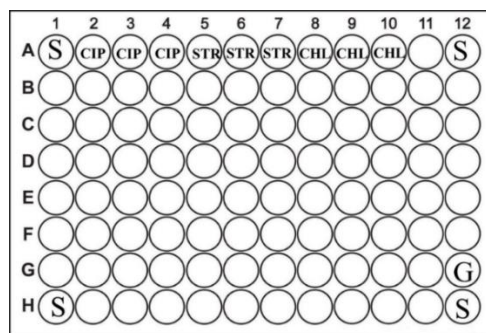
S : Sterility Control

G : Growth Control

CIP : Positive Control (Ciprofloxacin)

STR : Positive Control (Streptomycin)

CHL : Positive Control (Chloramphenicol)

Figure 3.4: Arrangement of 96 well plates for positive controls.

Table 3.5: Concentration of NAH derivative compounds in the 96- well plates.

Row	Concentration of NAH Derivative Compound (µg/mL)
A	250.00
B	125.00
C	62.50
D	31.25
E	16.63
F	7.81
G	3.91
H	1.95
I	0.98

Table 3.6: Concentration of NAH derivative compounds, streptomycin, chloramphenicol, and ciprofloxacin in the 96- well plates.

Row	Concentration of NAH derivative compounds (µg/mL)	Concentration of ciprofloxacin (µg/mL)	Concentration of streptomycin (µg/mL)	Concentration of chloramphenicol (µg/mL)
A	25.00	1.56	25.00	6.25
B	12.50	0.78	12.50	3.13
C	6.25	0.39	6.25	1.56
D	3.13	0.20	3.13	0.78
E	1.56	0.10	1.56	0.39
F	0.78	0.05	0.78	0.20
G	0.39	0.03	0.39	0.10
H	0.20	0.02	0.20	0.05
I	0.10	0.01	0.10	0.03

Table 3.7: Concentration of NAH derivative compounds and antibiotic- adjuvant combinations in the 96- well plates.

Row	Antibiotic-Adjuvant Combinations					
	Ciprofloxacin		Streptomycin		Chloramphenicol	
	Concentration of NAH Derivative Compound in Combination with Ciprofloxacin (µg/mL)	Concentration of Ciprofloxacin in Combination with NAH Derivative (µg/mL)	Concentration of NAH Derivative Compound in Combination with Streptomycin (µg/mL)	Concentration of Streptomycin in Combination with NAH Derivative (µg/mL)	Concentration of NAH Derivative Compound in Combination with Chloramphenicol (µg/mL)	Concentration of Chloramphenicol in Combination with NAH Derivative (µg/mL)
A	125.00	0.78	125.00	12.50	125.00	3.13
B	62.50	0.39	62.50	6.25	62.50	1.56
C	31.25	0.20	31.25	3.13	31.25	0.78
D	15.63	0.10	15.63	1.56	15.63	0.39
E	7.81	0.05	7.81	0.78	7.81	0.20
F	3.91	0.03	3.91	0.39	3.91	0.10
G	1.95	0.02	1.95	0.20	1.95	0.05
H	0.98	0.01	0.98	0.10	0.98	0.03
I	0.49	0.005	0.49	0.05	0.49	0.015

3.3.11 Minimum Inhibitory Concentration (MIC) Assay

After incubating for 24 hours at 37°C, 10 µL of Iodonitrotetrazolium (INT) chloride dye was added into each well in the 96-well plate. Following that, the plate was sealed using parafilm and incubated for 20 min at 37°C. The subsequent color changes were observed and recorded. Wells showing no visible color changes during this process were recorded as MIC (Barnes, et al., 2023).

3.3.12 Minimum Bactericidal Concentration (MBC) Assay

An amount of 10 µL was pipetted from the selected wells that showed no color changes after MIC assay to the labelled sterile MH agar plates. The chosen wells were positioned above the well displaying a color change. For example, if well 2F exhibited a red coloration after the MIC determination, the wells selected for MBC determination were 2C, 2D, and 2E. Subsequently, the inoculum was spread evenly in sterile MH agar plates by using a cotton swab. The MH agar plates were sealed with parafilm as well as incubated for 24 h at 37°C. After incubation, the bacteria colonies were determined. Wells contained fewer than 5 colonies were recorded as MBC signifying that more than 99.9% of the initial microbial was eliminated by the antimicrobial agent in lowest concentration (Petrus, et al., 2011).

3.3.13 Fractional Inhibitory Concentration (FIC) Index

After determining MIC values of each NAH derivatives compounds and adjuvants, FIC index was calculated to determine the synergism and interaction of NAH derivative compounds as adjuvants by using the formula below (Sadiki, et al., 2014):

$$\textbf{FICI} = \textbf{FIC (A)} + \textbf{FIC (B)}$$

Where,

$$\text{FIC (A)} = \frac{\text{MIC}_{AB}}{\text{MIC}_A}$$

And

$$\text{FIC (B)} = \frac{\text{MIC}_{BA}}{\text{MIC}_B}$$

MIC_{AB} : MIC of drug A tested in combination

MIC_A : MIC of drug A tested individually

MIC_{BA} : MIC of drug B tested in combination

MIC_{AB} : MIC of drug B tested individually

CHAPTER 4

RESULT

4.1 Minimum Inhibitory Concentration (MIC)

4.1.1 NAH Derivative Compounds

Table 4.1 shows the Minimum Inhibitory Concentration (MIC) of NAH derivative Compounds 1–9 against *B. cereus* (ATCC 13061), *B. subtilis* subsp. *spizizenni (*ATCC 6633), *S. aureus* (ATCC 6538), *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853), *S.* Typhimurium (ATCC 14028), Methicillin-resistance *S. aureus* (ATCC 33591) and Methicillin-resistance *S. aureus* (ATCC 43300). Minimum Inhibitory Concentration (MIC) is the lowest concentration of a drug required to restrain the microorganism's growth after 24-hour incubation period (Andrew, 2001). In accordance with the findings of Gu, et al. (2012), A MIC value less than 7.8 µg/mL is classified as highly active, while a MIC value ranging from 15.6–100 µg/mL is considered moderately active. Conversely, a MIC value exceeding 100 µg/mL is considered inactive.

From Table 4.1, Compound 1 (3,4-Cl$_2$) showed highly active antibacterial activity with the MIC value of 3.91 µg/mL against *B. cereus* (ATCC 13061) and 7.81 µg/mL against Methicillin-resistance *S. aureus* (ATCC 43300). However, Compound 1 obtained a MIC value of 31.25 µg/mL against *S. aureus* (ATCC 6538) showed moderately active. Additionally, Compound 1 demonstrated

inactive antibacterial activity against *B. subtilis* subsp. *spizizenni (*ATCC 6633), *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853), *S.* Typhimurium (ATCC 14028) and Methicillin-resistance *S. aureus* (ATCC 33591), characterized by MIC value of 125 µg/mL. Moreover, Compound 4 (4CH$_3$) had a MIC value of 62.50 µg/mL against *S. aureus* (ATCC 6538) showed moderately active. In contrast, Compound 4 showed inactive antibacterial activity against *B. cereus* (ATCC 13061), *B. subtilis* subsp. *spizizenni* (ATCC 6633), *E*. coli (ATCC 25922), *P. aeruginosa* (ATCC 27853) and *S.* Typhimurium (ATCC 14028) which obtained a MIC value of 125 µg/mL. On top of that, Compound 4 also presented inactive antibacterial activity against Methicillin-resistance *S. aureus* (ATCC 33591) and *Methicillin-resistance S. aureus* (ATCC 43300) with a MIC value of 250 µg/mL. In addition, Compound 6 (4F), Compound 8 (4OCH$_3$) and Compound 9 (H) showed moderately active with a MIC value of 62.50 µg/mL against *S. aureus* (ATCC 6538). However, these compounds were inactive against *B. cereus* (ATCC 13061), *B. subtilis* subsp. *Spizizenni (*ATCC 6633), *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853), *S.* Typhimurium (ATCC 14028), Methicillin-resistance *S. aureus* (ATCC 33591) and Methicillin-resistance *S. aureus* (ATCC 43300) with the MIC value of 125 µg/mL to 250 µg/mL. Furthermore, Compound 2 (3,4-OCH$_3$), Compound 3 (4Br), Compound 5 (4Cl) and Compound 7 (4NO$_2$) were inactive against the total 8 bacteria strains with the MIC values ranging from 125 µg/mL–250 µg/mL. As a result of the low MIC finding, statistical analysis was not conducted in this project. The results of this project were subjected to replication until consistency was achieved (Kowalska and Dudek-Wicher, 2021).

Table 4.1: Minimum Inhibitory Concentration (MIC) of NAH derivative Compounds 1–9 against selected bacteria.

Bacteria Strains	MIC (µg/mL)								
	NAH Derivatives Compounds								
	1	2	3	4	5	6	7	8	9
	3,4-Cl$_2$	3,4-OCH$_3$	4Br	4CH$_3$	4Cl	4F	4NO$_2$	4OCH$_3$	H
B. cereus (ATCC 13061)	3.91	125.00	125.00	125.00	125.00	125.00	125.00	125.00	125.00
B. subtilis subsp. *spizizenni* (ATCC 6633)	125.00	250.00	125.00	125.00	250.00	250.00	125.00	250.00	250.00
S. aureus (ATCC 6538)	31.25	125.00	125.00	62.50	125.00	62.50	125.00	62.50	62.50
E. coli (ATCC 25922)	125.00	125.00	125.00	125.00	125.00	125.00	125.00	125.00	125.00
P. aeruginosa (ATCC 27853)	125.00	125.00	125.00	125.00	125.00	125.00	125.00	125.00	125.00
S. Typhimurium (ATCC 14028)	125.00	125.00	125.00	125.00	125.00	125.00	125.00	125.00	125.00
Methicillin-resistance *S. aureus* (ATCC 33591)	125.00	250.00	250.00	250.00	250.00	125.00	250.00	125.00	250.00
Methicillin-resistance *S. aureus* (ATCC 43300)	7.81	250.00	125.00	250.00	250.00	250.00	125.00	250.00	250.00

Bacillus cereus (ATCC 13061), *Bacillus subtilis* subsp. *spizizenni* (ATCC 6633), *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella* Typhimurium (ATCC 14028), Methicillin-resistance *Staphylococcus aureus* (ATCC 33591) and Methicillin-resistance *Staphylococcus aureus* (ATCC 43300)

4.1.2 Positive Controls

Table 4.2 illustrates Minimum Inhibitory Concentration (MIC) of positive controls including ciprofloxacin, streptomycin and chloramphenicol against *B. cereus* (ATCC 13061), *B. subtilis* subsp. *spizizenni* (ATCC 6633), *S. aureus* (ATCC 6538), *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853), *S.* Typhimurium (ATCC 14028), Methicillin-resistance *S. aureus* (ATCC 33591) and Methicillin-resistance *S. aureus* (ATCC 43300). According to Sfeir (2020), the clinical breakpoint of ciprofloxacin against *Salmonella* spp. is ranging from MIC value 0.06 to 1 µg/mL. For *Enterobacteriaceae* other than *Salmonella* spp., the clinical breakpoint of ciprofloxacin is ranging from MIC value 0.25 to 1 µg/mL. Additionally, for *P. aeruginosa*, the clinical breakpoint of ciprofloxacin is ranging from MIC value 0.50 µg/mL to 2 µg/mL. Furthermore, Kronvall (2000) mentioned that the clinical breakpoint of ciprofloxacin is ranging from MIC value 0.06 to 4.0 µg/mL. The bacteria that have MIC value higher than clinical breakpoint considered as high resistance to the antibiotic whereas lower than clinical breakpoint known as susceptible to antibiotic (Gaur, et al., 2023). Based on the result in Table 4.2, *E. coli* (ATCC 25922) showed a MIC value at 0.005 µg/mL followed by *S.* Typhimurium (ATCC 14028) exhibited 0.01 µg/mL of MIC value, *B. subtilis* subsp. *spizizenni* (ATCC 6633) had a MIC value of 0.03 µg/mL and *B. cereus* (ATCC 13061) showed a MIC value of 0.10 µg/mL. Moreover, *P. aeruginosa* (ATCC 27853) presented a MIC value of 0.10 µg/mL. All bacteria strains mentioned above susceptible to ciprofloxacin. On the other hand, Methicillin-resistant *S. aureus* (ATCC 33591 and ATCC 43300) and *S. aureus* (ATCC 6538) exhibited an intermediate antibacterial activity against ciprofloxacin 0.20 µg/mL and 0.78 µg/mL MIC values, respectively.

In the accordance with the findings from Hu, et al. (2017), the clinical breakpoint of streptomycin is ranging from MIC value 32 to 64 µg/mL. Based on the results in Table 4.2, *B. subtilis* subsp. *spizizenni* (ATCC 6633) exhibited a MIC of 0.78 µg/mL, while *B. cereus* (ATCC 13061) and *E. coli* (ATCC 25922) demonstrated a MIC of 6.25 µg/mL. Both *S.* Typhimurium (ATCC 14028) and *P. aeruginosa* (ATCC 27853) displayed an MIC value of 12.50 µg/mL. *S. aureus* (ATCC 6538) and Methicillin-resistant *S. aureus* (ATCC 43300) both showed an MIC of 25 µg/mL. All of the aforementioned bacterial strains were susceptible to streptomycin. In contrast, Methicillin-resistant *S. aureus* (ATCC 33591) demonstrated 800 µg/mL MIC value, indicating resistance to streptomycin.

In addition, MacGowan and Wase (2001) mentioned that the clinical breakpoint of chloramphenicol is ranging from MIC value 2 to 4 µg/mL. In accordance with the results in Table 4.2, *B. cereus* (ATCC 13061), *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853), and Methicillin-resistance *S. aureus* (ATCC 43300) showed susceptibility to chloramphenicol with the MIC value of 1.56 µg/mL. While *B. subtilis* subsp. *spizizenni* (ATCC 6633) and *S.* Typhimurium (ATCC 14028) exhibited intermediate susceptibility to chloramphenicol with the MIC value of 3.13 µg/mL. In contrast, *S. aureus* (ATCC 6538) and Methicillin-resistance *S. aureus* (ATCC 33591) both showed resistance to chloramphenicol with the MIC value of 6.25 µg/mL and 50 µg/mL respectively.

Table 4.2: Minimum Inhibitory Concentration (MIC) of positive controls against selected bacteria.

Bacteria Strains	MIC (µg/mL)		
	Positive Controls		
	Ciprofloxacin	Streptomycin	Chloramphenicol
B. cereus (ATCC 13061)	0.10	6.25	1.56
B. subtilis subsp. *spizizenni* (ATCC 6633)	0.03	0.78	3.13
S. aureus (ATCC 6538)	0.78	25.00	6.25
E. coli (ATCC 25922)	0.005	6.25	1.56
P. aeruginosa (ATCC 27853)	0.10	12.50	1.56
S. Typhimurium (ATCC 14028)	0.01	12.50	3.13
Methicillin-resistance *S. aureus* (ATCC 33591)	0.20	800.0	50.0
Methicillin-resistance *S. aureus* (ATCC 43300)	0.20	25.00	1.56

*Bacillus cereus (ATCC 13061), *Bacillus subtilis* subsp. *spizizenni* (ATCC 6633), *Staphylococcus aureus (*ATCC 6538)*, Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853*), Salmonella* Typhimurium (ATCC 14028), Methicillin-resistance *Staphylococcus aureus* (ATCC 33591) and Methicillin-resistance *Staphylococcus* aureus (ATCC 43300)

4.1.3 NAH Derivative Compounds in Combination with Ciprofloxacin as Adjuvants

Table 4.3 illustrated Minimum Inhibitory Concentration (MIC) of NAH derivative Compounds 1 to 9 in combination with ciprofloxacin against *B. cereus* (ATCC 13061), *B. subtilis* subsp. *spizizenni* (ATCC 6633), *S. aureus* (ATCC 6538), *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853), *S.* Typhimurium (ATCC 14028), Methicillin-resistance *S. aureus* (ATCC 33591) and Methicillin-resistance *S. aureus* (ATCC 43300).

A total of 9 adjuvants exhibited highly to moderately active antibacterial activity against Gram-positive bacteria, Gram-negative bacteria and resistance bacteria.

The adjuvants showed MIC value ranging from 1.95 to 15.63 µg/mL against Gram-positive bacteria including *B. cereus* (ATCC 13061), *B. subtilis* subsp. *spizizenni* (ATCC 6633) and *S. aureus* (ATCC 6538). Furthermore, the adjuvants exhibited ranging from 0.25–15.63 µg/mL MIC values against Gram-negative bacteria involving *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853) and *S.* Typhimurium (ATCC 14028). On the other hand, the adjuvants showed MIC values ranging from 7.81 to 31.25 µg/mL against resistance bacteria including Methicillin-resistance *S. aureus* (ATCC 33591) and Methicillin-resistance *S. aureus* (ATCC 43300).

Table 4.3: Minimum Inhibitory Concentration (MIC) of NAH derivative Compounds 1–9 in combination with ciprofloxacin against selected bacteria.

Bacteria Strains	MIC (μg/mL)																	
	NAH Derivative Compounds in Combination with Ciprofloxacin (CIP)																	
	1		2		3		4		5		6		7		8		9	
	3,4-Cl$_2$		3,4-OCH$_3$		4Br		4CH$_3$		4Cl		4F		4NO$_2$		4OCH$_3$		H	
	NAH	CIP	NAH	CIP	NAH	CIP	NAH	CIP	NAH	CIP	NAH	CIP	NAH	CIP	NAH	CIP	NAH	CIP
B. cereus (ATCC 13061)	7.81	0.05	7.81	0.05	7.81	0.05	7.81	0.05	7.81	0.05	7.81	0.05	7.81	0.05	7.81	0.05	7.81	0.05
B. subtilis subsp. *spizizenni* (ATCC 6633)	3.91	0.03	3.91	0.03	3.91	0.03	3.91	0.03	1.95	0.02	1.95	0.02	1.95	0.02	1.95	0.02	1.95	0.02
S. aureus (ATCC 6538)	3.91	0.03	7.81	0.05	7.81	0.05	7.81	0.05	7.81	0.05	7.81	0.05	15.63	0.10	7.81	0.05	15.63	0.10
E. coli (ATCC 25922)	0.25	0.0025	0.49	0.005	0.49	0.005	0.49	0.005	0.49	0.005	0.49	0.005	0.49	0.005	0.25	0.0025	0.25	0.0025
P. aeruginosa (ATCC 27853)	15.63	0.10	31.25	0.20	31.25	0.20	31.25	0.20	31.25	0.20	31.25	0.20	31.25	0.20	15.63	0.10	15.63	0.10
S. Typhimurium (ATCC 14028)	1.95	0.02	1.95	0.02	1.95	0.02	1.95	0.02	1.95	0.02	1.95	0.02	1.95	0.02	1.95	0.02	1.95	0.02
Methicillin-resistance *S. aureus* (ATCC 33591)	7.81	0.05	31.25	0.20	31.25	0.20	31.25	0.20	31.25	0.20	31.25	0.20	31.25	0.20	31.25	0.20	31.25	0.20
Methicillin-resistance *S. aureus* (ATCC 43300)	7.81	0.05	31.25	0.20	31.25	0.20	31.25	0.20	62.50	0.39	62.50	0.39	31.25	0.20	31.25	0.20	31.25	0.20

Bacillus cereus (ATCC 13061), *Bacillus subtilis* subsp. *spizizenni* (ATCC 6633), *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella* Typhimurium (ATCC 14028), Methicillin-resistance *Staphylococcus aureus* (ATCC 33591) and Methicillin-resistance *Staphylococcus aureus* (ATCC 43300)

4.1.4 NAH Derivative Compounds in Combination with Streptomycin as Adjuvants

Table 4.4 illustrated Minimum Inhibitory Concentration (MIC) of NAH derivative Compounds 1 to 9 in combination with streptomycin against *B. cereus* (ATCC 13061), *B. subtilis* subsp. *spizizenni* (ATCC 6633), *S. aureus* (ATCC 6538), *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853), *S.* Typhimurium (ATCC 14028), Methicillin-resistance *S. aureus* (ATCC 33591) and Methicillin-resistance *S. aureus* (ATCC 43300).

A total of 9 adjuvants exhibited highly active to inactive antibacterial activity against Gram-positive bacteria including *B. cereus* (ATCC 13061), *B. subtilis* subsp. *spizizenni* (ATCC 6633) and *S. aureus* (ATCC 6538), characterized MIC values ranging from 1.95–62.50 µg/mL. In contrast, the adjuvants exhibited moderate active to inactive antibacterial against Gram-negative bacteria including *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853), *S.* Typhimurium (ATCC 14028) with the MIC value ranging from 7.81–125 µg/mL. Furthermore, all adjuvants obtained MIC value of 1.95 to 125 µg/mL Methicillin-resistant *S. aureus* (ATCC 33591) and Methicillin-resistant *S. aureus* (ATCC 43300), suggesting highly active to inactive antibacterial activity against these resistance bacteria.

Table 4.4: Minimum Inhibitory Concentration (MIC) of NAH derivative Compounds 1–9 in combination with streptomycin against selected bacteria.

Bacteria Strains	MIC (µg/mL)																	
	NAH Derivative Compounds in Combination with Streptomycin (STR)																	
	1		2		3		4		5		6		7		8		9	
	3,4-Cl₂		3,4-OCH₃		4Br		4CH₃		4Cl		4F		4NO₂		4OCH₃		H	
	NAH	STR	NAH	STR	NAH	STR	NAH	STR	NAH	STR	NAH	STR	NAH	STR	NAH	STR	NAH	STR
***B. cereus* (ATCC 13061)**	1.95	0.20	31.25	3.13	31.25	3.13	15.63	1.56	15.63	1.56	62.50	6.25	62.50	6.25	15.63	1.56	15.63	1.56
***B. subtilis* subsp. *spizizenni* (ATCC 6633)**	15.63	1.56	31.25	3.13	7.81	0.78	15.63	1.56	7.81	0.78	7.81	0.78	7.81	0.78	15.63	1.56	62.50	6.25
***S. aureus* (ATCC 6538)**	1.95	0.20	31.25	3.13	15.63	1.56	15.63	1.56	15.63	1.56	7.81	0.78	15.63	1.56	31.25	3.13	15.63	1.56
***E. coli* (ATCC 25922)**	62.50	6.25	62.50	6.25	62.50	6.25	62.50	6.25	62.50	6.25	62.50	6.25	62.50	6.25	62.50	6.25	62.50	6.25
***P. aeruginosa* (ATCC 27853)**	15.63	1.56	7.81	0.78	7.81	0.78	7.81	0.78	7.81	0.78	15.63	1.56	15.63	1.56	15.63	1.56	15.63	1.56
***S. Typhimurium* (ATCC 14028)**	125.00	12.5	125.00	12.50	125.00	12.50	125.00	12.50	125.00	12.50	125.00	12.50	125.00	12.50	125.00	12.50	125.00	12.50
Methicillin-resistance *S. aureus* (ATCC 33591)	1.95	12.5	125.00	800.00	125.00	800.00	125.00	800.00	125.00	800.00	125.00	800.00	125.00	800.00	125.00	800.00	125.00	800.00
Methicillin-resistance *S. aureus* (ATCC 43300)	1.95	0.20	62.50	6.25	62.50	6.25	62.50	6.25	62.50	6.25	62.50	6.25	62.50	6.25	62.50	6.25	31.25	3.13

*Bacillus cereus (ATCC 13061), *Bacillus subtilis* subsp. *spizizenni* (ATCC 6633), *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella* Typhimurium (ATCC 14028), Methicillin-resistance *Staphylococcus aureus* (ATCC 33591) and Methicillin-resistance *Staphylococcus aureus* (ATCC 43300)

4.1.5 NAH Derivative Compounds in Combination with Chloramphenicol as Adjuvants

Table 4.5 illustrated Minimum Inhibitory Concentration (MIC) of NAH derivative Compounds 1–9 in combination with chloramphenicol against *B. cereus* (ATCC 13061), *B. subtilis* subsp. *spizizenni* (ATCC 6633), *S. aureus* (ATCC 6538), *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853), *S.* Typhimurium (ATCC 14028), Methicillin-resistance *S. aureus* (ATCC 33591) and Methicillin-resistance *S. aureus* (ATCC 43300).

A total of 9 adjuvants exhibited highly active to inactive antibacterial activity against Gram-positive bacteria including *B. cereus* (ATCC 13061), *B. subtilis* subsp. *spizizenni* (ATCC 6633) and *S. aureus* (ATCC 6538), characterized MIC values with the range of 3.91–62.50 µg/mL. In contrast, the adjuvants exhibited moderate active to inactive antibacterial against Gram-negative bacteria, *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853), *S.* Typhimurium (ATCC 14028) (7.81–125 µg/mL). Furthermore, all adjuvants obtained MIC value of 3.91–125 µg/mL Methicillin-resistant *S. aureus* (ATCC 33591) and Methicillin-resistant *S. aureus* (ATCC 43300), suggesting highly active to inactive antibacterial activity against these resistance bacteria.

Table 4.5: Minimum Inhibitory Concentration (MIC) of NAH derivative Compounds 1 – 9 in combination with chloramphenicol against selected bacteria.

Bacteria Strains	MIC (µg/mL)																	
	NAH Derivative Compounds in Combination with Chloramphenicol (CHL)																	
	1		2		3		4		5		6		7		8		9	
	3,4-Cl$_2$		3,4-OCH$_3$		4Br		4CH$_3$		4Cl		4F		4NO$_2$		4OCH$_3$		H	
	NAH	CHL	NAH	CHL	NAH	CHL	NAH	CHL	NAH	CHL	NAH	CHL	NAH	CHL	NAH	CHL	NAH	CHL
B. cereus (ATCC 13061)	31.25	0.78	31.25	0.78	31.25	0.78	125.00	3.13	31.25	0.78	62.50	1.56	62.50	1.56	31.25	0.78	31.25	0.78
B. subtilis subsp. *spizizenni* (ATCC 6633)	15.63	0.39	125.00	3.13	31.25	0.78	62.50	1.56	62.50	1.56	62.50	1.56	62.50	1.56	62.50	1.56	62.50	1.56
S. aureus (ATCC 6538)	3.91	0.10	62.50	1.56	15.63	0.39	31.25	0.78	15.63	0.39	62.50	1.56	31.25	0.78	62.50	1.56	31.25	0.78
E. coli (ATCC 25922)	62.50	1.56	62.50	1.56	62.50	1.56	62.50	1.56	62.50	1.56	62.50	1.56	62.50	1.56	62.50	1.56	62.50	1.56
P. aeruginosa (ATCC 27853)	15.63	0.39	7.81	0.20	7.81	0.20	7.81	0.20	31.25	0.78	31.25	0.78	31.25	0.78	31.25	0.78	31.25	0.78
S. Typhimurium (ATCC 14028)	62.50	1.56	62.50	1.56	62.50	1.56	62.50	1.56	62.50	1.56	62.50	1.56	62.50	1.56	62.50	1.56	62.50	1.56
Methicillin-resistance *S. aureus* (ATCC 33591)	3.91	1.56	125.00	50.00	125.00	50.00	125.00	50.00	125.00	50.00	125.00	50.00	125.00	50.00	125.00	50.00	125.00	50.00
Methicillin-resistance *S. aureus* (ATCC 43300)	62.50	1.56	125.00	3.13	125.00	3.13	125.00	3.13	125.00	3.13	125.00	3.13	62.50	1.56	125.00	3.13	125.00	3.13

*Bacillus cereus (ATCC 13061), *Bacillus subtilis* subsp. *spizizenni* (ATCC 6633), *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella* Typhimurium (ATCC 14028), Methicillin-resistance *Staphylococcus aureus* (ATCC 33591) and Methicillin-resistance *Staphylococcus aureus* (ATCC 43300)

4.2 Minimum Bactericidal Concentration (MBC)

4.2.1 NAH Derivative Compound 1 (3,4-Cl$_2$)

Table 4.6 illustrated NAH derivative compound 1 against selected bacteria whereas Table 4.7 demonstrated MBC/MIC ratio of NAH Compound 1 against selected bacteria. Huang, et al. (2021) mentioned that a drug is classified as having bactericidal activity when MBC/MIC ratio is less than or equal to 4. Conversely, as MBC/MIC ratio is greater than or equal to 8, the drug is bacteriostatic. In accordance with the results of Table 4.6, Compound 1 had a MBC/MIC ratio of 2.00. This result proved that Compound 1 exhibited bactericidal activity against *B. cereus* (ATCC 13061).

Table 4.6: Minimum Bactericidal Concentration (MBC) of NAH derivative Compound 1 against selected bacteria.

Bacterial Strains	MBC of NAH Derivative Compound ((µg/mL)
	1
	3,4-Cl$_2$
B. cereus (ATCC 13061)	7.81
B. subtilis subsp. *spizizenni* (ATCC 6633)	-
S. aureus (ATCC 6538)	-
E. coli (ATCC 25922)	-
P. aeruginosa (ATCC 27853)	-
S. Typhimurium (ATCC 14028)	-
Methicillin-resistance *S. aureus* (ATCC 33591)	-
Methicillin-resistance *S. aureus* (ATCC 43300)	-

-: No MBC observed

*Bacillus cereus (ATCC 13061), *Bacillus subtilis* subsp. *spizizenni* (ATCC 6633), *Staphylococcus aureus (*ATCC 6538*), Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853*), Salmonella* Typhimurium (ATCC 14028), Methicillin-resistance *Staphylococcus aureus* (ATCC 33591) and Methicillin-resistance *Staphylococcus* aureus (ATCC 43300)

Table 4.7: Minimum Bactericidal Concentration (MBC)/Minimum Inhibitory Concentration (MIC) ratio of NAH derivative Compound 1 against selected bacteria.

Bacterial Strains	MBC/MIC Ratio of NAH Derivative Compound (µg/mL)
	1
	3,4-Cl$_2$
B. cereus (ATCC 13061)	2.00
B. subtilis subsp. *spizizenni* (ATCC 6633)	-
S. aureus (ATCC 6538)	-
E. coli (ATCC 25922)	-
P. aeruginosa (ATCC 27853)	-
S. Typhimurium (ATCC 14028)	-
Methicillin-resistance *S. aureus* (ATCC 33591)	-
Methicillin-resistance *S. aureus* (ATCC 43300)	-

-: No MBC observed

*Bacillus cereus (ATCC 13061), *Bacillus subtilis* subsp. *spizizenni* (ATCC 6633), *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella* Typhimurium (ATCC 14028), Methicillin-resistance *Staphylococcus aureus* (ATCC 33591) and Methicillin-resistance *Staphylococcus* aureus (ATCC 43300)

4.2.2 NAH Derivative Compounds in Combination with Ciprofloxacin as Adjuvants

Table 4.8 shown MBC of NAH derivative compounds 1–9 in combination with ciprofloxacin as adjuvants against selected bacteria, while Table 4.9 displayed MBC/MIC ratio of NAH derivative Compounds 1–9 in combination with ciprofloxacin as adjuvants against selected bacteria. From Table 4.9, adjuvant compound 1–5 and 7–9 obtained MBC/MIC ratio ranging from 1.00–2.00 against *B. cereus* (ATCC 13061), demonstrated their bactericidal activity. Similarly, adjuvant compound 1,2,4,5 and 8 showed bactericidal activity against *B. subtilis* subsp. *spizizenni* (ATCC 6633) with MBC/MIC ratio ranging from 1.00–2.01. In addition, adjuvant compound 1 obtained MBC/MIC ratio of 2.00 against Methicillin-resistance *S. aureus* (ATCC 33591) represented bactericidal activity.

Table 4.8: Minimum Bactericidal Concentration (MBC) of NAH derivative Compounds 1–9 in combination with ciprofloxacin as adjuvants against selected bacteria.

Bacteria Strains	MBC (µg/mL)																	
	NAH Derivative Compounds in Combination with Ciprofloxacin (CIP)																	
	1		2		3		4		5		6		7		8		9	
	3,4-Cl$_2$		3,4-OCH$_3$		4Br		4CH$_3$		4Cl		4F		4NO$_2$		4OCH$_3$		H	
	NAH	CIP	NAH	CIP	NAH	CIP	NAH	CIP	NAH	CIP	NAH	CIP	NAH	CIP	NAH	CIP	NAH	CIP
B. cereus (ATCC 13061)	7.81	0.05	15.63	0.10	7.81	0.05	15.63	0.10	7.81	0.05	-	-	15.63	0.10	7.81	0.05	7.81	0.05
B. subtilis subsp. *spizizenni* (ATCC 6633)	3.91	0.03	7.81	0.05	-	-	7.81	0.05	1.95	0.02	-	-	-	-	3.91	0.03	-	-
S. aureus (ATCC 6538)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E. coli (ATCC 25922)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P. aeruginosa (ATCC 27853)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S. Typhimurium (ATCC 14028)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methicillin-resistance *S. aureus* (ATCC 33591)	15.63	0.10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methicillin-resistance *S. aureus* (ATCC 43300)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

-: No MBC observed

*Bacillus cereus (ATCC 13061), *Bacillus subtilis* subsp. *spizizenni* (ATCC 6633), *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella* Typhimurium (ATCC 14028), Methicillin-resistance *Staphylococcus aureus* (ATCC 33591) and Methicillin-resistance *Staphylococcus aureus* (ATCC 43300)

Table 4.9: Minimum Bactericidal Concentration (MBC)/Minimum Inhibitory Concentration (MIC) of NAH derivative Compounds 1–9 in combination with ciprofloxacin as adjuvants against selected bacteria.

Bacteria Strains	MBC/MIC Ratio (µg/mL)																	
	NAH Derivative Compounds in Combination with Ciprofloxacin (CIP)																	
	1		2		3		4		5		6		7		8		9	
	3,4-Cl$_2$		3,4-OCH$_3$		4Br		4CH$_3$		4Cl		4F		4NO$_2$		4OCH$_3$		H	
	NAH	CIP	NAH	CIP	NAH	CIP	NAH	CIP	NAH	CIP	NAH	CIP	NAH	CIP	NAH	CIP	NAH	CIP
B. cereus (ATCC 13061)	1.00	1.00	2.00	2.00	1.00	1.00	2.00	2.00	1.00	1.00	-	-	2.00	2.00	1.00	1.00	1.00	1.00
B. subtilis subsp. *spizizenni* (ATCC 6633)	1.00	1.00	2.00	1.67	-	-	2.00	1.67	1.00	1.00	-	-	-	-	2.01	1.50	-	-
S. aureus (ATCC 6538)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E. coli (ATCC 25922)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P. aeruginosa (ATCC 27853)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S. Typhimurium (ATCC 14028)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methicillin-resistance *S. aureus* (ATCC 33591)	2.00	2.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methicillin-resistance *S. aureus* (ATCC 43300)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

-: No MBC observed

*B. cereus (ATCC 13061), *B. subtilis* subsp. *spizizenni* (ATCC 6633), *S. aureus* (ATCC 6538), *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853), *S. Typhimurium* (ATCC 14028), Methicillin-resistance *S. aureus* (ATCC 33591) and Methicillin-resistance *S. aureus* (ATCC 43300)

4.2.3 NAH Derivative Compounds in Combination with Streptomycin as Adjuvants

Table 4.10 shown MBC of NAH derivative compounds 1–9 in combination with streptomycin as adjuvant against selected bacteria, while Table 4.11 represents MBC/MIC ratio of NAH derivative Compounds 1–9 in combination with streptomycin as adjuvants against selected bacteria.

Table 4.11 revealed adjuvant compound 1 exhibited bactericidal activity against *B. cereus* (ATCC 13061) with MBC/MIC ratio of 1.00. Likewise, adjuvant compound 3, 6 and 7 also exhibited bactericidal activity against *B. subtilis* subsp. *spizizenni* (ATCC 6633) with MBC/MIC ratio of 1.00.

Table 4.10: Minimum Bactericidal Concentration (MBC) of NAH derivative Compounds 1–9 in combination with streptomycin against selected bacteria.

Bacteria Strains	MBC (µg/mL)																	
	NAH Derivative Compounds in Combination with Streptomycin (STR)																	
	1		2		3		4		5		6		7		8		9	
	3,4-Cl$_2$		3,4-OCH$_3$		4Br		4CH$_3$		4Cl		4F		4NO$_2$		4OCH$_3$		H	
	NAH	STR	NAH	STR	NAH	STR	NAH	STR	NAH	STR	NAH	STR	NAH	STR	NAH	STR	NAH	STR
B. cereus (ATCC 13061)	1.95	0.20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B. subtilis subsp. *spizizenni* (ATCC 6633)	-	-	-	-	7.81	0.78	-	-	-	-	7.81	0.78	7.81	0.78	-	-	-	-
S. aureus (ATCC 6538)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E. coli (ATCC 25922)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P. aeruginosa (ATCC 27853)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S. Typhimurium (ATCC 14028)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methicillin-resistance *S. aureus* (ATCC 33591)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methicillin-resistance *S. aureus* (ATCC 43300)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

-: No MBC observed

Bacillus cereus (ATCC 13061), *Bacillus subtilis* subsp. *spizizenni* (ATCC 6633), *Staphylococcus aureus (*ATCC 6538*), Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853*), Salmonella* Typhimurium (ATCC 14028), Methicillin-resistance *Staphylococcus aureus* (ATCC 33591) and Methicillin-resistance *Staphylococcus* aureus (ATCC 43300)

Table 4.11: Minimum Bactericidal Concentration (MBC)/Minimum Inhibitory Concentration (MIC) of NAH derivative Compounds 1–9 in combination with streptomycin against selected bacteria.

Bacteria Strains	MBC/MIC Ratio (µg/ML)																	
	NAH Derivative Compounds in Combination with Streptomycin (CIP)																	
	1		2		3		4		5		6		7		8		9	
	3,4-Cl$_2$		3,4-OCH$_3$		4Br		4CH$_3$		4Cl		4F		4NO$_2$		4OCH$_3$		H	
	NAH	STR	NAH	STR	NAH	STR	NAH	STR	NAH	STR	NAH	STR	NAH	STR	NAH	STR	NAH	STR
B. cereus (ATCC 13061)	1.00	1.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B. subtilis subsp. *spizizenni* (ATCC 6633)	-	-	-	-	1.00	1.00	-	-	-	-	1.00	1.00	1.00	1.00	-	-	-	-
S. aureus (ATCC 6538)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E. coli (ATCC 25922)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P. aeruginosa (ATCC 27853)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S. Typhimurium (ATCC 14028)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methicillin-resistance *S. aureus* (ATCC 33591)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methicillin-resistance *S. aureus* (ATCC 43300)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

-: No MBC observed

Bacillus cereus (ATCC 13061), *Bacillus subtilis* subsp. *spizizenni* (ATCC 6633), *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella* Typhimurium (ATCC 14028), Methicillin-resistance *Staphylococcus aureus* (ATCC 33591) and Methicillin-resistance *Staphylococcus aureus* (ATCC 43300)

4.3 Fractional Inhibitory Concentration (FIC) Index

4.3.1 NAH Derivative Compounds in Combination with Ciprofloxacin as Adjuvants

Table 4.12 demonstrates FIC Index of NAH derivative Compounds 1–9 in combination with ciprofloxacin as adjuvants against *B. cereus* (ATCC 13061), *B. subtilis* subsp. s*pizizenni* (ATCC 6633), *S. aureus* (ATCC 6538), *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853), *S.* Typhimurium (ATCC 14028), Methicillin-resistance *S. aureus* (ATCC 33591) and Methicillin-resistance *S. aureus* (ATCC 43300). According to Botelho (2000), when FIC index value falls below 0.5, it signifies synergism, while the value greater than 0.5 and equal or less than 4 is indicative of no significant difference. Conversely, a FIC index value exceeding 4.0 suggests antagonism. Based on the result in Table 4.12, most of the adjuvant compound did not exhibit significant interaction with ciprofloxacin when tested against the 8 selected bacteria, as indicated by FIC index values with the range of 0.56–2.50. Nonetheless, adjuvant compound 1 demonstrated synergistic interaction against Methicillin-resistance *S. aureus* (ATCC 33591) with FIC index of 0.31. On top of that, adjuvant compound 1, 8 and 9 exhibited synergistic interaction against *E. coli* (ATCC 25922) with FIC index of 0.50, whereas all compounds represented synergistic interaction against *S. aureus* (ATCC 6538) with FIC index values ranging from 0.13–0.50.

Table 4.12: Fractional Inhibitory Concentration (FIC) Index of NAH derivative Compounds 1–9 in combination with ciprofloxacin against selected bacteria.

Bacteria Strains	FIC Index (µg/ML)								
	NAH Derivatives Compounds in Combination with Ciprofloxacin								
	1	2	3	4	5	6	7	8	9
	3,4-Cl$_2$	3,4-OCH$_3$	4Br	4CH$_3$	4Cl	4F	4NO$_2$	4OCH$_3$	H
B. cereus (ATCC 13061)	2.50	0.56	0.56	0.56	0.56	0.56	0.56	0.56	0.56
B. subtilis subsp. *spizizenni* (ATCC 6633)	1.03	1.02	1.03	1.03	0.67	0.67	0.68	0.67	0.67
S. aureus (ATCC 6538)	0.16	0.13	0.13	0.19	0.13	0.19	0.25	0.19	0.38
E. coli (ATCC 25922)	0.50	1.00	1.00	1.00	1.00	1.00	1.00	0.50	0.50
P. aeruginosa (ATCC 27853)	0.63	1.25	1.25	1.25	1.25	1.25	1.25	0.63	0.63
S. Typhimurium (ATCC 14028)	1.02	1.02	1.02	1.02	1.02	1.02	1.02	1.02	1.02
Methicillin-resistance *S. aureus* (ATCC 33591)	0.31	1.13	1.13	1.13	1.13	1.25	1.13	1.25	1.13
Methicillin-resistance *S. aureus* (ATCC 43300)	1.25	1.13	1.25	1.13	2.20	2.20	1.25	1.13	1.13

Bacillus cereus (ATCC 13061), *Bacillus subtilis* subsp. *spizizenni* (ATCC 6633), *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella* Typhimurium (ATCC 14028), Methicillin-resistance *Staphylococcus aureus* (ATCC 33591) and Methicillin-resistance *Staphylococcus aureus* (ATCC 43300)

4.3.2 NAH Derivative Compounds in Combination with Streptomycin as Adjuvants

Table 4.13 exhibits FIC Index value of NAH derivative Compounds 1–9 in combination with streptomycin as adjuvants against *B. cereus* (ATCC 13061), *B. subtilis* subsp. *spizizenni* (ATCC 6633), *S. aureus* (ATCC 6538), *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853), *S.* Typhimurium (ATCC 14028), Methicillin-resistance *S. aureus* (ATCC 33591) and Methicillin-resistance *S. aureus* (ATCC 43300). Table 4.13 show that all adjuvant compound exhibited either synergism, no significant difference, or antagonism against 8 selected bacteria.

Adjuvant compound that shown synergistic interaction ranging from 0.03 to 0.50 including Adjuvant compound 4–5 and 8–9 against *B. cereus* (ATCC 13061) while Adjuvant compound 1–7 and 9 expressed synergistic interactions with *S. aureus* (ATCC 6538). Adjuvant compound 1 also exhibited a synergistic interaction with Methicillin-resistant *S. aureus* (ATCC 33591), and several adjuvant compound, including 1–2, 4–6 and 8–9, displayed synergism with Methicillin-resistant *S. aureus* (ATCC 43300). Furthermore, all 9-adjuvant compound showed synergistic interactions with *P. aeruginosa* (ATCC 27853). On the other hand, adjuvant compound 2 and 9 demonstrated antagonistic interactions with *B. subtilis* subsp. *spizizenni* (ATCC 6633), obtained FIC index values 4.14 and 8.04. The remaining adjuvant compound did not show significant interactions against the 8 selected bacteria, with FIC index values ranging from 0.53 to 2.13.

Table 4.13: Fractional Inhibitory Concentration (FIC) Index of NAH derivative Compounds 1–9 in combination with streptomycin against selected bacteria.

Bacteria Strains	FIC Index (µg/ML)								
	NAH Derivatives Compounds in Combination with Streptomycin								
	1	2	3	4	5	6	7	8	9
	3,4-Cl$_2$	3,4-OCH$_3$	4Br	4CH$_3$	4Cl	4F	4NO$_2$	4OCH$_3$	H
B. cereus (ATCC 13061)	0.53	0.75	0.75	0.37	0.37	1.50	1.50	0.37	0.37
B. subtilis subsp. *spizizenni* (ATCC 6633)	2.13	4.14	1.06	2.13	1.03	1.03	1.06	2.06	8.04
S. aureus (ATCC 6538)	0.07	0.38	0.19	0.31	0.19	0.16	0.19	0.63	0.31
E. coli (ATCC 25922)	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50
P. aeruginosa (ATCC 27853)	0.25	0.12	0.12	0.12	0.12	0.25	0.25	0.25	0.25
S. Typhimurium (ATCC 14028)	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Methicillin-resistance *S. aureus* (ATCC 33591)	0.03	1.50	1.50	1.50	1.50	2.00	1.50	2.00	1.50
Methicillin-resistance *S. aureus* (ATCC 43300)	0.26	0.50	0.75	0.50	0.50	0.50	0.75	0.50	0.50

Bacillus cereus (ATCC 13061), *Bacillus subtilis* subsp. *spizizenni* (ATCC 6633), *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella* Typhimurium (ATCC 14028), Methicillin-resistance *Staphylococcus aureus* (ATCC 33591) and Methicillin-resistance *Staphylococcus aureus* (ATCC 43300)

4.3.3 NAH Derivative Compounds in Combination with Chloramphenicol as Adjuvants

Table 4.14 illustrates FIC Index of NAH derivative Compounds 1–9 in combination with chloramphenicol as adjuvants against *B. cereus* (ATCC 13061), *B. subtilis* subs. *spizizenni* (ATCC 6633), *S. aureus* (ATCC 6538), *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853), *S.* Typhimurium (ATCC 14028), Methicillin-resistance *S. aureus* (ATCC 33591) and Methicillin-resistance *S. aureus* (ATCC 43300).

Table 4.14 revealed that a total of 9 adjuvant compound exhibited synergistic, antagonistic, or no significant interactions. adjuvant compound that represented synergistic interaction against selected bacteria with FIC index value ranging from 0.06 to 0.50, Specifically, adjuvant compound 1 and 3 demonstrated synergistic interaction against *B. subtilis* subsp. *spizizenni* (ATCC 6633), while adjuvant compound 1, 3, 5, and 7 exhibited synergistic interactions with *S. aureus* (ATCC 6538). Moreover, adjuvant compound 1– 4 showed synergism with *P. aeruginosa* (ATCC 27853), and adjuvant compound 1 also displayed synergism with Methicillin-resistant *S. aureus* (ATCC 33591).

On the other hand, adjuvant compound 1 displayed antagonistic interaction with *B. cereus* (ATCC 13061) and Methicillin-resistance *S. aureus* (ATCC 43300), obtained FIC index values 8.49 and 9.00, respectively. The remaining adjuvant compound did not show significant interactions with the 8 selected bacteria with FIC index values ranging from 0.62–3.01.

Table 4.14: Fractional Inhibitory Concentration (FIC) Index of NAH derivative Compounds 1–9 in combination with chloramphenicol against selected bacteria.

Bacteria Strains	FIC Index (µg/ML)								
	NAH Derivatives Compounds in Combination with Chloramphenicol								
	1	2	3	4	5	6	7	8	9
	3,4-Cl$_2$	3,4-OCH$_3$	4Br	4CH$_3$	4Cl	4F	4NO$_2$	4OCH$_3$	H
B. cereus (ATCC 13061)	8.49	0.75	0.75	3.00	0.75	1.50	1.50	0.75	0.75
B. subtilis subsp. *spizizenni* (ATCC 6633)	0.25	1.50	0.50	1.00	0.75	0.75	1.00	0.75	0.75
S. aureus (ATCC 6538)	0.14	0.75	0.19	0.62	0.19	1.25	0.37	1.25	0.62
E. coli (ATCC 25922)	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50
P. aeruginosa (ATCC 27853)	0.38	0.19	0.19	0.19	0.75	0.75	0.75	0.75	0.75
S. Typhimurium (ATCC 14028)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Methicillin-resistance *S. aureus* (ATCC 33591)	0.06	1.50	1.50	1.50	1.50	2.00	1.50	2.00	1.50
Methicillin-resistance *S. aureus* (ATCC 43300)	9.00	2.51	3.01	2.51	2.51	2.51	3.01	2.51	2.51

Bacillus cereus (ATCC 13061), *Bacillus subtilis* subsp. *spizizenni* (ATCC 6633), *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella* Typhimurium (ATCC 14028), Methicillin-resistance *Staphylococcus aureus* (ATCC 33591) and Methicillin-resistance *Staphylococcus aureus* (ATCC 43300)

CHAPTER 5

DISCUSSION

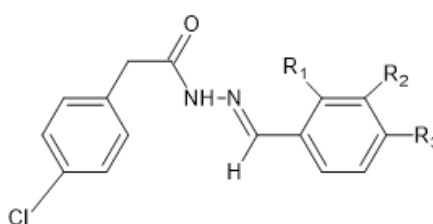### 5.1 Chemical Structure of NAH Derivative Compounds and Their Antibacterial Activity

Figure 5.1: *N*-acylhydrazones (NAH) derivative Compounds 1–9.

In accordance with Table 4.1, NAH derivative Compound 1 (3,4-Cl$_2$) represented highly active antibacterial activity against *B. cereus* (ATCC 13061) and Methicillin-resistance to (ATCC 43300). In contrast, Compounds 2–9 with the substituent groups 3,4-OCH$_3$, 4Br, 4CH$_3$, 4Cl, 4F, 4NO$_2$, 4OCH$_3$ and H respectively, exhibited moderate active and inactive antimicrobial activity against the 8 bacteria strains. According to Faleye, et al. (2024), the inclusion of halogens group in antimicrobial drugs is predominantly driven by the formation of halogen bonds, which are significant contributors to the antibacterial properties. Halogenated substances exhibit the capability to engage in multiple covalent interactions with ligands, therefore demonstrating both electrophilic and nucleophilic characteristics. This is matched with the research from Gu, et al. (2012) mentioned that the antibacterial activity suggested that adding of

halogen atom at the 4n- position generally increase the antibacterial activity. However, Compound 1 demonstrated an inactive antibacterial activity against *B. subtilis* subsp. *spizizenni* (ATCC 6633), *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853), *S.* Typhimurium (ATCC 14028) as well as Methicillin-resistance *S. aureus* (ATCC 33591) with MIC value of 125 μg/mL. Also, Compound 5 showed an inactive antibacterial activity (125 and 250 μg/mL) against 8 bacteria strains. Although both Compounds 1 and 5 consist of halogen group which is chloro (Cl) substituted group, they revealed insignificant antimicrobial activity. According to the findings from Gu, et al. (2012), the NAH derivative compounds featuring a 4-Cl substitution in the phenyl group demonstrated active antibacterial activity against *S. aureus*, *B. subtilis* and *E. coli* which indicates a difference from the previously mentioned results. This might be due to the difference in structure and position of Cl substitution. In accordance with Desai, Bhatt, Somani and Trivedi (2013), the position of electron withdrawing group attached to aromatic ring can impact the molecules' lipophilicity and therefore affect their antibacterial activity.

Generally, the results can be supported by structure-activity relationships (SAR) studies. Ahmad, Elisha, Vuuren and Viljoen (2021) revealed that SAR is a method that involves in linking the chemical structure quantitatively to biological activity. Hence, it is crucial aspect for drug design. Gu, et al. (2012) mentioned that compounds containing electron-withdrawing substituents like halogens and nitro groups in the aromatic ring demonstrated stronger antibacterial effects compared to those containing electron-donating groups like methyl and methoxy. This could be attributed to the fact that electron-

70

withdrawing substituents such as Cl and nitro improve the compounds' lipophilicity, caused greater partitioning of the compounds into the lipophilic phase of a microbial membrane (Polović, et al., 2019). Consequently, this affects the electronic properties of the chlorine substituents and electron attraction or repulsion, as well as steric interference with nearby amino acid residues at the chlorine atom position (Fang, et al., 2019). Therefore, this could explain Compounds 1 that bearing with Cl substitution exhibited an active antibacterial activity. However, Compounds 2,3 and 5–8 contained methoxy, bromo, Cl, fluoro and nitro respectively shown insignificant antibacterial activity ranging from 32.50 to 250 µg/mL. According to the findings from Faleye, et al. (2024), as the size of halogens increases, leading to longer C–X bond lengths. The antimicrobial potency of halogens substituted compounds may be influenced by the size and substitution patterns of the halogens. Additionally, compounds with electron withdrawing substituents at the *para* position in phenyl ring exhibit the highest antibacterial activity, followed by those at *meta* and *ortho* positions, respectively (Janowska, et al., 2024). In accordance with the findings from Kolanadiyil, et al. (2017), this may be attributed to the steric hindrance found in the meta and ortho positions, especially in ortho substitution which reduce the biological activity.

On the other hand, Compounds 4 and 9 with the substituent groups of methyl and hydrogen group displayed moderate to insignificant antibacterial activity within the range of 62.50 to 250 µg/mL. The effectiveness of antibacterial activity is significantly influenced by the arrangement of substitutions on the phenyl ring. Generally, the existence of electron donating groups on the phenyl

71

ring led to a substantial reduce of antibacterial activity due to the presence of hydroxyl group that able to form hydrogen bond. As methyl group and hydrogen group are electron donating group, therefore the antibacterial activity is reduced (Desai, et al., 2013).

5.2 Spectrum of Antibacterial Activity of NAH Derivative Compounds against Selected Bacteria Strains

In accordance with Table 4.1, NAH derivative Compound 1 had shown broad spectrum antibacterial activity against *B. cereus* (ATCC 13061)*, S. aureus* (ATCC 6538) and Methicillin-resistance *S. aureus* (ATCC 43300). The results obtained were similar with the findings from Yao, et al. (2021) in which NAH derivative Compound 3d exhibited active antibacterial activity against Methicillin-resistance *S. aureus* (ATCC 43300) with MIC value of 8 μg/mL and Compound 3g from Yao, et al. (2021) showed moderately active antibacterial activity against *S. aureus* (ATCC 25923) with MIC value of 32 μg/mL. Demeke, et al. (2021) stated that when discussing antibacterial activity, the term "broad-spectrum" refers to the capability of antibiotic to effectively combat a broad-spectrum bacterium such as Gram-positive and Gram-negative, as well as potentially anaerobic bacteria. Despite these broad-spectrum antibacterial compounds are essential in treating bacterial infections, they contribute some weaknesses such as encouraging resistance across multiple bacterial species and potentially damaging the host. However, the use of specific-species antibacterial agents, that effective against specific bacteria and are more specific in their action, may help alleviate some of these issues, as suggested by Melander,

Zurawski, and Melander (2018). Additionally, Alm and Lahiri (2020) have reported that over 50% of these narrow-spectrum compounds specifically target *S. aureus*. Therefore, this is matched with the results in which Compounds 4, 8 and assumed to be specific-species antibacterial compounds since they are only showed antibacterial activity against *S. aureus* (ATCC 6538). The results are similar with the findings from Yao, et al. (2021) stated that Compounds 3a, 3c and 3e (Figure 5.2) demonstrated a moderately active antibacterial activity against *S. aureus* (ATCC 25923), characterized by MIC value of 64 µg/mL. Due to Compounds 4 and 8 bearing to methoxy group, it exhibited a moderately active antimicrobial activity (Yao, et al., 2021).
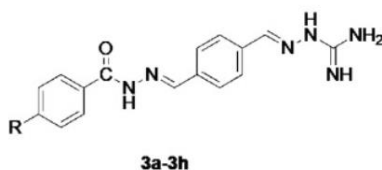
3a-3h

Figure 5.2: NAH derivative Compounds 3a–3h (Yao, et al., 2021).

In comparison, all NAH derivative compounds showed inactive antibacterial activity against Methicillin-resistance *S. aureus* (ATCC 33591) and Methicillin-resistance *S. aureus* (ATCC 43300) except for Compound 1. Theoretically, Methicillin-resistance *S. aureus* demonstrates broad resistance to beta-lactam antibiotics that not hydrolysed by beta-lactamase including methicillin and oxacillin. According to the findings from Lee, et al. (2018) in which Methicillin-resistant *S. aureus* is distinguished from other *S. aureus* strains by the presence of PBP2a. This can be supported by the findings from Haddadin, et al. (2002) stated that the prevalent mechanism of methicillin resistance is mediated by

*mec*A gene. It encodes for an additional penicillin-binding protein with low affinity to all beta-lactam antibiotics. Hence, it is assumed that Compound 1 is not affected by the specific resistance mechanism. Additionally, all NAH derivative compounds in this project showed inactive antibacterial activity against Gram-negative bacteria including *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853) and *S.* Typhimurium (ATCC 14028). According to the findings from Gu, et al., 2012, the results obtained were similar with the Compounds 4 and 8 in this project. Compounds 4b and 4f were inactive antibacterial activity (100 µg/mL) against *E. coli* (ATCC 25922). Similarly, all NAH compounds did not exhibit active antibacterial activity against *B. subtilis* subsp. *spizizenni* (ATCC 6633). This matched with the findings from Gu, et al. (2012) in which Compound 4i exhibited inactive antibacterial activity against *B. subtilis* (>100 µg/mL). Although Brejiyeh, et al. (2020) mentioned that Gram-negative bacteria supposed to be greater resistant compared to Gram-positive bacteria since they consist unique structure, the antibacterial activities of the synthesized compounds were significantly impacted by different aromatic substitutions (Gu, et al., 2012). Therefore, the result will be affected. Besides, Brauner, et al. (2016) proposed that the bacterial status displayed various characteristics like susceptibility, resistance, tolerance and persistence that will produce different results.
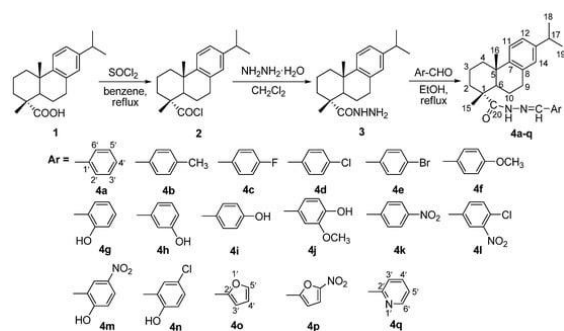
Figure 5.3: NAH derivative Compounds 4a–4q (Gu, et al., 2012).

On trend in Table 4.1 and 4.2, most NAH derivatives compounds showed less effective compared to standard antibiotics including ciprofloxacin, streptomycin and chloramphenicol against Gram-positive and Gram-negative bacteria. Kowalska-Krochmal and Dudek-Wicher (2021) proposed that higher MIC values indicate greater effectiveness and susceptibility to the bacteria. However, Compound 1 demonstrated greater effectiveness than streptomycin against resistant bacteria, and all NAH derivative compounds were more effective than streptomycin against Methicillin-resistance *S. aureus* (ATCC 33591). On the other hand, the NAH derivative compounds were less effective than ciprofloxacin and chloramphenicol against resistant bacteria. This is in line with Li, et al. (2017) who suggested that antibacterial activity of a compound will be affected by bacterial status, inoculum size and antibiotic concentrations.

5.3 Susceptibility of Gram-positive, Gram-negative and Resistance Bacteria against Selected Antibiotics

With reference to Table 4.3, Table 4.4 and Table 4.5, Gram-positive and Gram-negative bacteria displayed different susceptibility towards the NAH derivative compounds in combination of ciprofloxacin, streptomycin, and chloramphenicol. Besides, based on Table 4.3, NAH derivative compounds in combination with ciprofloxacin demonstrated highly active antibacterial activity towards Gram-positive bacteria (3.91–7.81 μg/mL) and moderately active to highly active antibacterial activity against Gram-negative bacteria (0.25–31.25 μg/mL). Tentatively, Gram-negative should display higher resistance against antibiotics due to the presence of outer membrane (OM) porins, whereas fluoroquinolone can pass through Gram-positive via passive diffusion (Yang, 2022). These proteins are highly prevalent in the Gram-negative bacteria OM and can be divided into non-specific and specific porins based on their function (Koebnik, et al., 2000). According to Lyer, et al. (2018), OM porins are important for maintaining the structural integrity of OM in Gram-negative bacteria. Specifically, some antibiotics like beta-lactams and fluoroquinolones, can pass through the outer membrane via the non-specific porin OmpF, as noted by Mach, et al. (2008). Consequently, mutations in OmpF can lead to resistance to certain beta-lactam antibiotics in certain Gram-negative bacteria. However, Table 4.3 revealed that Gram-negative bacteria obtained a lower MIC value range compared to Gram-positive bacteria. Especially *E. coli* (ATCC 25922) and *S.* Typhimurium (ATCC 14028) displayed MIC values with 0.25–1.95 μg/mL. This means that Gram-positive bacteria displayed a higher resistance against ciprofloxacin as compared to Gram-negative bacteria. The results obtained were

reliable with the research of Khalid et al. (2023) which indicated that ciprofloxacin exhibited greater sensitivity towards Gram-negative bacteria (28%) compared to Gram-positive bacteria (25%). Based on the findings from Card, et al. (2015), ciprofloxacin showed effectiveness against aerobic Gram-negative and Gram-positive bacteria, but it has limited effectiveness against most of the anaerobic bacteria. It is therefore assumed that *E. coli* and *S.* are anaerobic bacteria which demonstrated a lower antibacterial activity (Lim, Yoon and Hovde, 2010).

On trend observed in Table 4.4, NAH derivative compounds in combination with streptomycin exhibited highly active to moderate antibacterial activity against Gram-positive bacteria (1.95–62.50 µg/mL), as well as highly active to inactive antibacterial activity against Gram-negative bacteria (7.81–125 µg/mL). Vardanyan, et al. (2006) proposed that streptomycin is effective for most of the bacteria, including Gram-positive and acid-fast bacteria, as well as Gram-negative bacteria. On top of that, the results illustrate that Gram-negative bacteria obtained higher MIC values range than Gram-positive bacteria which purport that Gram-negative bacteria are more resistance to streptomycin in comparison to Gram-positive bacteria. Collet, et al. (2020) revealed Gram-negative bacteria are characterized by their multi-layered macromolecular structure known as the cell envelope. This envelope comprises three primary components arranged from outer to inner parts including periplasmic membrane (OM), peptidoglycan layer as well as cytoplasmic membrane (IM). Studies has found that peptidoglycan layer is a crucial feature of the bacterial cell envelope to form a scaffold-like complex around the bacterial IM (Gauba and Rahman,

2023). Gram-negative bacteria consist of thinner layer compared to Gram-positive. Moreover, the findings from Breijyeh, Jubeh and Karaman (2020) revealed that the OM of Gram-negative bacteria acts as a key factor in conferring resistance against broad spectrum of antibiotics. For instance, beta-lactams, quinolones and colistins. Unlike Gram-negative bacteria, the absence of this protective OM in Gram-positive bacteria rendering it less resistant to antibiotics (Miller, 2016).

Furthermore, Table 4.5 has illustrated NAH derivative compounds in combination with chloramphenicol demonstrated a broad-spectrum antibacterial activity, from highly active to moderately active antibacterial activity against the Gram-positive bacteria (3.91–62.50 µg/mL) and Gram-negative bacteria (7.81–62.50 µg/mL). The results in Table 4.5 indicates that Gram-negative bacteria are more resistance towards chloramphenicol since Gram-positive bacteria exhibited lower MIC value. Studies have shown that chloramphenicol more effective against 96% of Gram-positive bacteria than 59% of Gram-negative bacteria (Khalid, et al., 2023). In accordance with Moffa, et al. (2015), chloramphenicol is a potent inhibitor that can bind to the 50S subunit of the bacterial ribosome of Gram-positive and Gram-negative bacteria reversibly to inhibit protein synthesis. It also prevents the extension of transfer RNA (tRNA) to the A site on 50S ribosome (Sood, 2016). Generally, the increase of inherent resistance in Gram-negative bacteria is largely attributed to resistance-nodulation-division efflux pumps, which can extrude antibiotics from bacterial cell. Empirical evidence has indicated that the deactivation of one or more components of efflux pumps is linked to promote the susceptibility to antibiotics.

In summary, the results demonstrate that streptomycin exhibited the most prominent efficacy against Gram-positive bacteria (1.95 µg/mL), whereas ciprofloxacin displays the highest susceptibility against Gram-negative bacteria (0.25 µg/mL). A lower MIC value signifies the requirement of fewer drugs to inhibit bacterial growth. As the MIC value decreases, antibiotics demonstrate increased susceptibility to bacteria (Kowalska-Krochmal and Dudek-Wicher, 2021).

On the other hand, Table 4.3 illustrates that the adjuvants compounds in combination with ciprofloxacin showed highly to moderately active antibacterial activity towards resistance bacteria (7.81–31.25 µg/mL). Alternatively, Table 4.4 and 4.5 have shown that adjuvant compound of streptomycin (1.95–125 µg/mL) and chloramphenicol (3.91–125 µg/mL) represented highly active to inactive antibacterial activity against resistance bacteria. Hence, it is postulated that ciprofloxacin is more effective against resistance bacteria compared to streptomycin and chloramphenicol, with the exception of adjuvant compound 1. Based on the results, only adjuvant compound 1 demonstrated highly active antibacterial activity towards resistance bacteria, influenced by the presence of various genes in the bacteria can impact the ability of the resistance genes, as suggested by Levy (2002). Despite it illustrated a rapid increase in fluoroquinolone resistance in *S. aureus* as well as Methicillin-resistance *S. aureus*, ciprofloxacin is often specified for treating Methicillin-resistance *S. aureus* infections among fluoroquinolone antibiotics (Mirzaie, et al., 2020). This can be explained by the research from Kemung, et al. (2018), in which Methicillin-resistant *S. aureus* is resistant towards most beta-lactam antibiotics.

Therefore, ciprofloxacin will not be affected by the resistance mechanism. However, resistance bacteria exhibited resistance against streptomycin and chloramphenicol showed different.

Besides, it is notable that most of the Methicillin-resistant *S. aureus* strains are relatively resistance compared to Gram-positive and Gram-negative bacteria against adjuvant compound since the presence of SCC*mec* types II and III in the MRSA strains for this project. This can be further explained by the resistance to methicillin will occur when the bacteria consist of *mec*A gene, which found within the staphylococcal cassette chromosome *mec* (SCC*mec*) element. (Katayama, Ito and Hiramatsu, 2000). In accordance with Robinson and Enright (2003), Isolates of SCC*mec* types II and III have been found to have higher survival rates which challenging healthcare settings due to their additional genes that confer resistance to heavy metals and non-beta-lactam drugs. The majority of SCC*mec* types III and IIIA isolates have shown resistance to certain antibiotics including azithromycin, ciprofloxacin, cotrimoxazole and erythromycin (Fatholahzadeh, et al., 2008).

5.4 Antibacterial Activity of NAH Derivative Compounds and NAH Derivative Compounds in Combination with Selected Antibiotics as Adjuvants with Reference to MIC/MBC Ratio

Bernatova, et al. (2013) suggested that compound and adjuvant compound with bactericidal activity represented that it could kill the selected bacteria, whereas bacteriostatic activity will delay the bacteria growth as well as maintain the

stationary phase of bacteria. Moreover, Pankey and Sabath (2004) proposed that certain bactericidal antibacterial agents are demonstrating bactericidal activity towards selected bacteria depends on *in vitro* determination of MBC/MIC ratio values. Table 4.7 revealed that Compound 1 exhibited bactericidal activity against *B. cereus* (ATCC 13061). In accordance with Fang, et al. (2019), most of the drugs in combination with Cl substitution showed higher antibacterial activity. Cl will undergo electronic effect with surrounding amino acid which alter the ability of amino acid to adhere at active site, therefore affect the biological activity (Naumman, 2000).

Furthermore, Table 4.9 displayed most of the NAH derivative compounds in combination with ciprofloxacin as adjuvant showed bactericidal against *B. cereus* (ATCC 13061), *B. subtilis* subsp. *spizizenni* (ATCC 6633) and Methicillin-resistance *S. aureus* (ATCC 33591). Similarly, Table 4.11 illustrates adjuvant compound of streptomycin represented bactericidal against *B. cereus* (ATCC 13061) and *B. subtilis* subsp. *spizizenni* (ATCC 6633). In accordance with Drlica (1999), fluoroquinolones have the potential to demonstrate both bacteriostatic and bactericidal activities. Hawkey (2003) posited that the bactericidal activity of ciprofloxacin caused the chromosomal DNA fragmentation when DNA gyrase-quinolone complexes release the free DNA ends. Moreover, findings from Chen et al. (1996) indicate that the quinolone antimicrobials entrap DNA gyrase (topoisomerase II) and topoisomerase IV during DNA cleavage and inhibit the strains from rejoining to disrupt the chromosomal topology.

Generally, bactericidal antibiotics including aminoglycosides, quinolones and beta-lactams boost the production of lethal hydroxyl radicals in bacteria with different drug-target interactions (Kohanski, Dwyer and Collins, 2010). Bernatová et al. (2013) revealed that the hydroxyl radical aids in promoting the efficacy which culminating in bacterial cell death. As the hydroxyl radicals present a highly toxic characteristic, this makes them adept at causing damage to proteins, membrane lipids and DNA against their target (Tamayo, et al., 2009). Therefore, it is proposed that ciprofloxacin and streptomycin are able to controbute to DNA fragmentation and increase the rate of cell death. In contrast, adjuvant compound in combination with chloramphenicol did not obtain MBC/MIC ratio due to the no MBC value obtained. Parvekar, et al. (2020) suggested that Minimum Inhibitory Concentration (MBC) is the ability of an antimicrobial agent to eliminate 99.9% of the bacteria at lowest concentration. Therefore, it is proposed that adjuvant compound of chloramphenicol able to inhibit instead of killing the bacteria.

5.5 Synergism Effect of NAH Derivative Compounds in Combination with Selected Antibiotics as Adjuvants.

Moellering (1979) mentioned that synergism effect is the combination of two or more antimicrobial drugs produced a better effect compared used alone. In accordance with Ni, et al. (2015), FIC index of an antibiotic-adjuvant less than or equal to 0.5 is known as synergy, more than 4.0 known as antagonism. Allen and Brown (2019) define adjuvants as compounds utilized alongside antibiotics

to boost their antimicrobial effectiveness. When compound combined with antibiotics to form adjuvant, it is possible to reduce the bacterial mutation rates, thereby slowing resistance development due to the highly conserved bacterial target. Table 4.12 shows that certain adjuvant compound have a synergistic effect with ciprofloxacin against Gram-positive and Gram-negative bacteria. Similarly, adjuvant compound 1, 8, and 9 demonstrated synergy against *E. coli* (ATCC 25922). Moreover, Table 4.13 demonstrates that certain adjuvant compound also exhibited synergy with streptomycin against 8 bacteria. Adjuvant compound 4–5 and 8–9 demonstrated synergism against *B. cereus* (ATCC 13061). Furthermore, all 9 adjuvant compound showed synergistic interactions with *P. aeruginosa* (ATCC 27853). According to Table 4.14, the adjuvant compound also demonstrated synergistic interaction with chloramphenicol against 8 bacteria. adjuvant compound 1 and 3 showed synergy with *B. subtilis* subsp. *spizizenni* (ATCC 6633) and Compounds 1–4 showed synergism with *P. aeruginosa* (ATCC 27853).

Based on the results obtained in Table 4.12, 4.13 and 4.14, certain adjuvant compound have a synergistic effect with ciprofloxacin, streptomycin and chloramphenicol against resistance bacteria and *S. aureus* (ATCC 6538). adjuvant compound 1 exhibited synergistic interaction with three antibiotics against Methicillin-resistance *S. aureus* (ATCC 33591) and *S. aureus* (ATCC 6538). In addition, adjuvant compound 1–7 and 9 expressed synergistic interactions with streptomycin as well as adjuvant compound 1, 3, 5 and 7 exhibited synergistic interactions with chloramphenicol against *S. aureus* (ATCC 6538). On top of that, all adjuvant compound represented synergistic interaction

with ciprofloxacin against *S. aureus* (ATCC 6538). Moreover, adjuvant compound 1–2, 4–6, and 8–9, displayed synergism with streptomycin against Methicillin-resistant *S. aureus* (ATCC 43300). In short, it is proposed that antibiotic adjuvants mentioned above displayed a multi-drug resistance and retain the effectiveness of current antibiotics (Gill, Franco, and Hancock, 2015). Moreover, Turnidge and Paterson (2007) also propose that synergistic combinations are preferable for developing therapies, as they can enhance treatment efficiency and reduce patient toxicity.

In contrast, adjuvant compound 2 and 9 demonstrated antagonistic interactions with streptomycin against *B. subtilis* subsp. *spizizenni (*ATCC 6633). Similarly, adjuvant compound 1 exhibited antagonism with chloramphenicol towards *B. cereus* (ATCC 13061) and Methicillin-resistance *S. aureus* (ATCC 43300). Therefore, it is assumed that the adjuvant compound hindered the antibacterial effect of streptomycin and chloramphenicol against the selected bacteria. The statement can be supported by Ocampo, et al. (2014) where antagonism is defined as the effect of one drug interference with each other. Generally, drug antagonism is counted unfavourable from a clinical viewpoint. On the other hand, most of the adjuvant compound did not show significant interactions with ciprofloxacin, streptomycin, and chloramphenicol against the 8 selected bacteria. Johnson et al. (2004) recommended the additivity is indicated by the FIC index ranging from 0.5 to 4.0. Borisy et al. (2003) suggested that additively effect is caused by lack of substantial interaction between antibiotics and adjuvant compound, hence the compounds do not display antibacterial properties.

5.6 Potential Application of NAH

Generally, structure of NAH is widely recognized as significant due to its presence in numerous bioactive compounds. Studies have shown that NAH offer benefits in animal health, contributing to reduced morbidity and mortality rates and promoting healthier animal populations. Research by Costa et al. (2020) highlighted the potential of Compound (E)-3-amino-N'-((3,5-dimethyl-1-phenyl-1H-pyrazol-4-yl) methylene) benzofuran-2-carbohydrazide (LASSBio-2090) in regulating glucose handling, lowering blood pressure and enhancing cardiovascular function in animals. This compound is believed to primarily function by blocking TNF-α, similar to its predecessor LASSBio-1425. Lima and Barreiro (2005) proposed that LASSBio-2090 is specifically developed to match with the main structural of dipeptidyl peptidase 4 (DPP4) inhibitors (Figure 2.16). Moreover, study has shown that NAH are generally assumed safe to be utilized in animals intended for human consumption. According to Cukierman (2017), research in healthy Wistar rats showed that NAH can penetrate through the blood-brain barrier without changing GSH as well as biometal levels in the brain, liver, kidneys and heart under standard homeostasis conditions. Therefore, it is assumed that the use of NAH as antibiotics in food animals will not impact the safety of food and the health of consumers.
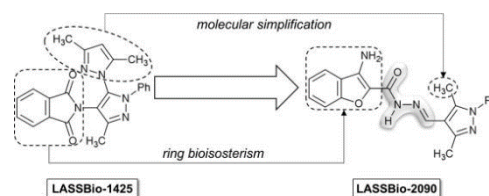
Figure 5.4: Structure of 3-amino-*N'*-((3,5-dimethyl-1-phenyl-1*H*-pyrazol-4-yl) methylene) benzofuran-2-carbohydrazide (LASSBio-2090) (Costa, et al., 2020).

Furthermore, Chen and colleagues (2014) suggested that NAH are a compelling group of chelating ligands with diverse biological effects, such as effective against HIV, hepatitis A, and influenza virus. Based on Carcelli, et al.'s (2016) research, a range of NAH have shown anti-influenza activity, with 50% effective concentration (EC50) values between 3–20 μM. This was detected via enzymatic assay with PA-Nter endonuclease and cell-based influenza viral ribonucleoprotein (vRNP) reconstitution as well as virus yield assays.

5.7 Study Limitation and Future Recommendations

In this project, one of the challenges encountered is the insignificance of antibacterial activity exhibited by certain compounds when tested against selected bacteria. The NAH derivative compounds investigated in this study demonstrated inactive antibacterial activity against Gram-negative bacteria, specifically *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853), and *S.* Typhimurium (ATCC 14028). In order to overcome this limitation, it is advisable to incorporate different Gram-negative strains in subsequent studies to assess the efficacy of NAH derivative compounds against Gram-negative bacteria. For

example, *P. fluorescens*, as referenced in Gu, et al. (2012) findings, could serve as a suitable prospect.

Moreover, the current project encountered a limitation in the adjuvant screening process involving ciprofloxacin, streptomycin, and chloramphenicol, as the outcomes were predominantly negative. Hence, enhancing the screening process by incorporating Moxifloxacin is recommended, given its additional activity against anaerobes and Gram-positive organisms, unlike ciprofloxacin who only showed effectiveness against aerobic Gram-positive and Gram-negative bacteria (Baggio and Ananda-Rajah, 2021).

Furthermore, the evaluation of the bactericidal and synergistic effects of antibiotics and antibiotic-adjuvant combinations can be further clarified through time-kill curves (Fadwa, Albarag, Alkoblan, and Mateen, 2021). Utilizing this method will facilitate the comparative analysis of antibacterial potential among various combinations. Additionally, the checkerboard method, which utilizes the FIC index to represent bacterial growth inhibition, can be employed to determine the synergy between two or more drugs for further study (Martinez-Irujo, Villahermosa, Alberdi, and Santiago, 1996).

CHAPTER 6

CONCLUSION

The objectives in this project were archived in which the *in vitro* antibacterial effect of NAH derivative compounds individually as well as in combination with ciprofloxacin, streptomycin and chloramphenicol against selected Gram-positive, Gram-negative bacteria, and Methicillin-resistant strains determined through minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).

Compound 1 (3,4-Cl2), a derivative of NAH, exhibited strong antibacterial activity against *B. cereus* (ATCC 13061) and Methicillin-resistant *S. aureus* (ATCC 43300). In contrast, Compounds 2–9 displayed varying degrees of moderate to inactive antimicrobial activity against the eight bacterial strains tested. However, Compound 1 showed inactive antibacterial activity against *B. subtilis* subsp. *spizizenni* (ATCC 6633), *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853), *S.* Typhimurium (ATCC 14028), and methicillin-resistant *S. aureus* (ATCC 33591), with a minimum inhibitory concentration (MIC) of 125 µg/mL. Similarly, Compound 5 was inactive against all eight bacterial strains (125 and 250 µg/mL). Despite both Compounds 1 and 5 containing a halogen (chloro) group, their antimicrobial activity was insignificant, suggesting that the position of an electron-withdrawing group on the aromatic ring can influence the lipophilicity of compound, and its antibacterial effectiveness. Additionally,

Compounds 4 and 9, containing methyl and hydrogen substituents, exhibited moderate to insignificant antibacterial activity (62.50 –250 µg/mL). Overall, the presence of electron-donating groups on the phenyl ring led to a significant reduction in antibacterial activity.

Additionally, NAH derivative Compound 1 demonstrated broad-spectrum antibacterial activity against *B. cereus* (ATCC 13061), *S. aureus* (ATCC 6538), and methicillin-resistant *S. aureus* (ATCC 43300). Conversely, Compounds 4, 8, and 9 appear to be species-specific antibacterial agents, as they only showed activity against *S. aureus* (ATCC 6538). Although Gram-negative bacteria are typically greater resistance compared to Gram-positive bacteria due to their unique cell structure, all NAH compounds failed to exhibit significant antibacterial activity against *B. subtilis* subsp. *spizizenni* (ATCC 6633). Furthermore, most NAH derivative compounds were less effective compared to standard antibiotics (ciprofloxacin, streptomycin, and chloramphenicol). On top of that, all NAH derivatives were less potent than ciprofloxacin and chloramphenicol against resistant bacteria. However, Compound 1 was more effective than streptomycin against resistant bacteria, and all NAH derivatives outperformed streptomycin in activity against methicillin-resistant *S. aureus* (ATCC 33591).

Moreover, when NAH derivative compounds were combined with ciprofloxacin formed antibiotic adjuvants, a reduction in antibacterial potency was observed against Gram-negative, Gram-positive, and resistant bacteria. Typically, the OM

of Gram-negative bacteria confers greater resistance, as most antibiotics required to penetrate this barrier to reach their targets. However, in this project, Gram-negative bacteria showed greater susceptibility, likely due to the bacterial strains were anaerobic, while ciprofloxacin is particularly effective against aerobic bacteria. Conversely, the combination of NAH derivative compounds with streptomycin resulted in a decrease in antibacterial activity against Gram-positive, Gram-negative, and resistant bacteria. Similarly, combining NAH derivatives with chloramphenicol led to a broad reduction in antibacterial effectiveness across Gram-positive, Gram-negative and resistant bacteria. In summary, ciprofloxacin was proved that more effective against resistant bacteria compared to streptomycin and chloramphenicol.

Compound 1 displayed strong bactericidal activity against *B. cereus* (ATCC 13061) by interacting with surrounding amino acids and affecting the ability of binding to the active site, thus impacting the biological activity. Additionally, majority of NAH derivative compounds, when combined with ciprofloxacin as an adjuvant, exhibited bactericidal effects against *B. cereus* (ATCC 13061), *B. subtilis* subsp. *spizizenii* (ATCC 6633) and Methicillin-resistant *S. aureus* (ATCC 33591). Similarly, NAH derivative compounds combined with streptomycin showed bactericidal effects against *B. cereus* (ATCC 13061) and *B. subtilis* subsp. *spizizenii* (ATCC 6633). However, the adjuvant compound combined with chloramphenicol did not produce a minimum bactericidal concentration to minimum inhibitory concentration (MBC/MIC) ratio due to the absence of an MBC value, suggesting that the adjuvant compound of chloramphenicol may inhibit the bacteria rather than kill them.

In addition, NAH derivative compounds in combination with ciprofloxacin demonstrated a synergistic effect against *S. aureus* (ATCC 6538), *E. coli* (ATCC 25922) and Methicillin-resistance *S. aureus* (ATCC 33591). In contrast, the combination of NAH derivative compounds with streptomycin and chloramphenicol showed both antagonistic and synergistic effects towards several bacterial strains, indicating interference with the antibacterial activity of these antibiotics. Additionally, most adjuvant compound did not significantly interact with ciprofloxacin, streptomycin, and chloramphenicol, suggesting limited additive effects. Hence, the study suggests that ciprofloxacin, streptomycin, and chloramphenicol might not be suitable for further investigation.

In terms of safety and the use of NAH, it is generally considered safe for animals intended for human consumption. Furthermore, it has positive effects on animal health, leading to lower rates of sickness and death and promoting healthier animal populations. To enhance this project, bactericidal and synergistic effects of antibiotics and antibiotic-adjuvant combinations could be further study using time-kill curves and the checkerboard method. Additionally, the adjuvant screening process could be improved by testing other fluoroquinolones such as Moxifloxacin, as it has additional activity against anaerobes and Gram-positive organisms. Moreover, significance of antibacterial activity against Gram-negative bacteria also could enhance by testing with different bacteria strains.

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Appendix A
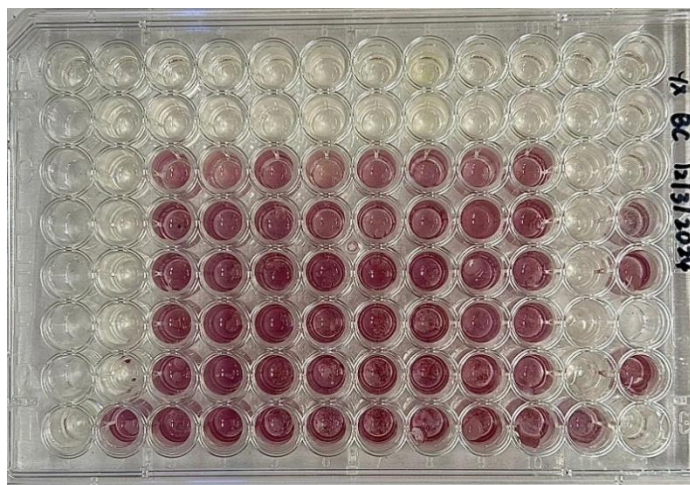
Figure A.1: MIC for NAH derivative compounds against *Bacillus cereus* (ATCC 13061).
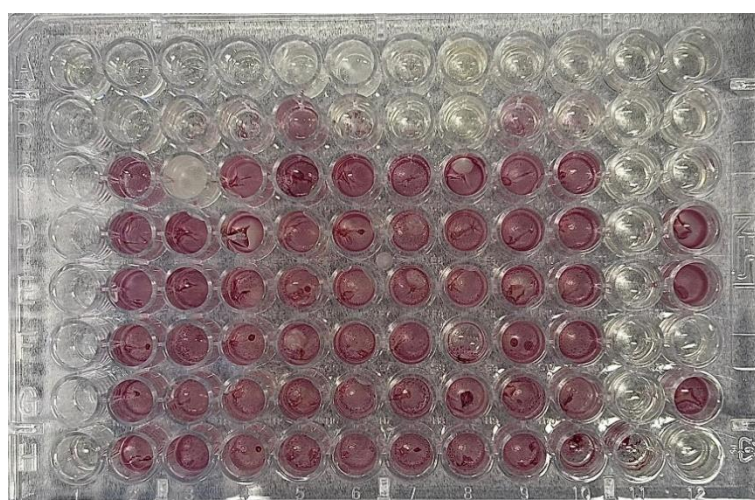
Figure A.2: MIC for NAH derivative compounds against *Bacillus subtilis* subsp. *spizizenni* (ATCC 6633).
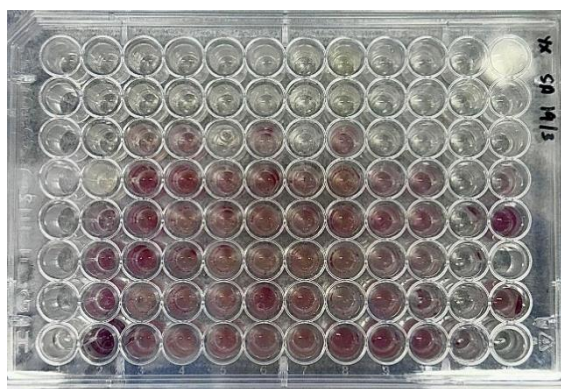
Figure A.3: MIC for NAH derivative compounds against *Staphylococcus aureus* (ATCC 6538).
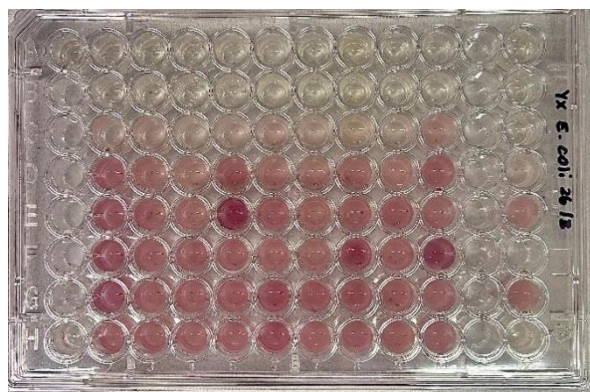
Figure A.4: MIC for NAH derivative compounds against *Escherichia coli* (ATCC 25922).
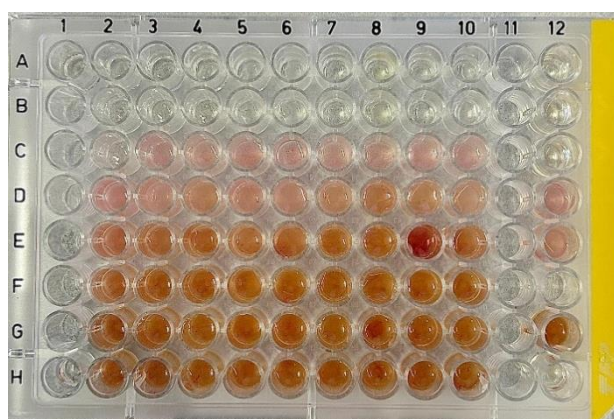
Figure A.5: MIC for NAH derivative compounds against *Pseudomonas aeruginosa* (ATCC 27853).
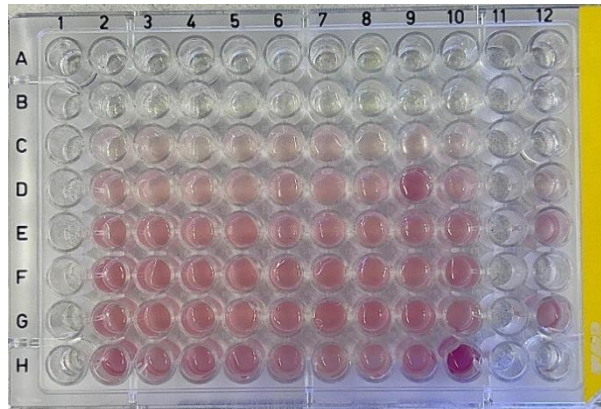
Figure A.6: MIC for NAH derivative compounds against *Salmonella* Typhimurium (ATCC 14028).
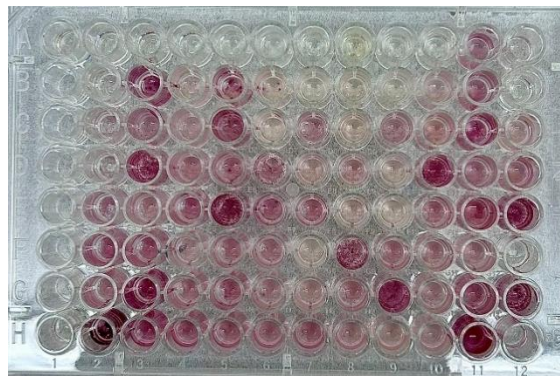
Figure A.7: MIC for NAH derivative compounds against Methicillin-resistance *Staphylococcus aureus* (ATCC 33591).
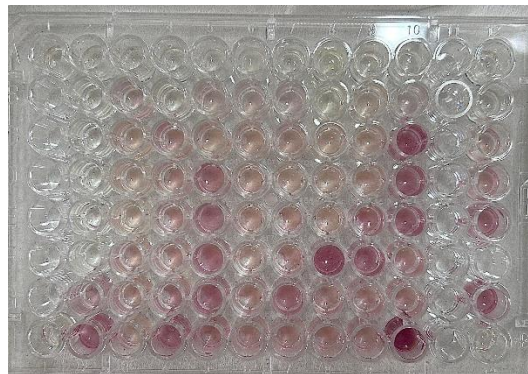
Figure A.8: MIC for NAH derivative compounds against Methicillin-resistance *Staphylococcus aureus* (ATCC 43300).

Figure B.1: MIC replicate streptomycin (Wells 2–4), ciprofloxacin (Wells 5–7) and chloramphenicol (Wells 8–10) as positive controls against *Bacillus cereus* (ATCC 13061).
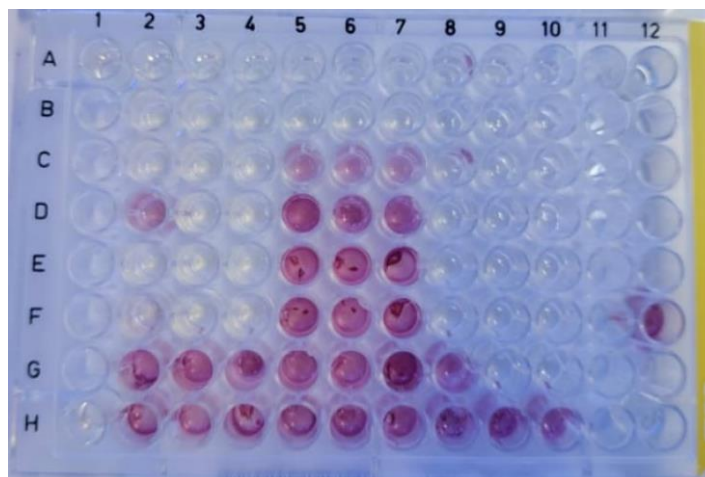
Figure B.2: MIC replicate for streptomycin (Wells 2–4), chloramphenicol (Wells 5–7) and ciprofloxacin (Wells 8–10) as positive controls against *Bacillus subtilis* subsp. *spizizenni* (ATCC 6633).
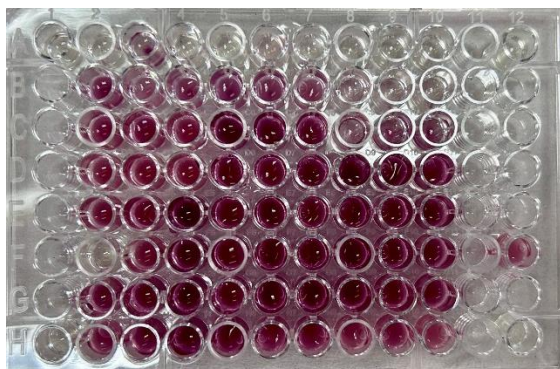
Figure B.3: MIC replicate for streptomycin (Wells 2–4), chloramphenicol (Wells 5–7) and ciprofloxacin (Wells 8–10) as positive controls against *Staphylococcus aureus* (ATCC 6538).
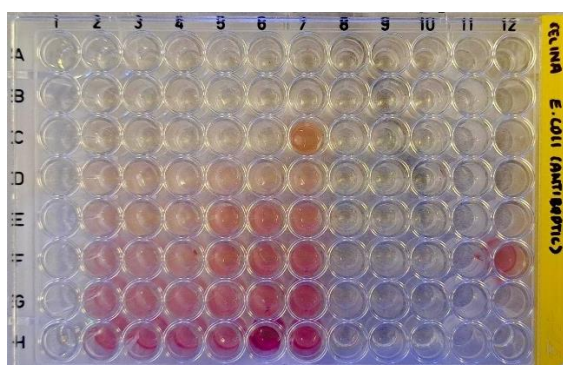
Figure B.4 (A): MIC replicate for streptomycin (Wells 2–4) and chloramphenicol (Wells 5–7) as positive controls against *Escherichia coli* (ATCC 25922).
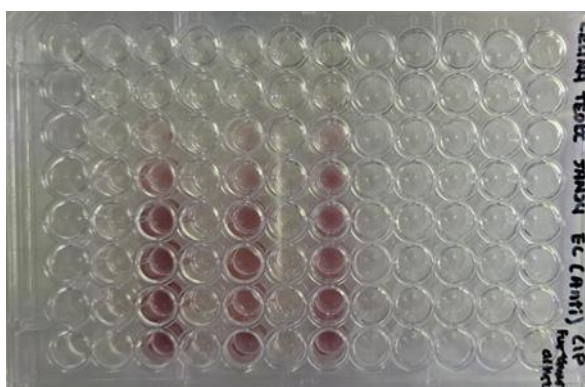
Figure B.4 (B): MIC replicate for ciprofloxacin (Wells 2–7) as positive controls against *Escherichia coli* (ATCC 25922).
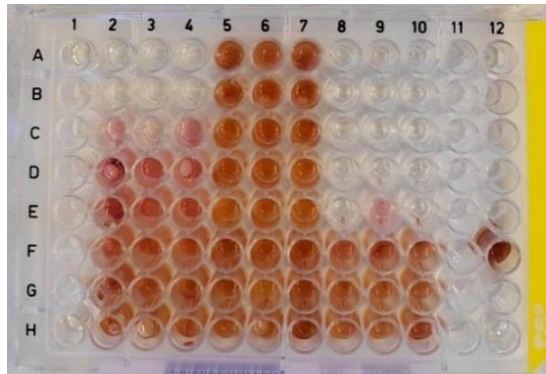
Figure B.5 (A): MIC replicate for streptomycin (Wells 2–4) and ciprofloxacin (Wells 8–10) as positive controls against *Pseudomonas aeruginosa* (ATCC 27853).
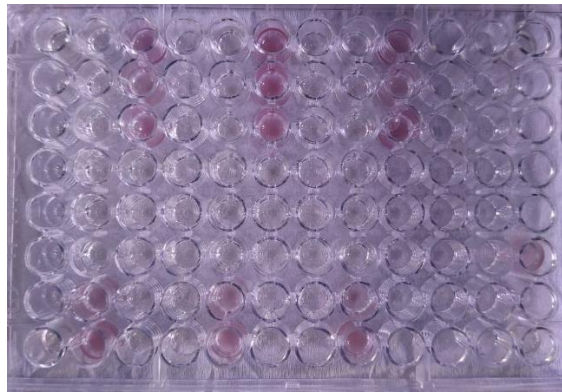
Figure B.5 (B): MIC replicate for chloramphenicol (wells 2–10) as positive controls against *Pseudomonas aeruginosa* (ATCC 27853).
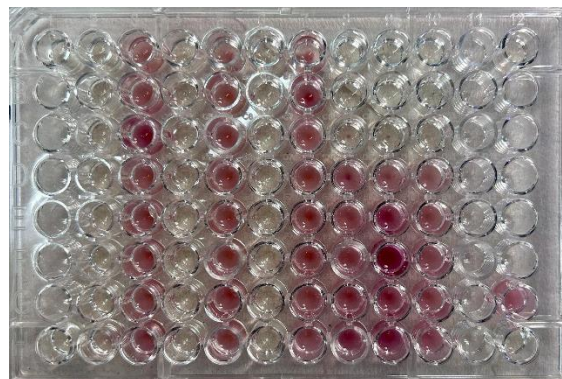
Figure B.6 (A): MIC replicate for ciprofloxacin (wells 2–7) and chloramphenicol (wells 8–10) as positive controls against *Salmonella* Typhimurium (ATCC 14028).
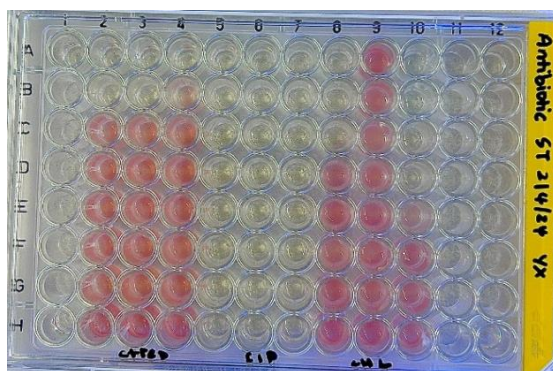
Figure B.6 (B): MIC replicate for streptomycin (wells 2–4) as positive controls against *Salmonella* Typhimurium (ATCC 14028).
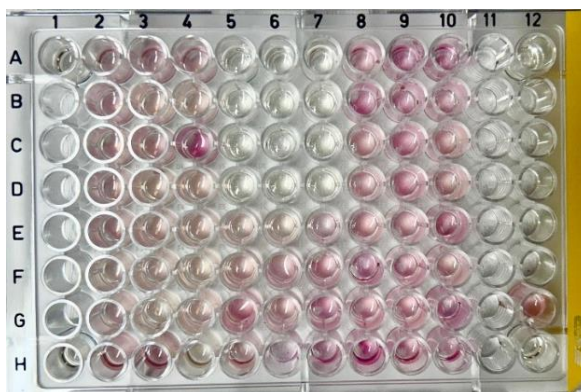
Figure B.7 (A): MIC replicate for ciprofloxacin (wells 5–7) as positive controls against as adjuvants against Methicillin-resistance *Staphylococcus aureus* (ATCC 33591).
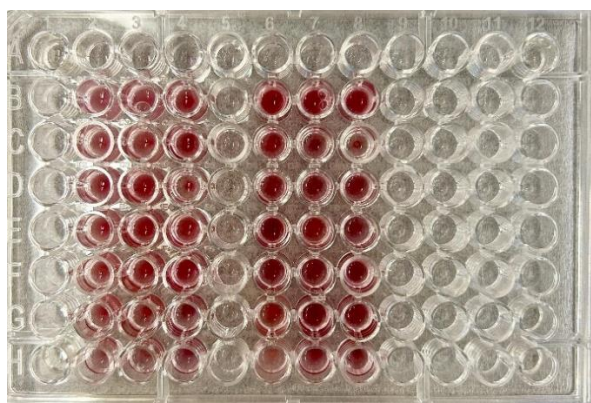
Figure B.7 (B): MIC replicate for streptomycin (wells 2–4) and chloramphenicol (wells 6–8) as positive controls against Methicillin-resistance *Staphylococcus aureus* (ATCC 33591).

Figure B.8: MIC replicate for streptomycin (wells 2–4), chloramphenicol (wells 5–7) and ciprofloxacin (wells 8–10) as positive controls against Methicillin-resistance *Staphylococcus aureus* (ATCC 43300).
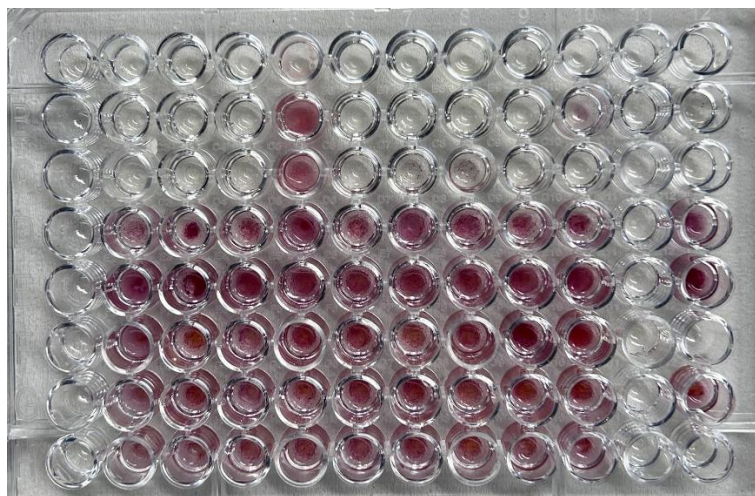
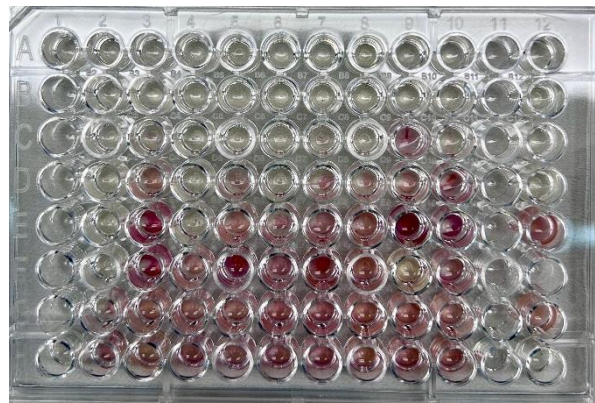**Figure C.1(A)**: MIC for NAH derivative compounds in combination with chloramphenicol as adjuvants against *Bacillus cereus* (ATCC 13061).
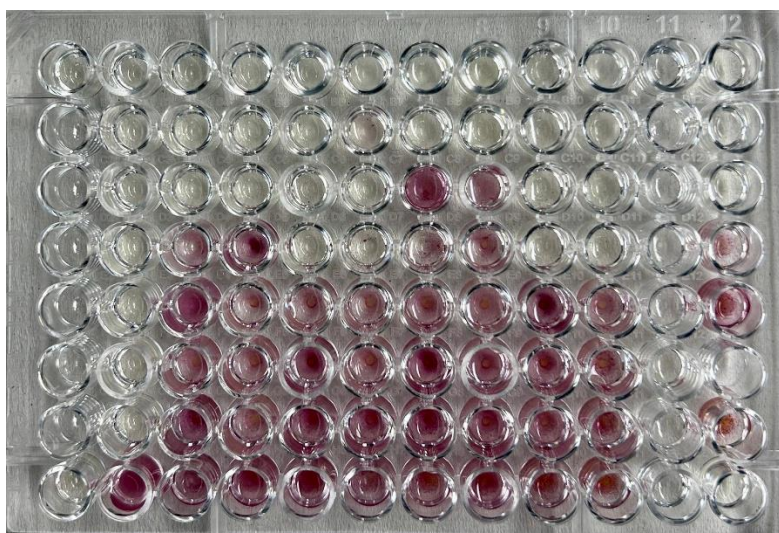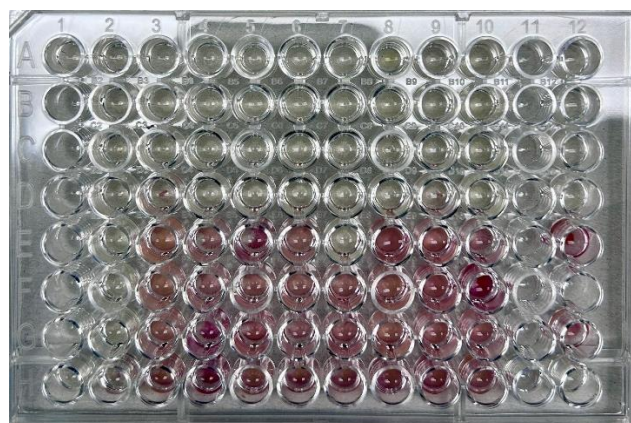

Figure C.1(B): MIC for NAH derivative compounds in combination with streptomycin as adjuvants against *Bacillus cereus* (ATCC 13061).
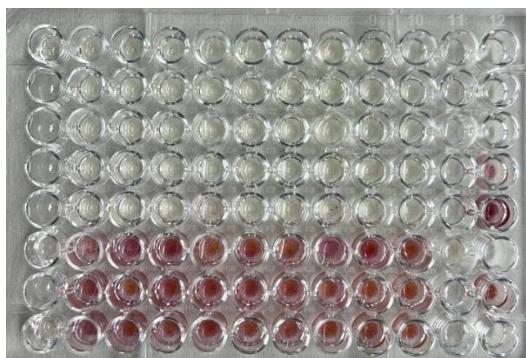
Figure C1(C): MIC for NAH derivative compounds in combination with ciprofloxacin as adjuvants against *Bacillus cereus* (ATCC 13061).
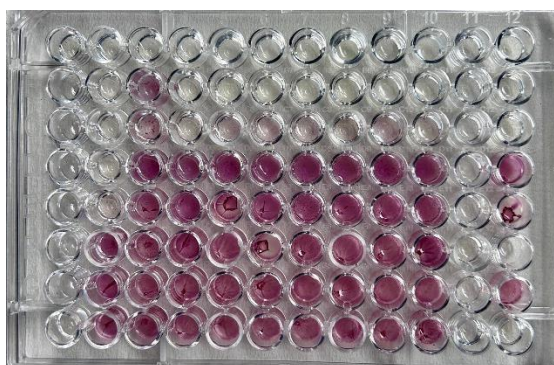
Figure C.2(A): MIC replicate for NAH derivative compounds in combination with chloramphenicol as adjuvants against *Bacillus subtilis* subsp. *spizizenni* (ATCC 6633).
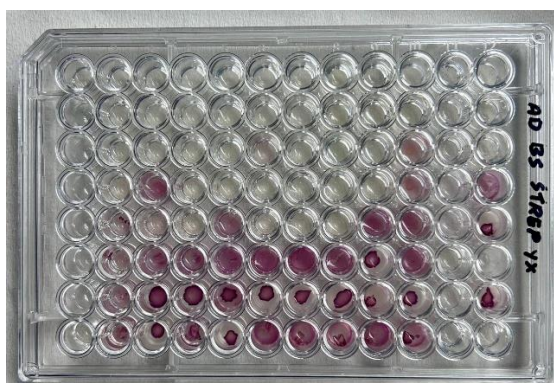
Figure C.2(B): MIC replicate for NAH derivative compounds in combination with streptomycin as adjuvants against *Bacillus subtilis* subsp. *spizizenni* (ATCC 6633).
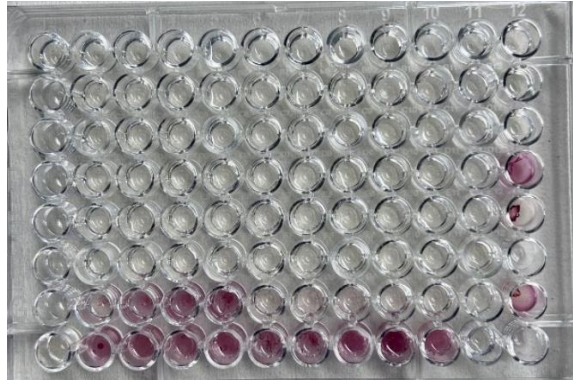
Figure C.2(C): MIC replicate for NAH derivative compounds in combination with ciprofloxacin as adjuvants against *Bacillus subtilis* subsp. *spizizenni* (ATCC 6633).

Figure C.3(A): MIC replicate for NAH derivative compounds in combination with chloramphenicol as adjuvants against *Staphylococcus aureus* (ATCC 6538).
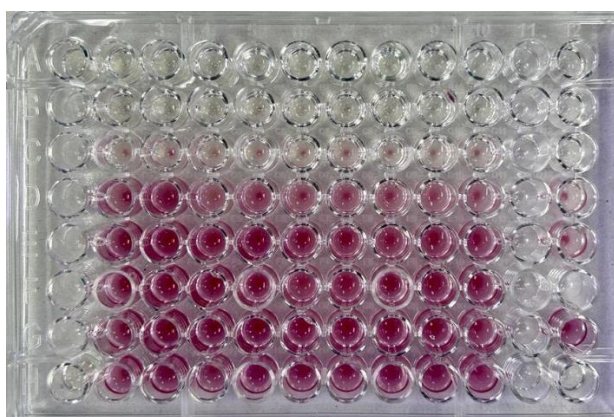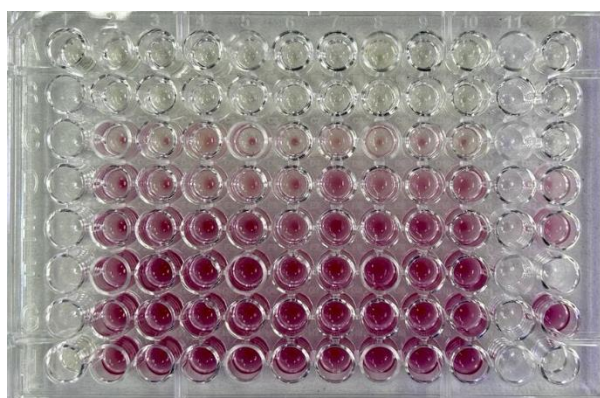

Figure C.3(B): MIC replicate for NAH derivative compounds in combination with streptomycin as adjuvants against *Staphylococcus aureus* (ATCC 6538).

Figure C.3(C): MIC replicate for NAH derivative compounds in combination with ciprofloxacin as adjuvants against *Staphylococcus aureus* (ATCC 6538).

Figure C.4 (A): MIC replicate for NAH derivative compounds in combination with chloramphenicol as adjuvants against *Escherichia coli* (ATCC 25922).
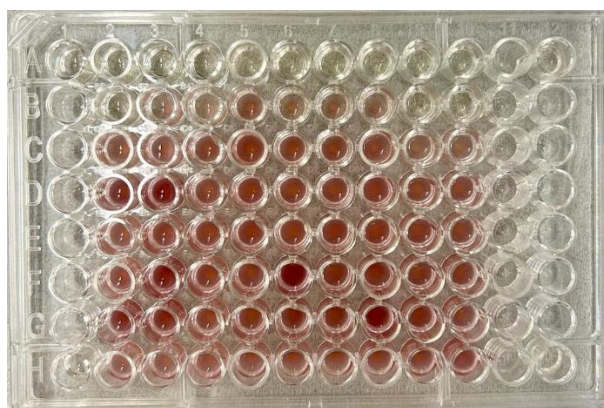

Figure C.4 (B): MIC replicate for NAH derivative compounds in combination with streptomycin as adjuvants against *Escherichia coli* (ATCC 25922).

C1

Figure C.4(C1) & (C2): MIC replicate for NAH derivative compounds in combination with ciprofloxacin as adjuvants against *Escherichia coli* (ATCC 25922).
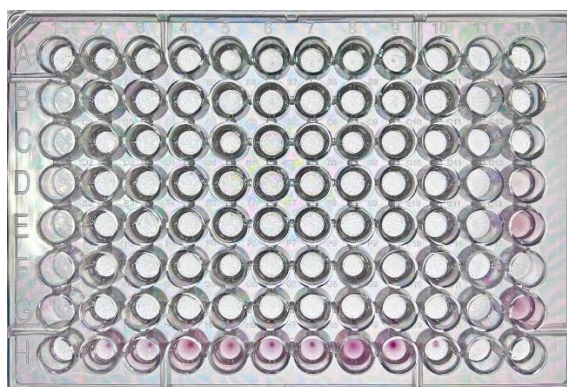

C2

Figure C.5(A): MIC replicate for NAH derivative compounds in combination with ciprofloxacin as adjuvants against *Salmonella* Typhimurium (ATCC 6538).
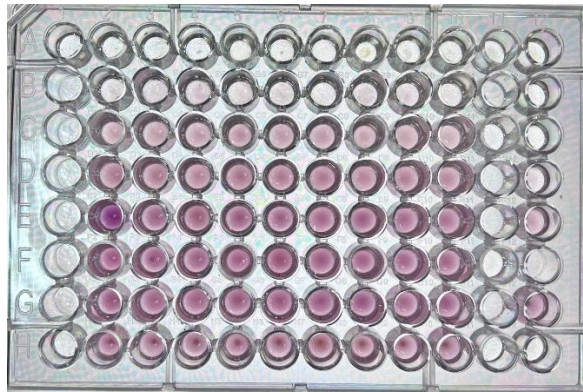
Figure C.5(B): MIC replicate for NAH derivative compounds in combination with streptomycin as adjuvants against *Salmonella* Typhimurium (ATCC 6538).
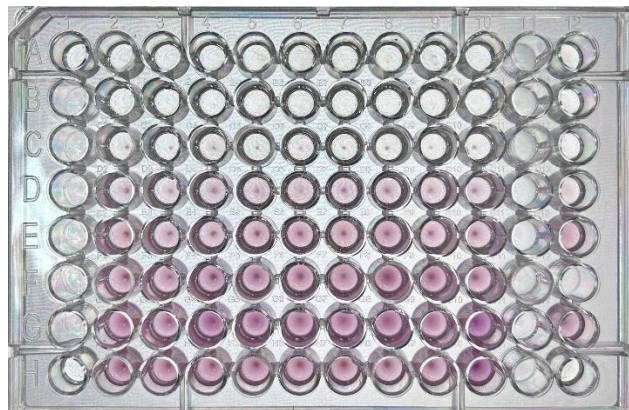

Figure C.5(C): MIC replicate for NAH derivative compounds in combination with ciprofloxacin as adjuvants against *Salmonella* Typhimurium (ATCC 6538).
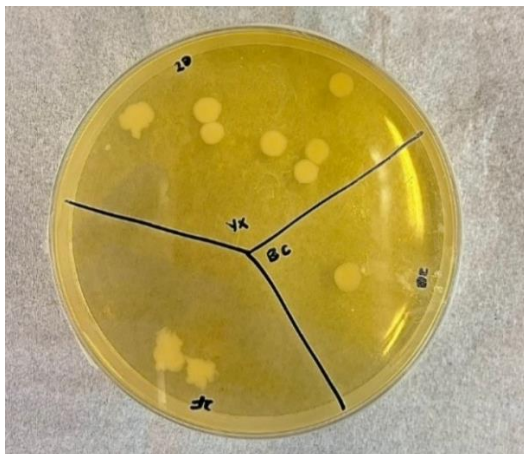
Figure D.1: MBC for NAH derivative compound 1 against *Bacillus cereus* (ATCC 13061).
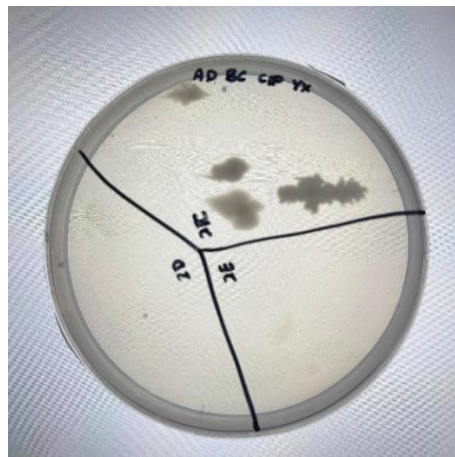
Figure D.2: MBC for NAH derivative compound 1 in combination with ciprofloxacin against *Bacillus cereus* (ATCC 13061).

Figure D.3: MBC for NAH derivative compound 5 in combination with ciprofloxacin against *Bacillus cereus* (ATCC 13061).

Figure D.4: MBC for NAH derivative compound 7 in combination with ciprofloxacin against *Bacillus cereus* (ATCC 13061).
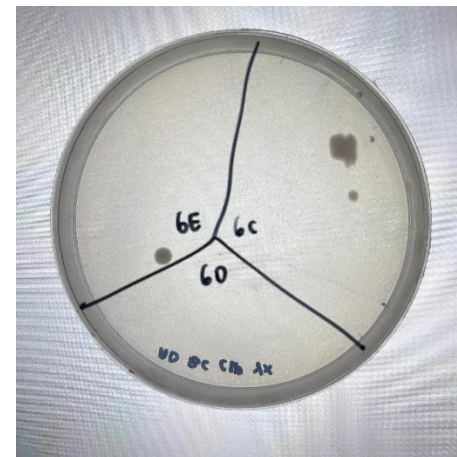

Figure D.5: MBC for NAH derivative compound 8 in combination with ciprofloxacin against *Bacillus cereus* (ATCC 13061).
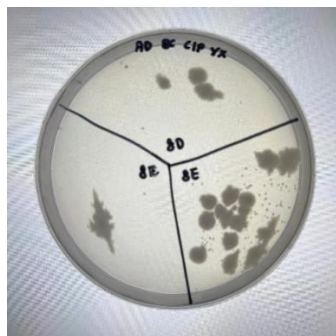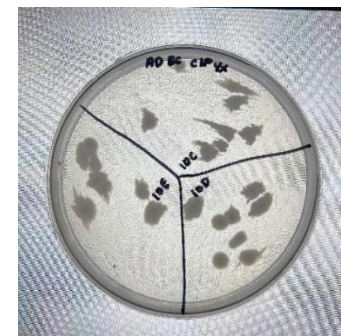

Figure D.6: MBC for NAH derivative compound 9 in combination with ciprofloxacin against *Bacillus cereus* (ATCC 13061).

Figure D.7: MBC for NAH derivative compound 1 in combination with streptomycin against *Bacillus cereus* (ATCC 13061).

Figure D.8: MBC for NAH derivative compound 1 in combination with ciprofloxacin against *Staphylococcus aureus* (ATCC 6538).
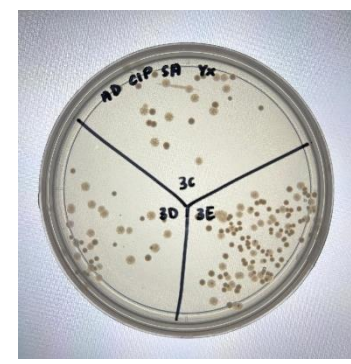

Figure D.9: MBC for NAH derivative compound 2 in combination with ciprofloxacin against *Staphylococcus aureus* (ATCC 6538).

136

Figure D.10: MBC for NAH derivative compound 4 in combination with ciprofloxacin against *Staphylococcus aureus* (ATCC 6538).

Figure D.11: MBC for NAH derivative compound 5 in combination with ciprofloxacin against *Staphylococcus aureus* (ATCC 6538).

Figure D.12: MBC for NAH derivative compound 8 in combination with ciprofloxacin against *Staphylococcus aureus* (ATCC 6538).

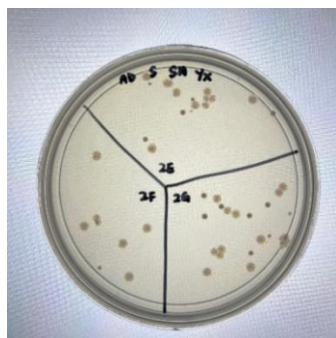**Figure D.13:** MBC for NAH derivative compound 1 in combination with streptomycin against *Staphylococcus aureus* (ATCC 6538).

Figure D.14: MBC for NAH derivative compound 3 in combination with Streptomycin against *Staphylococcus aureus* (ATCC 6538).
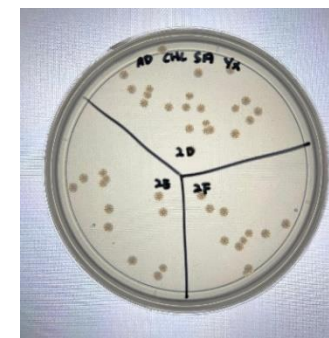

Figure D.15: MBC for NAH derivative compound 1 in combination with chloramphenicol against *Staphylococcus aureus* (ATCC 6538).

137

Figure D.16: MBC for NAH derivative compound 1 in combination with ciprofloxacin against *Escherichia coli* (ATCC 25922).

Figure D.17: MBC for NAH derivative compound 2 in combination with ciprofloxacin against *Escherichia coli* (ATCC 25922).

Figure D.18: MBC for NAH derivative compound 3 in combination with ciprofloxacin against *Escherichia coli* (ATCC 25922).

Figure D.19: MBC for NAH derivative compound 4 in combination with ciprofloxacin against *Escherichia coli* (ATCC 25922).

Figure D.20: MBC for NAH derivative compound 5 in combination with ciprofloxacin against *Escherichia coli* (ATCC 25922).
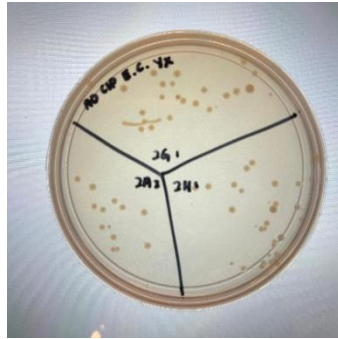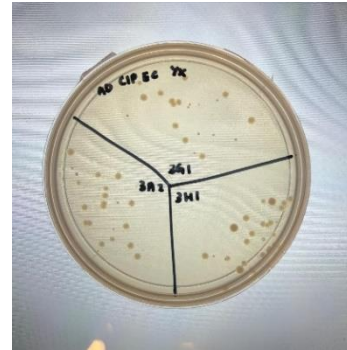

Figure D.21: MBC for NAH derivative compound 6 in combination with ciprofloxacin against *Escherichia coli* (ATCC 25922).
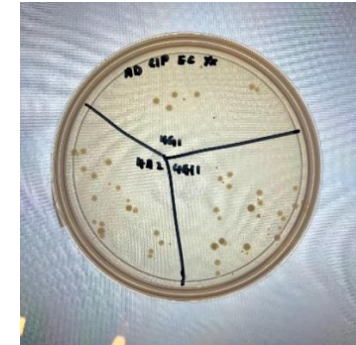
Figure D.22: MBC for NAH derivative compound 1 in combination with ciprofloxacin against *Salmonella* Typhimurium (ATCC 6538).

Figure D.23: MBC for NAH derivative compound 2 in combination with ciprofloxacin against *Salmonella* Typhimurium (ATCC 6538).

Figure D.24: MBC for NAH derivative compound 3 in combination with ciprofloxacin against *Salmonella* Typhimurium (ATCC 6538).

Figure D.25: MBC for NAH derivative compound 4 in combination with ciprofloxacin against *Salmonella* Typhimurium (ATCC 6538).

Figure D.26: MBC for NAH derivative compound 6 in combination with ciprofloxacin against *Salmonella* Typhimurium (ATCC 6538).
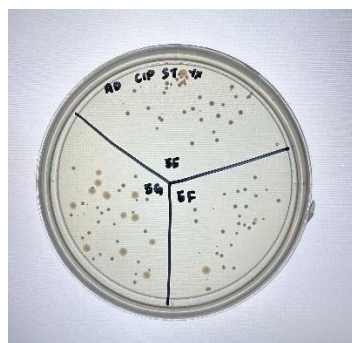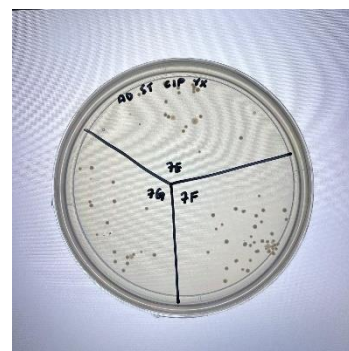

Figure D.27: MBC for NAH derivative compound 7 in combination with ciprofloxacin against *Salmonella* Typhimurium (ATCC 6538).

Universiti Tunku Abdul Rahman
Form Title: Supervisor's Comments on Originality Report Generated by Turnitin for Submission of Final Year Project Report (for Undergraduate Programmes)

Form Number: FM-IAD-005	Rev No.: 0	Effective Date: 01/10/2013	Page No.: 1of 1

FACULTY OF SCIENCE

Full Name(s) of Candidate(s)	Long Yi Xian
ID Number(s)	2002199
Programme / Course	Bachelor of Science (HONOURS) Food Science
Title of Final Year Project	Antibacterial Activity of 5- Chloro Substituted Phenyl N-Acylhydrazone Derivatives with Aromatic Substitution at *Meta*- and *Para*- Directors as Potential Adjuvants

Similarity	**Supervisor's Comments** **(Compulsory if parameters of originality exceed the limits approved by UTAR)**
Overall similarity index: 15 **% Similarity by source** Internet Sources: **1 2** % Publications: **1 4** % Student Papers: **6** %	N/A
Number of individual sources listed of more than 3% similarity: 0	N/A

Parameters of originality required and limits approved by UTAR are as follows:
 (i)   **Overall similarity index is 20% and below, and**
 (ii) **Matching of individual sources listed must be less than 3% each, and**
 (iii) **Matching texts in continuous block must not exceed 8 words**
Note: Parameters (i) – (ii) shall exclude quotes, bibliography and text matches which are less than 8 words.

Note Supervisor/Candidate(s) is/are required to provide softcopy of full set of the originality report to Faculty/Institute
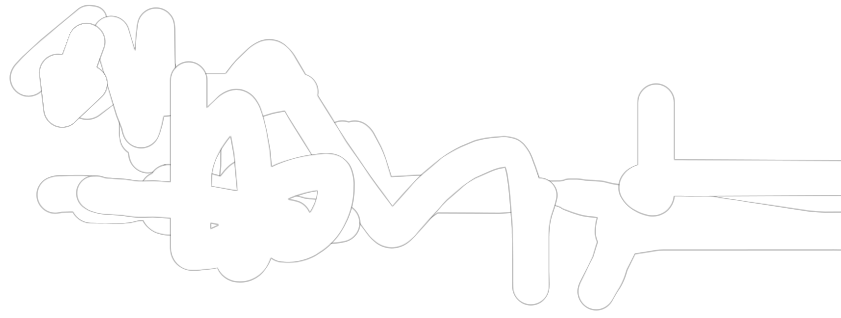
Based on the above results, I hereby declare that I am satisfied with the originality of the Final Year Project Report submitted by my student(s) as named above.

KahCheng

Signature of Supervisor

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