ANTIBACTERIAL ACTIVITY OF 5-CHLORO SUBSTITUTED PHENYL *N*-ACYLHYDRAZONES WITH AROMATIC SUBSTITUTION AT ORTHO- AND PARA-DIRECTORS AS POTENT ADJUVANTS

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ABSTRACT

ANTIBACTERIAL ACTIVITY OF 5-CHLORO SUBSTITUTED PHENYL *N*-ACYLHYDRAZONES WITH AROMATIC SUBSTITUTION AT ORTHO- AND PARA-DIRECTORS AS POTENT ADJUVANTS

Chan Yao Xiang

The emergence of antimicrobial-resistant pathogens has led to a decline in the availability of effective medications for clinical use, which urges the development of new drugs. *N*-acylhydrazone (NAH) with its versatile chemical moiety becomes essential in designing new drugs as its derivatives have been approved as therapeutics. This project studied the *in vitro* antibacterial activity of 7 NAH derivative compounds with different aromatic substitutions at *ortho* and *para* directors individually and in combination with 3 standard drugs as adjuvants against 8 bacterial strains using the broth microdilution method to determine Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). The results revealed that Compounds 1 (2-Br), 4 (H), 6 (2-OCH_3) and 7 (2,4-Cl_2) exhibited moderately active antibacterial activity against *Staphylococcus aureus* (ATCC 6538) with a MIC value of 62.5 µg/mL, showing species-specific antibacterial activity. With the combination of antibiotics as adjuvants, the NAH derivative compounds demonstrated enhanced antibacterial activities. Notably, the ciprofloxacin-NAH adjuvant combinations obtained

MBC/MIC ratio ranging from 1 to 4, suggesting the bactericidal effects. Nevertheless, the Fractional Inhibitory Concentration (FIC) index calculated revealed that neither of the antibiotic-NAH adjuvant combinations was suitable for further studies although synergism was observed because most of the combinations recorded insignificant interactions, indicating the antibacterial activity was contributed by the antibiotic itself.

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DECLARATION

I hereby declare that this final year project report is based on my 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.

CHAN YAO XIANG

APPROVAL SHEET

This final year project entitled **"ANTIBACTERIAL ACTIVITY OF 5-CHLORO SUBSTITUTED PHENYL *N*-ACYLHYDRAZONES WITH AROMATIC SUBSTITUTION AT ORTHO- AND PARA-DIRECTORS AS POTENT ADJUVANTS"** was prepared by CHAN YAO XIANG and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (Honours) Food Science at Universiti Tunku Abdul Rahman.

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SUBMISSION OF FINAL YEAR PROJECT

I, **CHAN YAO XIANG** (ID No: **20ADB03747**) hereby certified that I have completed the final year project titled "**ANTIBACTERIAL ACTIVITY OF 5-CHLORO SUBSTITUTED PHENYL *N*-ACYLHYDRAZONES WITH AROMATIC SUBSTITUTION AT ORTHO- AND PARA-DIRECTORS AS POTENT ADJUVANTS**" under the supervision of Dr. Teo Kah Cheng (Supervisor) from the Department of Agricultural and Food Science, Faculty of Science.

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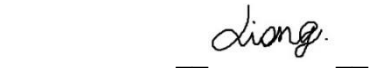
Yours truly,



CHAN YAO XIANG

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LIST OF ABBREVIATIONS

A. baumanni	*Acinetobacter baumanni*
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*
EPI	Efflux pump inhibitors
ESBL	Extended-spectrum-beta-lactamases
ESKAPE	*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanni*, *Pseudomonas aeruginosa* and *Enterobacter* species
F	Fluoro
FIC	Fractional Inhibitory Concentration
H	Hydrogen
INT	Iodonitrotetrazolium
K. pneumoniae	*Klebsiella pneumoniae*

MBC	Minimum Bactericidal Concentration
MH	Mueller-Hinton
MIC	Minimum Inhibitory Concentration
MOA	Mechanism of action
MRSA	Methicillin-resistant *Staphylococcus aureus*
MTT	(3- (4,5-dimethyl thiazol -2-yl)-2,5-di phenyl tetrazolium bromide
NAH	*N*-acylhydrazone
NO_2	Nitro
OCH_3	Methoxy
OD	Optical density
OM	Outer membrane
P. aeruginosa	*Pseudomonas aeruginosa*
P. putida	*Pseudomonas putida*
PAβN	Phenylalanyl arginyl β-napthylamide
PBPs	Penicillin-binding proteins
S. aureus	*Staphylococcus aureus*
S. Typhimurium	*Salmonella* Typhimurium
STR	Streptomycin
TMV	Tobacco mosaic viruses

CHAPTER 1

INTRODUCTION

1.1 Antibiotics

Antibiotics are chemical substances that can stop microbial growth or cause death to microbes. Antibiotics are classified into bacteriostatic which inhibits bacterial growth and bactericidal when the killing effect of bacterial cells is observed (Walsh, 2003). The drug discovery started in 1909 by Paul Ehrlich who developed salvarsan, the first sulfa drug used in treating syphilis. The period from 1940 to 1960 is often referred to as the golden age of antibiotic discovery, as many antibiotics developed at that time are still used in clinical treatment, despite the reduced efficacy of the antibiotics due to the emergence of antimicrobial resistance (AMR) (Hutchings, Truman and Wilkinson, 2019). These antibiotics include antibiotics produced naturally by actinomycetes, antibiotics isolated from fungal origin and manmade antibiotics (Pancu, et al., 2021). Antibiotics are commonly used in healthcare, including treating infections and surgery (Patel, et al., 2023). Additionally, antibiotics are widely used in the animal industry, particularly in husbandry, to prevent the spread of diseases among livestock which often live in crowded and unhygienic conditions (Kumar, et al., 2020).

1.2 Antibiotic Adjuvants

Due to the extensive usage of antibiotics, bacteria acquired resistance mechanisms and evolved into multidrug-resistant (MDR), extensively drug-resistant (XDR) and pan-drug-resistant (PDR) bacteria, rendering antibiotics ineffective against them (Magiorakos, et al., 2012). New approaches have been developed throughout the years to improve the performance of traditional antibiotics including combining antibiotic adjuvants with antibiotics which has been proven to be the most successful strategy (González-Bello, 2017; Kumar, et al., 2023). Antibiotic adjuvants are substances that potentiate the antimicrobial activity of an antibiotic when co-administered with the antibiotic despite lacking antibiotic properties themselves. Besides, these substances are also called adjuvants when they broaden the spectrum of activity or exhibit synergistic effects upon combining with the antibiotic (Bernal, et al., 2013). Antibiotic adjuvants heighten the antimicrobial activity of antibiotics by reducing the intrinsic resistance of the bacteria or directly blocking the bacterial resistance mechanism (Dhanda, Acharya and Haldar, 2023).

1.3 *N*-acylhydrazone (NAH)

N-acylhydrazone (NAH) is one of the small organic compounds with a bioactive scaffold, proven to be crucial and promising in drug design and medicinal chemistry (Thota, et al., 2018). NAH is represented by a general formula of R_1–NHN═CH–R_2. The R_1 and R_2 are substituted with different functional groups to investigate their pharmacological properties in developing new drug molecules for the future to tackle the problem of reduced effectiveness of antimicrobials due to

antimicrobial resistance (Biliz, et al., 2023). NAH-related drugs have been approved as therapeutics such as nitrofurazone, nitrofurantoin and carbazochrome. Additionally, NAH derivatives also exhibit a wide range of biological activities such as antiviral, anticancer, anti-inflammatory and so on (Gu, et al., 2012). Hence, the studies of NAH derivatives in terms of antibacterial activity increase due to their potential as new drugs to combat antimicrobial resistance.

1.4 Problem Statement: Development of Antibiotic Resistance

Antibiotics have been overprescribed in treating human diseases that in fact, do not require the help of antibiotics to be cured and can be healed by the immune system (Biggers, 2023). Antibiotics are also irrationally used in animal husbandry, with the purpose of preventing the spread of diseases among livestock (Shahid, et al., 2021). The overprescription and irrational usage of antibiotics cause antibiotic resistance, which is now a global concern issue. Antibiotic resistance leads to the emergence of antibiotic-resistant strains, hence limiting the effective drugs in clinical use (Biggers, 2023).

The objectives of this study are:

1. To determine the *in vitro* antibacterial properties of *N*-acylhydrazone derivative compounds against selected Gram-negative, Gram-positive and Methicillin-resistant bacteria strains.
2. To compare the effectiveness of *in vitro* antibacterial activity of *N*-acylhydrazone derivative compounds alone and *N*-acylhydrazone derivative compounds in combination with streptomycin, chloramphenicol and ciprofloxacin respectively as adjuvants against selected Gram-negative, Gram-positive and Methicillin-resistant bacteria strains.

CHAPTER 2

LITERATURE REVIEW

2.1 Antibiotic

2.1.1 Antibiotic Classification

Antibiotics can be classified based on the mechanism of action on bacteria which are i) suppression of cell wall synthesis, ii) disruption of protein synthesis, iii) inhibition of nucleic acid synthesis, iv) blocking of metabolic pathways and v) breakdown of cell membrane structure or function (Reygaert, 2018).

The antibiotics involved in inhibiting cell wall synthesis are β-lactams, penicillin, cephalosporins and carbapenems. The bacterial cell wall is made of peptidoglycan to provide a barrier against harsh environments. To strengthen the cell wall, the peptide chain in peptidoglycan is cross-linked with glycine residues with the aid of penicillin-binding proteins (PBPs). β-lactams primarily affect the activity of PBPs as β-lactams bind to the PBPs due to the presence of β-lactam ring structure to inhibit the synthesis of new peptidoglycan and cross-linking, resulting in the destruction of bacterial cells (Halawa, et al., 2023). Another antibiotic exhibiting the mechanism is glycopeptide which inhibits the production of peptidoglycan by binding to the peptidoglycan cross-linking enzymes, particularly transpeptidase and carboxypeptidase (Etebu and Arikekpar, 2016).

The synthesis of protein relies on the bacterial 70S ribosomes that is made up of 30S and 50S ribosomal subunits. The 30S and 50S ribosomal subunits are targeted by different antibiotics. The inhibitors of the 30S subunit include aminoglycosides and tetracycline. Providing the property of being positively charged, aminoglycosides bind to the negatively charged outer membrane of the bacteria, forming large pores to enter the cell by using the energy from the active transport of the bacteria (Jana and Deb, 2006; Halawa, et al., 2023). Once inside the cell, aminoglycosides bind to the 16S ribosomal RNA of the 30S subunit through hydrogen bonds. The binding causes the termination of mRNA translation, inhibiting the synthesis of protein (Kapoor, Saigal and Elongavan, 2017). It is noted that the uptake of aminoglycosides into the bacterial cell by active transport requires oxygen to function, hence aminoglycosides are more effective against aerobes compared to anaerobes (Kohanski, Dwyer and Collins, 2010). Chloramphenicol, macrolides and oxazolidinone are the antibiotics targeting the 50S ribosomal units, either stopping the initiation phase of protein translation or the elongation phase of protein synthesis, blocking the synthesis of protein (Etebu and Arikekpar, 2016).

The inhibition of nucleic acid synthesis involves the antibiotic class – fluoroquinolones which target DNA gyrase. DNA gyrase enzyme plays a role in the indirect unwinding of double helix structures of DNA during DNA replication. As the helicase enzyme creates positive supercoils continuously during the unwinding

of DNA, it produces tension. With the aid of DNA gyrase, it introduces negative supercoils to counteract the tension, allowing the continuous progression of DNA replication (Wise, 1999; Kapoor, Saigal and Elongavan, 2017). The fluoroquinolone inhibits the DNA gyrase by binding to the subunit A of DNA gyrase, disrupting its function to reseal the nicks during DNA replication. Apart from targeting DNA gyrase, fluoroquinolones also bind to topoisomerase IV which is essential to cutting and separating daughter DNA strands, hence the binding action leads to DNA breakage (Malik, Zhao and Drlica, 2006).

Furthermore, antibiotics sulfonamides and trimethoprim have a mechanism of action in inhibiting folic acid metabolism. Folic acid is an essential substrate for the production of nucleic acids and amino acids. Dihydropteroate synthase and dihydrofolate reductase act as enzymes in catalysing the pathway of folic acid metabolism. According to Talaro (2008), due to the similarities in the chemical structure of sulfonamides and tetrahydrofolate which is a substrate needed in the folic acid metabolic pathway, dihydropteroate synthase may not be able to discriminate between both substrates. Hence, sulfonamides can fit into the active site of dihydropteroate synthase and outcompete with tetrahydrofolate, leading to the inhibition of folic acid synthesis. Meanwhile, trimethoprim binds to the dihydrofolate reductase which also prevents the synthesis of folic acid (Yoneyama and Katsumata, 2006).

Moving on, the depolarization of the cell membrane is exhibited by daptomycin, a member of the lipopeptide antibiotic. It targets the bacterial cell membrane that is dependent on calcium for its function, changing the membrane potential and leading to cell death (Eyler and Shvets, 2019). Besides, polymyxins, a peptide antibiotic with narrow spectrum activity isolated from *Bacillus polymyxa* had detergent activity due to its unique fatty acid component. The presence of this fatty acid component allows it to bind to the lipopolysaccharide of the bacterial cell membrane, forming abnormal pores and causing the cell to become leaky (Talaro, 2008).

2.2 Antibiotic Resistance

2.2.1 Antimicrobial Resistance Mechanisms

Antimicrobial resistance mechanisms can be divided into 4 categories including regulating the uptake of a drug into the cell, activation of efflux pump, modification of drug target and enzymatic inactivation of drug. Gram-negative bacteria are capable of utilizing four mechanisms against antibiotics whereas limiting the uptake of drugs is rare in Gram-positive bacteria due to the absence of an outer membrane compared to Gram-negative bacteria (Reygaert, 2018).

The presence of the outer membrane made of lipopolysaccharide in Gram-negative bacteria acts as a barrier to selectively inhibit the entry of molecules that contribute

to antimicrobial resistance. Compared to hydrophilic drugs, hydrophobic drugs such as fluoroquinolones are easier to permeate into the bacterial cell membrane due to the high lipid content of the outer membrane (Kumar and Schweizer, 2005; Reygaert, 2018). Porin channels are found in large outer membranes of bacteria that act as a pathway for substances such as hydrophilic molecules to pass through. Due to evolution and mutations, the porins present in the membrane decrease and the selectivity of the porins is also altered, limiting the uptake of drugs into the cell. Mutation of porin channels has been observed in *Neisseria gonorrhoeae* which becomes more resistant to β-lactams and tetracycline (Gill, et al., 1998; Reygaert, 2018).

Apart from that, efflux pumps are involved in the antimicrobial resistance mechanism of bacteria. Efflux pumps refer to the membrane proteins found in cytoplasmic membranes that express toxic substances such as antibiotics from the cell to prevent intracellular damage by the toxic substances. Most efflux pumps are multidrug-resistant, conferring the ability to transport a wide range of antibiotics such as macrolides, tetracyclines and fluoroquinolones out of the cell (Kapoor, Saigal and Elongavan, 2017).

Moreover, the inactivation of antibiotics by the enzymes also leads to antibiotic resistance in bacteria. These enzymes include β-lactamases, aminoglycoside-modifying enzymes and chloramphenicol acetyltransferase. β-lactamases are

classified into 4 classes which are Class A (penicillinase), Class B (metallo-β-lactamases), Class C (cephalosporinases) and Class D (oxacillin hydrolyzing enzymes) (Kapoor, Saigal and Elongavan, 2017). Bacteria such as *Enterobacteriaceae*, *P. aeruginosa* and Gram-negative bacteria are capable of producing one of these classes of β-lactamases, rendering the effectiveness of antibiotics. For aminoglycoside-modifying enzymes, there are several examples such as phosphoryl-transferases, nucleotidyl-transferases and adenylyl-transferases found in *S. aureus* and *Streptococcus pneumoniae*. These enzymes affect the affinity of aminoglycosides to bind on the 30S ribosomal subunit of bacteria, limiting its antimicrobial activity (Strateva and Yordanov, 2009; Kapoor, Saigal and Elongavan, 2017). Chloramphenicol acetyltransferase functions by acetylating the hydroxyl groups in chloramphenicol, leading to the modification of chloramphenicol structure and rendering its binding ability to ribosomal 50S subunits (Tolmasky, 2000).

Lastly, antibiotic resistance can be achieved through modification of the drug target. This mechanism is observed in vancomycin-resistant *Enterococci* and methicillin-resistant *S. aureus* which exhibit vancomycin resistance. This is due to the presence of *van* genes that induce changes in the structure of peptidoglycan in its dipeptide precursors. The modified peptidoglycan precursors result in the reduction of the binding ability of vancomycin to the bacterial membrane to pass through it (Beceiro, Tomás and Bou, 2013). Ribosomal mutations also impact the antibiotics that target ribosomal subunits in bacteria. It has been identified that the *erm* genes encoded for

methyltransferases alter the ribosomal target sites, blocking the binding of antibiotics to ribosomes and providing inducible resistance to the bacteria (Mancuso, et al., 2021).

2.2.2 Global-Verified Bacteria Acquiring Antibiotic Resistance

ESKAPE pathogens have been verified as antimicrobial-resistant bacteria that pose harm to human health and, worse still, fatality. The acronym ESKAPE refers to six multidrug-resistant bacteria including *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanni*, *Pseudomonas aeruginosa* and *Enterobacter* spp (Mulani, et al., 2019). These bacteria are also categorized as critical-priority bacteria by the World Health Organization (WHO) due to their resistance to multidrug and cause fatal infectious diseases such as bloodstream infections and pneumonia (Mancuso, et al., 2021).

2.3 Classification of Antibiotic Adjuvants

Antibiotic adjuvants are classified into Class I and Class II depending on the mode of action and target of action. Class I antibiotic adjuvants focus on combating the bacterial resistance mechanisms whereas Class II antibiotic adjuvants enhance antibiotic activity by modulating host defence mechanisms (Tyres and Wright, 2019; Oliveira, et al., 2020). Class I antibiotic adjuvants can be further divided into Class IA, which directly targets the resistance mechanisms of pathogens, and Class IB,

also known as passive resistance inhibitors, which indirectly inhibit the intrinsic resistance mechanisms of bacteria (Wright, 2016).

2.3.1 Class IA Adjuvants

According to Dhanda, Acharya and Haldar (2023), Class IA antibiotic adjuvants aim to inhibit bacterial enzymes and efflux pump systems. Beta-lactamase inhibitor is a successful example of this class. Beta-lactamase antibiotics such as penicillin and amoxicillin contain a beta-lactam ring, conferring the antibiotic activity against bacteria. However, certain bacteria can produce serine beta-lactamases and metallo-beta-lactamases (Tooke, et al., 2019). Serine beta-lactamases comprise an active site encoded by serine residue that can hydrolyse beta-lactamase antibiotics upon binding. The inactivation of beta-lactamase antibiotics by metallo-beta-lactamases involves the activation of a water molecule by the Zn^{2+} atoms on the active site of these enzymes which then bind to the antibiotics and hydrolyse them (Wright, 2016). These enzymes enable bacteria to modify the beta-lactam ring, reducing the activity of this antibiotic. To combat the resistance of the beta-lactamase-producing bacteria, pairing beta-lactamase inhibitors and beta-lactamase antibiotics has been initiated in clinical treatment (Idowu, et al., 2019). One example of this combination is Augmentin which pairs clavulanic acid (an adjuvant) and amoxicillin (an antibiotic), shown to be active against a wide range of microbes (González-Bello, et al., 2020; Kumar, et al., 2023). Clavulanic acid, a natural product isolated from *Streptomyces clavuligerus*, displays poor antibacterial activity but shows high inactivation activity of serine beta-lactamases. Nevertheless,

the effectiveness of clavulanic acid decreases as the bacteria evolve and produce extended-spectrum-beta-lactamases (ESBLs) over decades. New antibiotic adjuvants such as the combination of diazabicyclooctanones (DBOs) with ceftazidime, tazobactam with piperacillin and so on are developed to counteract the emergence of ESBLs. These combinations have been studied to be capable of inhibiting ESBLs and carbapenemases (Yahav, et al., 2020, Kumar, et al., 2023).

2.3.2 Class IB Adjuvants

The inhibitory targets of Class IB adjuvants include bacterial efflux pumps which function as cellular transport proteins to excrete substances such as antibiotics in the cell, reducing the intracellular antibiotics levels and diminishing their effectiveness, thereby conferring antibiotic resistance to the bacteria. Efflux transporters are classified into several superfamilies including ATP binding cassette (ABC), small multidrug resistance (SMR), major facilitator (MF), resistance nodulation and cell division (RND) as well as proteobacterial antimicrobial compound efflux (PACE) superfamilies (Huang, et al., 2022). Therefore, efflux pump inhibitors (EPIs) are developed to reduce the antibiotic resistance imparted by the efflux pump. Lomovskaya, et al. (2001) successfully discovered a compound, namely MC-207,110 [phenylalanyl arginyl β-napthylamide (PAβN)] as an EPI. It was noticed that PAβN potentially increase the antibiotic activity when paired with different antibiotics against *P. aeruginosa* strains.

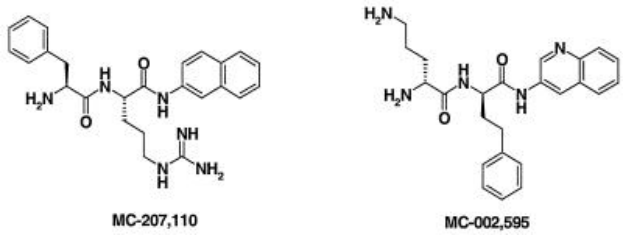
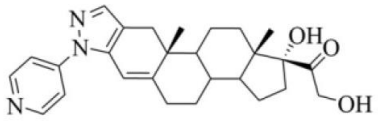


Figure 2.1: The chemical structure of EPIs studied including PAβN (Lomovskaya, et al., 2001).

Certain compounds in Class IB adjuvants are found to exhibit inhibitory effects on biofilms. Biofilms are produced by bacteria to allow survival in extreme environments by conferring antibiotic resistance and adaptability to different external stresses. Researchers have identified adjuvant molecules that act as antibiofilm agents to inhibit or destroy the biofilms. These adjuvant molecules include N-acetylcysteine, Tween 80, D-amino acid and DNase I (Kumar, et al., 2023). These adjuvants prevent biofilm formation by several methods such as preventing the attachment of bacteria to the surfaces and inhibiting biofilm maturation (Kumar, et al., 2023). A macromolecule namely QCybuAP has been studied and it exhibits activity in disrupting the maturation of biofilms of *E. coli* and *Acinetobacter baumanni* when paired with erythromycin. The bacteria level at the burn wound infected by *A. baumanni* is reduced noticeably when this combination is used (Uppu, et al., 2015; Dhanda, Acharya and Haldar, 2023).

Class IB adjuvants are also involved in inhibiting the functions of enzymes responsible for certain cellular processes. The adjuvant molecule involved is the

murgocil, a steroid-like core structure. Murgocil acts selectively by binding to the *S. aureus* MurG (SaMurG) enzyme through the formation of two hydrogen bonds between murgocil and *Sa*MurG. This binding action inhibits the conversion of lipid I to lipid II, which significantly reduces the synthesis of peptidoglycan that is essential for the formation of bacterial cell walls (Mann, et al., 2013). Apart from binding to *Sa*MurG, murgocil itself exhibited a certain degree of antibacterial activity against *S. aureus* (Dhanda, Archarya and Handlar, 2023).



Murgocil (44)

Figure 2.2: The chemical structure of murgocil (Dhanda, Archarya and Handlar, 2023).

2.3.3 Class II Adjuvants

Class II adjuvants are known as immune enhancers which enhance the host defense mechanisms. The research found that antimicrobial peptides function synergistically with antibiotics and potentiate the antibiotic activity in treating biofilms (Hancock, Nijnik and Philpott, 2012; Dhanda, Acharya and Haldar, 2023). For example, BAY 11-7082 which is an inhibitor of IkBα kinase heighten the macrophage activity in eradicating *Mycobacterium tuberculosis* by inactivating the nuclear factor-kappa B (Bai, et al., 2023, Kumar, et al., 2023). Streptazolin has also been discovered to exhibit enhancement of macrophage activity against *Streptococcus* mutans by stimulating the phosphoinositide 3-kinase pathway,

leading to the upregulation of nuclear factor-κB (Wright, 2016). Besides, another way of boosting the host defense mechanisms by the adjuvants is through modulating the reactive-oxygen species (ROS) and reactive-nitrogen species (RNS). These species are reactive radicals with the ability to damage cells, including bacteria, hence are used to treat wounds and food contamination by eliminating microbes (Li, et al., 2021; Kumar, et al., 2023). Dhanda, Archarya and Haldar (2023) reported that the EDC34 peptide demonstrated inhibitory activity against *E. coli* and *P. aeruginosa* when used with ceftazidime, an antibiotic under the cephalosporin class.

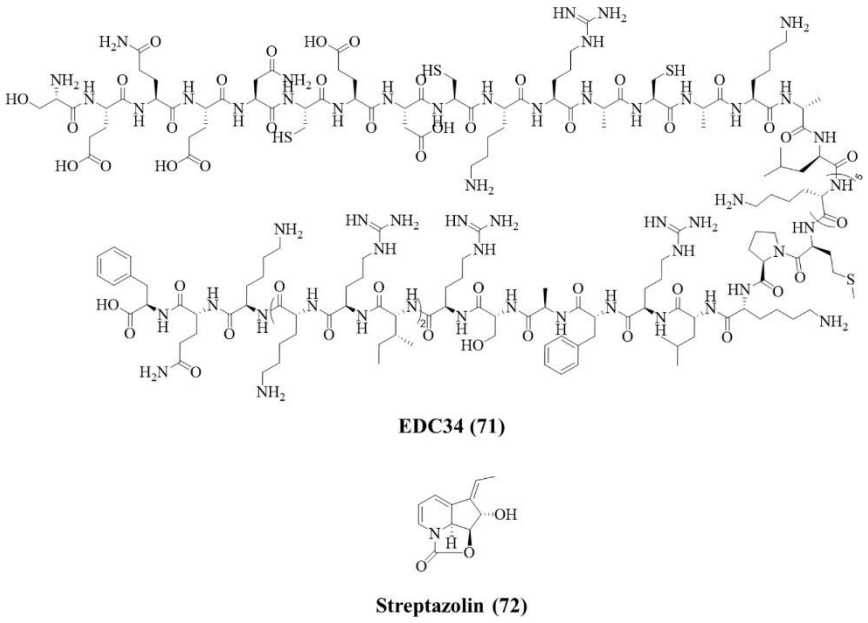


Figure 2.3: The adjuvants with the ability to modulate host response (Dhanda, Archaya and Haldar, 2023).

2.4 *N*-acylhydrazone (NAH)

2.4.1 Production of *N*-acylhydrazone (NAH)

According to Socea, et al. (2022), NAH is synthesized by condensing an aldehyde or ketone with a derivative of the class of hydrazides in the presence of an alcohol under reflux and acidic conditions. The condensation reaction between the quinoline ring and pyrazole scaffold in the acidic condition results in a yield of 82 – 99% of NAH compounds (Munir, et al., 2021).

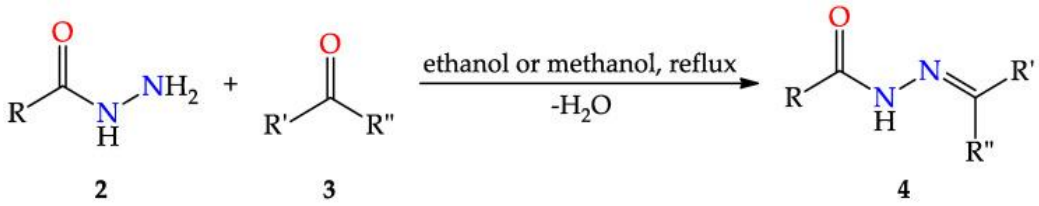


Figure 2.4: The general synthesis reaction of *N*-acylhydrazone (Socea, et al., 2022).

2.4.2 Biological Activity of *N*-acylhydrazone Derivatives

Due to the versatile moiety of NAH, different functional groups can be substituted to achieve different pharmacological and biological properties in the development of drugs. According to Biliz, et al. (2023), it is identified that NAH demonstrates a broad range of biological activities including antibacterial, antiviral and anticancer as shown in Figure 2.5. The chemical structure and the usage of approved NAH-related drugs such as nitrofurazone, nitrofurantoin and so on are shown in Table 2.1.

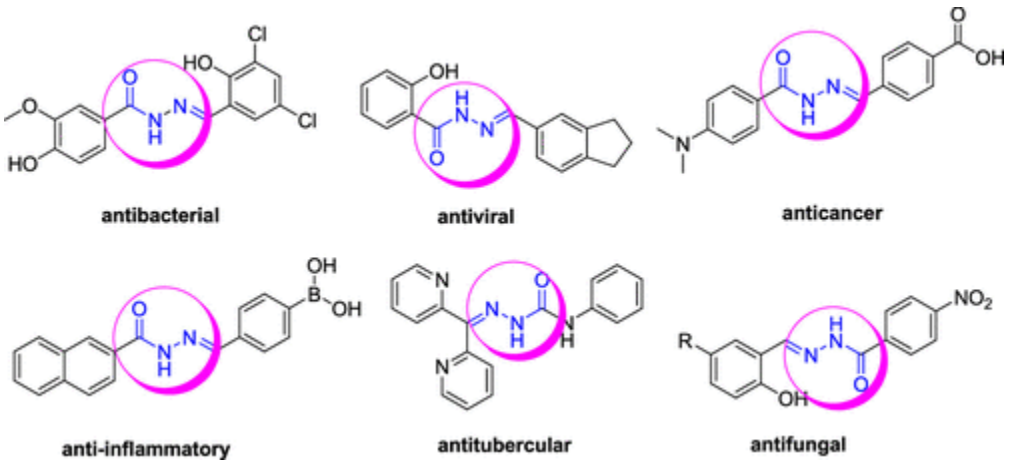


Figure 2.5: The biological activities of some NAH compounds (Biliz, et al., 2023).

Table 2.1: The chemical structure and application of approved NAH-based drugs.

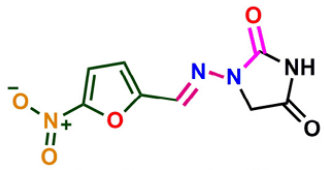
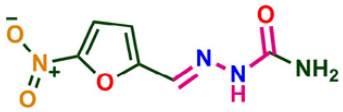
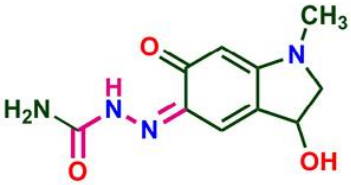
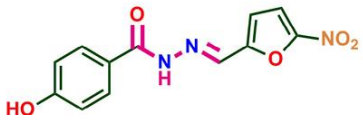
Drugs	Chemical structure	Usage	Reference
Nitrofurantoin	 **Figure 2.6**: The chemical structure of nitrofurantioin.	Oral antibacterial agent to treat genitourinary tract infections	Thota, et al., (2018)
Nitrofurazone	 **Figure 2.7**: The chemical structure of nitrofurazone.	Topical antibacterial agent	Thota, et al., (2018)
Carbazochrome	 **Figure 2.8**: The chemical structure of carbazochrome.	Hemostatic agent for capillary and parenchymal hemorrhage	Thota, et al., (2018)

Table 2.1 (continued): The chemical structure and application of approved NAH-based drugs.

Drugs	Chemical structure	Usage	Reference
Nifuroxazide	 Figure 2.9: The chemical structure of nifuroxazide.	Treat colitis and diarrhea in adults	Thota, et al. (2018)

2.4.3 Antibacterial Activity Possessed by *N*-acylhydrazone Derivatives

As the antibacterial activity of NAH derivative compounds is the interest of this project, the studies of the antibacterial activity of NAH derivatives are compared. It is identified that NAH derivative compounds (4a-q) generated from dehydroabietic acid exhibited antibacterial activity through the determination of minimum inhibitory concentration (MIC) by a modified microdilution method (Gu, et al., 2012). The MIC values exhibited by the NAH derivative compounds range from 1.9 to more than 100 μg/mL against *S. aureus*, *B. subtilis*, *E, coli* and *P. fluorescens*. Besides, it was observed that the NAH derivative compounds generated were relatively more effective against Gram-positive bacteria than Gram-negative bacteria. The observation was suggested by the substitution of different functional groups on aromatic rings having impacts on the antibacterial property of the synthesized compounds in the structure-activity relationship studies (Gu, et al., 2012).

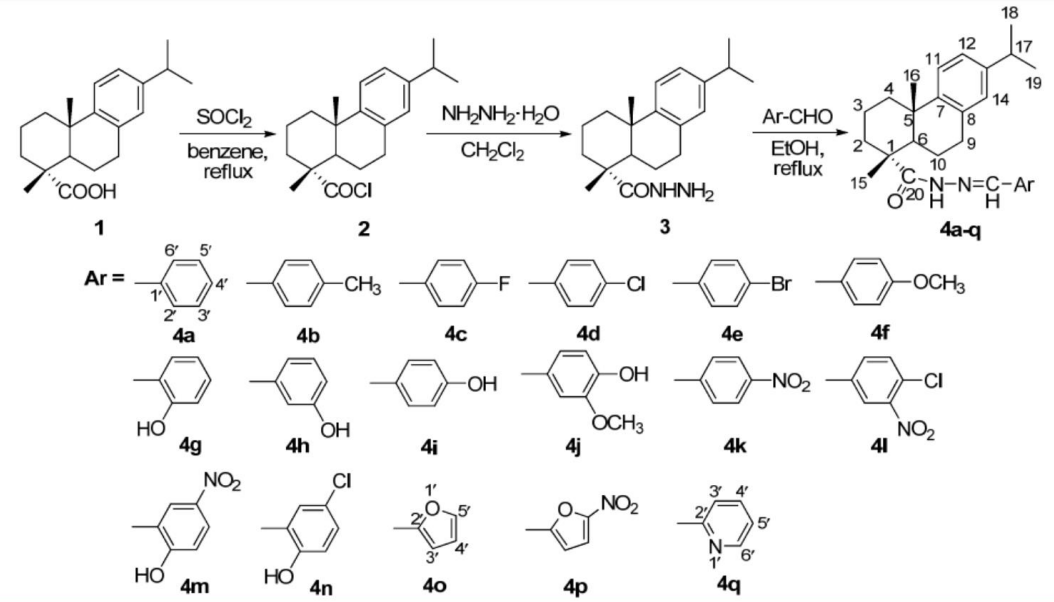


Figure 2.6: The pathway of synthesizing hydrazone derivatives (4a–q) from dehydroabietic acid (Gu, et al., 2012).

Moving on to another study by Aarjane, et al. (2020), the *in vitro* antibacterial activity of NAH derivative compounds (3a-k) synthesized from acridone against Gram-negative bacteria of *P. putida*, *K. pneumoniae* and *E.coli* as well as Gram-positive bacteria of *S. aureus* was tested using MIC determination. The MIC values obtained were ranging from 19.61 to 156.31 µg/mL. The results showed a similar trend with Gu, et al. (2012) results in which the compounds were more active against Gram-positive bacteria than Gram-negative bacteria, with Compound 3a as the most potent (19.61 µg/mL) compound against *S. aureus*. Apart from antibacterial studies, they also provided in-silico studies of these synthesized compounds against *S. aureus* and *P. putida*.

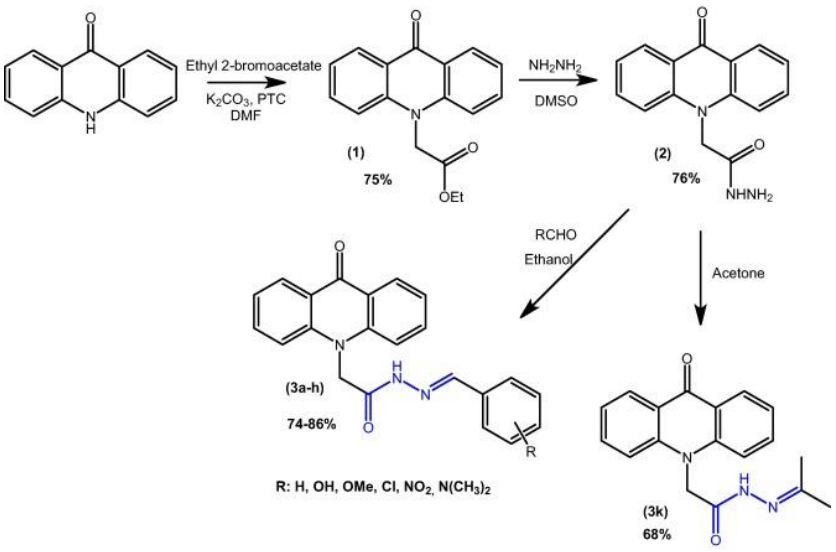


Figure 2.7: The pathway of synthesizing novel *N*-acylhydrazone derivatives from acridone (3a-k) (Aarjane, et al., 2020).

The antibacterial activities of ferrocenyl-*N*-acylhydrazones were studied by dos Santos Filho and de Souza Castro (2022) through MIC determination. The antibacterial studies displayed MIC values ranging from 31.25 µg/mL to no inhibitory effects against *B. subtilis*, *S. aureus*, *E. coli* and *K. pneumoniae*. A similar trend of the compounds being more effective against Gram-positive bacteria than Gram-negative bacteria was also noted, with Compound SintMed77 as the most active compound against *B. subtilis*, achieving a MIC value of 31.25 µg/mL. Most of the compounds are inactive against Gram-negative bacteria, particularly *E. coli* and *K. pneumoniae*.

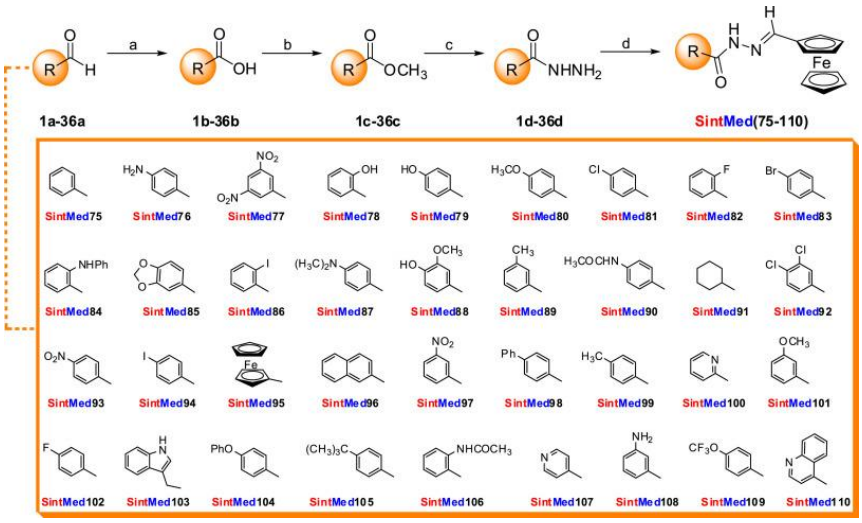


Figure 2.8: Synthetic route for the ferrocenyl *N*-acylhydrazone derivatives (dos Santos Filho and de Souza Castro, 2022).

CHAPTER 3

METHODOLOGY

3.1 Experimental Flowchart

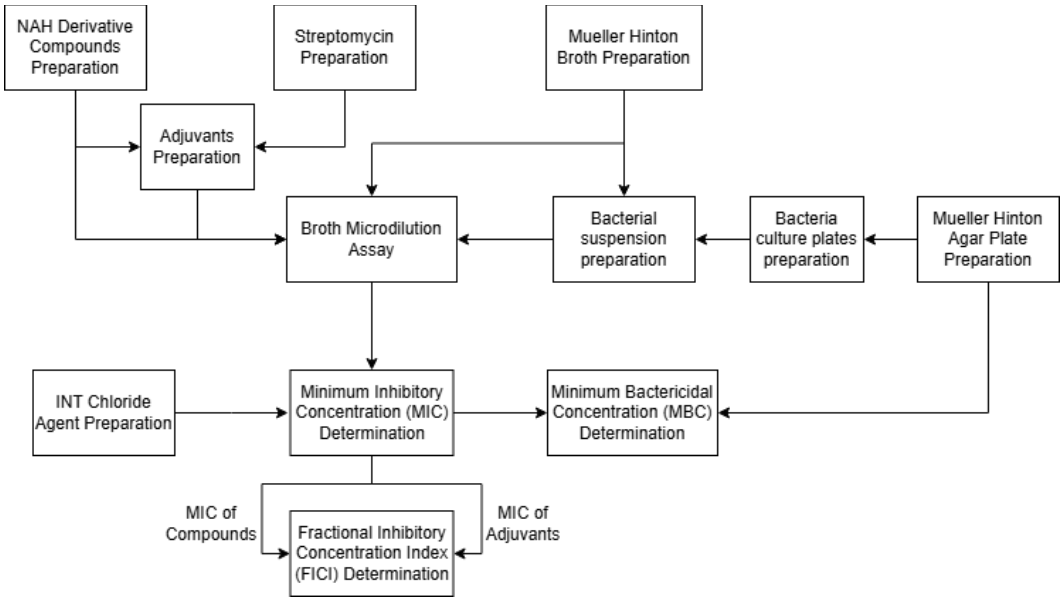


Figure 3.1: The overview of the experiment.

3.2 Materials, Apparatus and Equipment Used

3.2.1 *N*-acylhydrazone (NAH) Derivative Compounds

The NAH derivative compounds with aromatic substitution of different functional groups at ortho- and para-directors were provided by Dr Teo Kah Cheng, an

assistant professor from the Department of Agricultural and Food Science. The structure of the NAH derivative compounds was elucidated using nuclear magnetic resonance (NMR) spectroscopic analysis by Dr Sim Kooi Mow, an associate professor from the Department of Chemical Science. Both professors were from the Faculty of Science of Universiti Tunku Abdul Rahman (UTAR), Kampar. The core structure of the NAH derivatives and their aromatic substitution were illustrated in Figure 3.2 and Table 3.1 respectively.

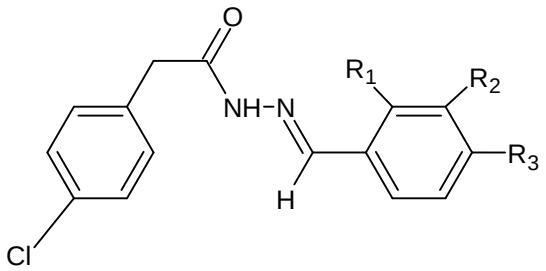


Figure 3.2: The chemical core structure of the 5-Chloro substituted *N*-acylhydrazone (NAH) derivative compounds with functional R groups.

Table 3.1: The NAH derivative compounds with different R substituents at respective positions.

NAH derivative compounds	R substituents at the respective position	R_1	R_2	R_3
1	2-Br	Br	H	H
2	2-Cl	Cl	H	H
3	2-F	F	H	H
4	H	H	H	H

Table 3.1 (continued): The NAH derivative compounds with different R substituents at respective positions.

NAH derivative compounds	R substituents at the respective position	R_1	R_2	R_3
5	2-NO_2	NO_2	H	H
6	2-OCH_3	OCH_3	H	H
7	2,4-Cl_2	Cl	H	Cl

3.2.2 Bacterial Strains

The bacterial strains tested were categorized and listed in Table 3.2.

Table 3.2: The list of bacterial strains used in the project.

Types of Bacteria	Bacterial Strains
Gram-positive bacteria	*Bacillus cereus* (ATCC 13061)
	Bacillus subtilis subsp. *spizizenni* (ATCC 6633)
	Staphylococcus aureus (ATCC 6538)
Gram-negative bacteria	*Escherichia coli* (ATCC 25922)
	Pseudomonas aeruginosa (ATCC 27853)
	Salmonella Typhimurium (ATCC 14028)
Methicillin-resistant bacteria	MRSA (ATCC 33591)
	MRSA (ATCC 43300)

MRSA: Methicillin-resistant *Staphylococcus aureus*

3.2.3 Chemical Reagents and Media

The chemical reagents and media used in the project were listed in Table 3.3 along with their manufacturer and country of origin.

Table 3.3: The list of chemical reagents and media used in this project including their manufacturer and country of origin.

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

3.2.4 Apparatus, Consumables and Glassware

The apparatus, consumables and glassware used in the project were tabulated in Table 3.4 along with their manufacturer and country of origin.

Table 3.4: The list of apparatus, consumables and glassware used, their manufacturer, and country of origin.

Apparatus/Consumables/Glassware	Manufacturer	Country of Origin
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 Scientific	United States
Micropipette tips (100 – 1000 µl)	Gilson	France
Micropipette tips (200 µl)	Gilson	France

Table 3.4 (continued): The list of apparatus, consumables and glassware used, their manufacturer, and country of origin.

Apparatus/Consumables/Glassware	Manufacturer	Country of Origin
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
Tissue culture plate 96 well	Premier Diagnostic	Malaysia
Weighing boat	-	-

3.2.5 Laboratory Instruments

The laboratory instruments used in the project were listed in Table 3.5 along with their manufacture and country of origin.

Table 3.5: The manufacturer and country of origin of the laboratory instruments used in the project.

Laboratory Instruments	Manufacturer	Country of Origin
Analytical balance	Mettler-Toledo	United States
Autoclave machine	Hirayama	Japan

Table 3.5 (continued): The manufacturer and country of origin of the laboratory instruments used in the project.

Laboratory Instruments	Manufacturer	Country of Origin
Drying oven	Binder	Germany
Freezer (-20˚C)	Liebherr	United States
Incubator	Memmert	Germany
Laminar air flow cabinet	ESCO	Singapore
Microbalance	Mettler-Toledo	United States
Micropipette (100 – 1000 µl)	Rainin	United States
Micropipette (20 – 200 µl)	Rainin	United States
Micropipette (2 - 20 µl)	Rainin	United States
Refrigerator (4˚C)	KIM	Malaysia
Ultra-low temperature freezer (-80˚C)	Eppendorf	Germany
UV-Vis Spectrophotometer	Thermo Fisher Scientific	United States
Vortex mixer	Scientific Industries	United States

3.3 Experimental Procedures

3.3.1 Preparation Steps of *N*-acylhydrazone (NAH) Derivative Compounds

Firstly, 3 mg of the NAH derivative compound (2-Br) was measured using a micro spatula and microbalance. The weighted compound was transferred into a sterile

glass sample vial tube. The weighing steps were repeated for the remaining 6 compounds. Next, 3 ml of dimethyl sulfoxide (DMSO) was added to each vial tube with respective compounds using a micropipette. The vial tubes were vortexed to dissolve the compounds. The vial tubes containing the compounds were stored at room temperature for further analysis.

3.3.2 Preparation Steps of Mueller-Hinton (MH) Agar and MH Broth

Firstly, 15.2 g of MH agar powder was weighed and added to a 500 ml Schott bottle. Next, 400 ml of distilled water was added to the Schott bottle containing the MH agar powder. The Schott bottle was heated mildly, stirred using a laboratory spatula to dissolve the powder, and autoclaved. The sterile MH agar solution was cooled to about 45˚C before pouring the solution onto the Petri dishes in the laminar flow hood. The Petri dishes were cooled to room temperature for solidification. The solidified agar plates were stored in the refrigerator at 4˚C in a plastic package.

To prepare MH broth, 4.2 g of MH broth powder was weighed into a 250 ml Schott bottle. Next, 200 ml of distilled water was added to the Schott bottle and shaken to dissolve the broth powder. The Schott bottle was autoclaved. The Schott bottle containing sterile MH broth was sealed with a parafilm after cooling and was stored in the refrigerator at 4˚C.

3.3.3 Preparation Steps of Antibiotics

Firstly, 1 mg of streptomycin powder was added to a sterile centrifuge tube after weighing with a microbalance. Next, 10 mL of sterile distilled water was added to the centrifuge tube and was vortexed to dissolve the powder (Barnes, et al., 2023). A streptomycin solution with a concentration of 100 µg/mL was obtained. The weighing and dissolving steps were repeated for chloramphenicol and ciprofloxacin (European Committee for Antimicrobial Susceptibility Testing, 2003).

To prepare 10 mL of 25 µg/mL chloramphenicol solution, 2.5 mL of 100 µg/mL chloramphenicol solution was transferred to a sterile centrifuge tube using a micropipette and diluted with 7.5 mL of sterile distilled water. To prepare 10 ml of 6.25 µg/mL ciprofloxacin solution, 0.625 mL of 100 µg/mL ciprofloxacin solution was transferred to a sterile centrifuge tube. Then, 9.375 mL of sterile distilled water was added to the centrifuge tube to dilute the ciprofloxacin solution. The volume required to dilute the antibiotics solution was calculated using:

$$M_1V_1 = M_2V_2$$

M_1 = original concentration of antibiotics (µg/mL)
V_1 = volume to the original concentration of antibiotics (mL)
M_2 = new concentration of antibiotics (µg/mL)
V_2 = volume of the new concentration of antibiotics (mL)

3.3.4 Preparation Steps of Adjuvant Solution

Firstly, 3 mL of the first dissolved NAH derivative compound (2-Br) was aliquoted into a sterile glass sample vial labelled. Next, 3 mL of 100 µg/mL streptomycin solution was transferred into the glass sample vial to produce streptomycin-adjuvant solutions. These steps were repeated for the remaining NAH derivative compounds. The streptomycin solution was replaced with 25 µg/mL chloramphenicol and 6.25 µg/mL ciprofloxacin to produce chloramphenicol-adjuvant and ciprofloxacin-adjuvant solutions. The adjuvant solutions were vortexed and stored in the refrigerator at 4˚C.

3.3.5 Preparation Steps of Iodonitrotetrazolium (INT) Chloride Dye

Firstly, 4 mg of INT chloride powder was weighed using a microbalance into a sterile centrifuge tube wrapped with aluminium foil. Next, 3 drops of absolute ethanol were added into the sterile centrifuge tube containing INT chloride powder. Then, 10 mL of sterile distilled water was added to the centrifuge tube to produce an INT chloride dye with a 0.4 mg/mL concentration. The dissolved INT chloride dye was stored in the refrigerator at 4˚C.

3.3.6 Preparation Steps of Bacterial Culture Plate

Aseptic techniques were applied throughout the preparation process. The glycerol stocks of the 8 bacterial strains were taken out of the ultra-low temperature freezer. The glycerol stocks were defrosted and shaken. The first bacterial strain from its

glycerol stock was streaked on the MH agar plate prepared using an inoculating loop. The streaking step was repeated for the remaining 7 bacterial strains. The bacterial culture plates were sealed with parafilm and incubated upside down at 37˚C for 24 hours. After incubation, the bacterial culture plates were stored in the refrigerator at 4˚C. Before usage, the refrigerated bacterial culture plates were incubated at 37˚C for 24 hours to reactivate the bacteria.

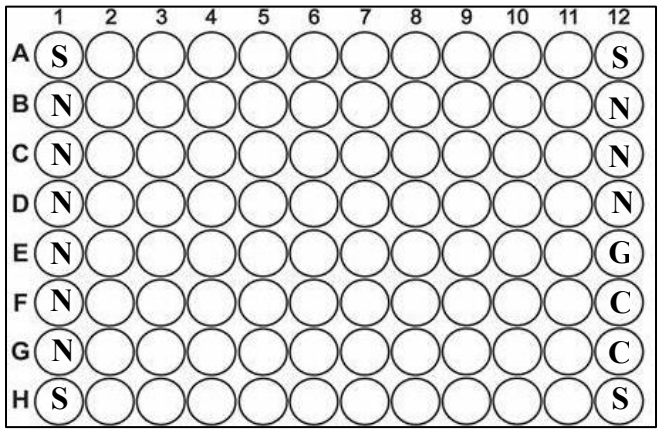
3.2.7 Preparation of Bacteria Suspension

Aseptic techniques were applied throughout the preparation process. Firstly, 5 mL of MH broth prepared was added to a sterile 15 mL centrifuge tube. Using an inoculating loop, the bacterial colonies were inoculated into the centrifuge tube containing the MH broth and then vortexed. Next, 1 mL bacterial suspension was pipetted into a cuvette. A cuvette containing 1 mL of sterile MH broth was used as the “blank”. The absorbance of the bacterial suspension was measured at 625 nm using a UV-Vis Spectrophotometer. The targeted optical density (OD) value to be obtained was in the range of 0.08A to 0.10A to represent the colony-forming unit of 1×10^8 CFU/mL. Bacteria colonies were added if the absorbance was lower than 0.08A while sterile MH broth was added if the absorbance was higher than 0.10A. Once the target OD was achieved, 50 µL of the bacterial suspension was aliquoted into a sterile centrifuge tube containing 4950 µL MH broth and then vortexed. A bacterial suspension with a final concentration of 1×10^6 CFU/mL was obtained (Clinical and Laboratory Standards Institute, 2017).

3.2.8 Broth Microdilution Assay

The placement of solutions in 96-well plates is illustrated in Figures 3.3 and 3.4 (Clinical and Laboratory Standards Institute, 2017). Firstly, 100 µL of MH broth was added to the wells of the four corners labelled as "S" to prepare sterility controls. DMSO control at 12.5% was prepared by adding 12.5 µL DMSO solution and 37.5 µL MH broth into the well at 12F. Then, 50 µL of the solution at 12F was transferred into 12G to prepare 6.25% DMSO control, mixed, and 50 µL of the mixed solution at 12G was discarded. Then, 50 µL of sterile MH broth was added to each remaining well. Fifty µL dissolved compounds 1 to 8 were added to their respective wells labelled as "N" to prepare negative controls. After that, 50 µL compound 1 (2-Br) was transferred into well 2A and mixed. Fifty µL of the solution at 2A was transferred into the subsequent well 2B and was mixed again. This step was repeated continuously down the wells from 2B to 2C, 2C to 2D until 2H in the same manner (Nigussie, et al., 2021). Fifty µL of the solution at 2H was discarded (Eloff, 1988). The steps were repeated for the remaining 6 compounds and antibiotic-adjuvant combinations into their respective column of wells following Figure 3.3. The steps were repeated for antibiotics as positive controls according to Figure 3.4. Then, 50 µL of bacterial suspension was added to every well that contained solutions except the sterility controls at four corners. The wells added with bacterial suspension produced a bacterial concentration of 5×10^5 CFU/mL (European Committee for Antimicrobial Susceptibility Testing, 2003). The 96-well plate was sealed with parafilm and incubated at 37˚C for 24 hours (Mogana, et al.,

2020). Each 96-well plate was used for a particular bacterial strain. The steps were repeated for the remaining bacterial strains. The concentration of the NAH derivative compounds, antibiotics and antibiotic-adjuvant combinations in respective wells was tabulated in Tables 3.6 and 3.7.



S: Sterility Control (100 µL MH broth)

N: Negative control (50 µL MH broth + 50 µL NAH compound)

G: Growth control (50 µL MH broth + 50 µL bacterial suspension)

C: DMSO control at 12.5% and 6.25%

Column 2: Compound 1 (2-Br) and its antibiotic-adjuvants **N**: 1B

Column 3: Compound 2 (2-Cl) and its antibiotic-adjuvants **N**: 1C

Column 4: Compound 3 (2-F) and its antibiotic-adjuvants **N**: 1D

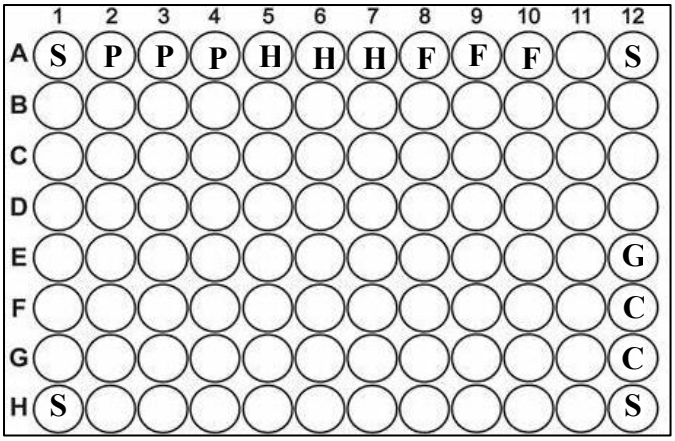
Column 5: Compound 4 (H) and its antibiotic-adjuvants **N**: 1E

Column 6: Compound 5 (2-NO_2) and its antibiotic-adjuvants **N**: 1F

Column 7: Compound 6 (2-OCH_3) and its antibiotic-adjuvants **N**: 1G

Column 8: Compound 7 (2,4-Cl_2) and its antibiotic-adjuvants **N**: 12B

Figure 3.3: The arrangement of solutions in the 96-well plate for NAH compounds and antibiotic-adjuvant combinations.



P: Streptomycin

H: Chloramphenicol

F: Ciprofloxacin

Figure 3.4: The arrangement of solution in the 96-well plate for positive controls.

Table 3.6: The concentration of NAH derivative compounds and antibiotics as positive controls in the 96-well plate.

Row	Concentration of NAH derivative compounds (µg/mL)	Concentration of streptomycin (µg/mL)	Concentration of chloramphenicol (µg/mL)	Concentration of ciprofloxacin (µg/mL)
A	250.00	25.00	6.25	1.56
B	125.00	12.50	3.13	0.78
C	62.50	6.25	1.56	0.39
D	31.25	3.13	0.78	0.20
E	15.63	1.56	0.39	0.10
F	7.81	0.78	0.20	0.05
G	3.91	0.39	0.10	0.03
H	1.95	0.20	0.05	0.02
I	0.98	0.10	0.03	0.01

Table 3.7: The concentration of different adjuvants in the 96-well plate.

Row	Adjuvant I		Adjuvant 2		Adjuvant 3	
	NAH	**Streptomycin**	**NAH**	**Chloramphenicol**	**NAH**	**Ciprofloxacin**
A	125.00	12.50	125.00	3.13	125.00	0.78
B	62.50	6.25	62.50	1.56	62.50	0.39
C	31.25	3.13	31.25	0.78	31.25	0.20
D	15.63	1.56	15.63	0.39	15.63	0.10
E	7.81	0.78	7.81	0.20	7.81	0.05
F	3.91	0.39	3.91	0.10	3.91	0.03
G	1.95	0.20	1.95	0.05	1.95	0.02
H	0.98	0.10	0.98	0.03	0.98	0.01
I	0.49	0.05	0.49	0.02	0.49	0.005

3.2.9 Minimum Inhibitory Concentration (MIC) Assay

After incubation for 24 hours, 20 µL INT chloride solution was added to each well. The 96-well plate was sealed with parafilm and incubated at 37˚C for 20 minutes. The bacterial growth in each well was observed after incubation. No colour changes indicated no bacterial growth while red colour showed bacterial growth (Perumal, et al., 2012). The first well showing no colour change above the coloured well was recorded as the MIC of the compounds, antibiotics or antibiotic-adjuvant solutions (Balouiri, Sadiki and Ibnsouda, 2016). The results were recorded, and a photo of the plate was taken.

3.2.10 Minimum Bactericidal Concentration (MBC) Assay

A Mueller-Hinton (MH) agar plate was labelled into 3 sections. Then, 10 µL of the solution from the well identified as MIC was aliquoted onto one section of the agar plate. The inoculum was spread using a sterile cotton swab. This step was repeated for the two wells above the MIC well (Mogana, et al., 2020). For example, if 4F

was identified as MIC well, solutions in 4E and 4D were inoculated and spread onto the other 2 sections labelled respectively. The plates were sealed with parafilm and incubated at 37˚C for 24 hours. The bacterial colony number was counted after incubation. The section containing less than 5 bacterial colonies was considered MBC (Serafim, et al., 2019). The results were recorded and a photo of the plate was taken.

3.2.11 Fractional Inhibitory Concentration (FIC) Index

The FIC index for the interaction between the antibiotics and NAH derivative compounds was calculated using the formula below (Meletiadis, et al., 2009):

$$FIC\ Index = \frac{MIC\ of\ compound\ in\ combination}{MIC\ of\ compound\ in\ individual} + \frac{MIC\ of\ antibiotic\ in\ combination}{MIC\ of\ antiobiotic\ in\ individual}$$

CHAPTER 4

RESULTS

4.1 Minimum Inhibitory Concentration (MIC)

MIC refers to the minimal level of an antimicrobial agent expressed in µg/mL to inhibit microbial growth. No observable colony can be seen after incubating overnight at a controlled temperature (Kowalska-Krochmal and Dudek-Wicher, 2021). Kalli, et al., (2021) categorized the antibacterial activity of the compounds into 3 with the MIC values which were active compounds (MIC lower than or equal to 25 µg/mL), moderately active (MIC fell between 25 to 100 µg/mL) and inactive (MIC equal to or higher than 100 µg/mL). Statistical analysis was not performed due to poor MIC values which indicated low antibacterial activity exhibited by the compounds (Almajan, et al., 2010).

4.1.1 NAH Derivative Compounds

The antibacterial activity of the NAH derivative compounds 1 to 7 with different functional groups through aromatic substitution at ortho- and para-directors, in terms of MIC values against 8 selected bacterial strains were tabulated in Table 4.1.

It was observed that Compound 1 with 2-Br aromatic substitution, Compound 4 with H aromatic substitution, Compound 6 with 2-OCH_3 aromatic substitution and

Compound 7 with 2,4-Cl_2 aromatic substitution displayed moderate antibacterial activity with MIC value of 62.50 µg/mL against *S. aureus* (ATCC 6538). However, Compounds 1, 4, 6 and 7 were inactive against the other 7 selected bacterial strains by exhibiting antibacterial activity in MIC values ranging from 125 to 250 µg/mL respectively. The remaining Compounds 2, 3 and 5 were interpreted as inactive compounds and had low antibacterial activity against 8 selected bacterial strains with MIC values ranging from 125 to 250 µg/mL, which reached the MIC breakpoint for inactive compounds.

Table 4.1: The Minimum Inhibitory Concentration (MIC) of *N*-acylhydrazone derivative compounds against 8 selected bacterial stains.

Bacterial Strains	**MIC (µg/mL)**						
	NAH Derivative Compounds						
	1	**2**	**3**	**4**	**5**	**6**	**7**
	2-Br	**2-Cl**	**2-F**	**H**	**2-NO_2**	**2-OCH_3**	**2,4-Cl_2**
***Bacillus cereus* (ATCC 13061)**	125.00	125.00	125.00	125.00	125.00	125.00	125.00
***Bacillus subtilis* subsp. *spizizenni* (ATCC 6633)**	250.00	250.00	125.00	250.00	125.00	250.00	250.00
***Staphylococcus aureus* (ATCC 6538)**	62.50	125.00	125.00	62.50	125.00	62.50	62.50
***Escherichia coli* (ATCC 25922)**	125.00	125.00	125.00	125.00	125.00	125.00	125.00
***Pseudomonas aeruginosa* (ATCC 27853)**	125.00	125.00	125.00	125.00	125.00	125.00	125.00
***Salmonella Typhimurium* (ATCC 14028)**	125.00	125.00	125.00	125.00	125.00	125.00	125.00
MRSA (ATCC 33591)	250.00	250.00	250.00	250.00	250.00	250.00	250.00
MRSA (ATCC 43300)	250.00	250.00	250.00	250.00	250.00	250.00	250.00

MRSA: Methicillin-resistant *Staphylococcus aureus*

4.1.2 NAH Derivative Compounds in Combination with Streptomycin as Adjuvants

Table 4.2 demonstrated the antibacterial activity of NAH derivative Compounds 1 to 7 in combination with streptomycin as adjuvants in terms of MIC values against the selected bacterial strains.

It was noticed that Compounds 1 to 7 possessed stronger antibacterial activity against 7 bacterial strains with MIC values ranging from 3.91 to 125 µg/mL after combined with streptomycin as streptomycin-adjuvants. Streptomycin-adjuvants 1 to 7 displayed MIC values ranging from 3.91 to 31.25 µg/mL against Gram-positive bacteria including *B. cereus* (ATCC13061*), B. subtilis* subsp. *spizizenni* (ATCC 6633) and *S. aureus* (ATCC 6538), showing high to moderate antibacterial activity. Meanwhile, the MIC values ranging from 31.25 to 125 µg/mL demonstrated that Compounds 1 to 7 in combination with streptomycin were considered moderately active to inactive against Gram-negative bacteria inclusive of *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853) and *S.* Typhimurium (ATCC 14028). The MIC values of Compounds 1 to 7 against *S.* Typhimurium (ATCC 14028) remained at 125 µg/mL upon the combination with streptomycin, suggesting that no effect on the antibacterial activity. Next, streptomycin-adjuvants 1 to 7 showed moderate to low antibacterial activity from the MIC values of 62.5 to 125 µg/mL against Methicillin-resistant *S. aureus* (ATCC 33591) and Methicillin-resistant *S. aureus* (ATCC 43300).

Table 4.2: The Minimum Inhibitory Concentration of adjuvants using streptomycin against 8 selected bacterial strains.

Bacterial strains	**MIC (µg/mL)**														
	NAH derivative compounds in combination with streptomycin														STR
	1		2		3		4		5		6		7		
	2-Br		2-Cl		2-F		H		2-NO_2		2-OCH_3		2, 4-Cl_2		
	NAH	STR	NAH	STR	NAH	STR	NAH	STR	NAH	STR	NAH	STR	NAH	STR	
***Bacillus cereus* (ATCC 13061)**	31.25	3.13	15.63	1.56	31.25	3.13	15.63	1.56	15.63	1.56	15.63	1.56	15.63	1.56	6.25
***Bacillus subtilis* subsp. *spizizenni* (ATCC 6633)**	7.81	0.78	7.81	0.78	7.81	0.78	15.63	1.56	3.91	0.39	7.81	0.78	7.81	0.78	0.78
***Staphylococcus aureus* (ATCC 6538)**	31.25	3.13	31.25	3.13	15.63	1.56	15.63	1.56	31.25	3.13	31.25	3.13	31.25	3.13	25.00
***Escherichia 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	6.25
***Pseudomonas aeruginosa* (ATCC 27853)**	31.25	100.00	31.25	100.00	31.25	100.00	31.25	100.00	31.25	100.00	31.25	100.00	31.25	100.00	12.50
***Salmonella* Typhimurium (ATCC 14028)**	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	12.50
MRSA (ATCC 33591)	125.00	400.00	125.00	400.00	125.00	400.00	125.00	400.00	125.00	400.00	125.00	400.00	125.00	400.00	800.00
MRSA (ATCC 43300)	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	25.00

MRSA: Methicillin-resistant *Staphylococcus aureus*

STR: Streptomycin

4.1.3 NAH Derivative Compounds in Combination with Chloramphenicol as Adjuvants

Table 4.3 demonstrated the antibacterial activity of NAH derivative Compounds 1 to 7 in combination with chloramphenicol as adjuvants in terms of MIC values against the selected bacterial strains.

Upon combining with chloramphenicol, 7 chloramphenicol-adjuvants exhibited moderate to low antibacterial potential with MIC values ranging from 31.25 to 125 µg/mL. Chloramphenicol-adjuvants 1 to 7 possessed moderate to low antibacterial activity against Gram-positive bacteria including *B. cereus* (ATCC13061*), B. subtilis* subsp. *spizizenni* (ATCC 6633) and *S. aureus* (ATCC 6538) with MIC values ranging from 31.25 to 125 µg/mL. Furthermore, Compounds 1 to 7 as chloramphenicol-adjuvants showed moderate antibacterial activity against Gram-negative bacteria which were *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853) and *S.* Typhimurium (ATCC 14028) as indicated by MIC values of 31.25 to 62.5 µg/mL. The MIC value of 125 µg/mL displayed by Compounds 1 to 7 in combination with chloramphenicol against Methicillin-resistant *S. aureus* (ATCC 33591) and Methicillin-resistant *S. aureus* (ATCC 43300) suggested a low antibacterial potential against these resistant bacterial strains.

Table 4.3: The Minimum Inhibitory Concentration of adjuvants using chloramphenicol against 8 selected bacterial strains.

Bacterial strains	**MIC (µg/mL)**														
	NAH derivative compounds in combination with chloramphenicol														CHL
	1		2		3		4		5		6		7		
	2-Br		2-Cl		2-F		H		2-NO_2		2-OCH_3		2, 4-Cl_2		
	NAH	CHL	NAH	CHL	NAH	CHL	NAH	CHL	NAH	CHL	NAH	CHL	NAH	CHL	
***Bacillus cereus* (ATCC 13061)**	31.25	0.78	31.25	0.78	62.50	1.56	31.25	0.78	31.25	0.78	31.25	0.78	31.25	0.78	1.56
***Bacillus subtilis* subsp. *spizizenni* (ATCC 6633)**	62.50	1.56	62.50	1.56	125.0	3.13	62.50	1.56	62.50	1.56	62.50	1.56	62.50	1.56	3.13
***Staphylococcus aureus* (ATCC 6538)**	62.50	1.56	62.50	1.56	62.50	1.56	62.50	1.56	62.50	1.56	62.50	1.56	31.25	0.78	6.25
***Escherichia 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	1.56
***Pseudomonas aeruginosa* (ATCC 27853)**	31.25	6.25	31.25	6.25	62.50	12.50	31.25	6.25	31.25	6.25	31.25	6.25	31.25	6.25	1.56
***Salmonella* Typhimurium (ATCC 14028)**	31.25	0.78	31.25	0.78	31.25	0.78	31.25	0.78	31.25	0.78	31.25	0.78	31.25	0.78	3.13
MRSA (ATCC 33591)	125.00	25.00	125.00	25.00	125.00	25.00	125.00	25.00	125.00	25.00	125.00	25.00	125.00	25.00	50.00
MRSA (ATCC 43300)	125.00	3.13	125.00	3.13	125.00	3.13	125.00	3.13	125.00	3.13	125.00	3.13	125.00	3.13	1.56

MRSA: Methicillin-resistant *Staphylococcus aureus*

CHL: Chloramphenicol

4.1.4 NAH Derivative Compounds in Combination with Ciprofloxacin as Adjuvants

Table 4.4 demonstrated the antibacterial activity of NAH derivative Compounds 1 to 7 in combination with ciprofloxacin as adjuvants in terms of MIC values against the selected bacterial strains.

It was evident that the 7 compounds generally demonstrated high to moderate antibacterial effects upon combining with ciprofloxacin, indicated by MIC values ranging from 0.98 to 31.25 μg/mL. From the MIC values of 3.91 to 15.63 μg/mL, ciprofloxacin-adjuvants 1 to 7 displayed highly active antibacterial activity against Gram-positive bacteria comprising of *B. cereus* (ATCC13061), *B. subtilis* subsp. *spizizenni* (ATCC 6633) and *S. aureus* (ATCC 6538). Moreover, Compounds 1 to 7 combined with ciprofloxacin obtained MIC values of 0.98 to 15.63 μg/mL against Gram-negative bacteria including *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853) and *S.* Typhimurium (ATCC 14028) which also suggested a high antibacterial potential. On the other hand, ciprofloxacin-adjuvants 1 to 7 exhibited moderate antibacterial activity against Methicillin-resistant *S. aureus* (ATCC 33591) and Methicillin-resistant *S. aureus* (ATCC 43300) with MIC value of 31.25 to 62.5 μg/mL. It can be observed that ciprofloxacin-adjuvants were more effective against these resistant bacterial strains compared to streptomycin and chloramphenicol.

Table 4.4: The Minimum Inhibitory Concentration of adjuvants using ciprofloxacin against 8 selected bacterial strains.

Bacterial strains	**MIC (µg/mL)**														
	NAH derivative compounds in combination with ciprofloxacin														CIP
	1		2		3		4		5		6		7		
	2-Br		2-Cl		2-F		H		2-NO_2		2-OCH_3		2, 4-Cl_2		
	NAH	CIP	NAH	CIP	NAH	CIP	NAH	CIP	NAH	CIP	NAH	CIP	NAH	CIP	
***Bacillus cereus* (ATCC 13061)**	15.63	0.10	7.81	0.05	15.63	0.10	7.81	0.05	7.81	0.05	7.81	0.05	7.81	0.05	0.10
***Bacillus subtilis* subsp. *spizizenni* (ATCC 6633)**	3.91	0.03	3.91	0.03	3.91	0.03	3.91	0.03	3.91	0.03	3.91	0.03	3.91	0.03	0.03
***Staphylococcus aureus* (ATCC 6538)**	31.25	0.20	15.63	0.10	15.63	0.10	15.63	0.10	15.63	0.10	15.63	0.10	15.63	0.10	0.78
***Escherichia coli* (ATCC 25922)**	1.95	0.02	1.95	0.02	1.95	0.02	0.98	0.01	0.98	0.01	0.98	0.01	1.95	0.02	0.005
***Pseudomonas aeruginosa* (ATCC 27853)**	15.63	0.10	15.63	0.10	15.63	0.10	15.63	0.10	15.63	0.10	15.63	0.10	15.63	0.10	0.10
***Salmonella* 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	0.01
MRSA (ATCC 33591)	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	0.20
MRSA (ATCC 43300)	31.25	0.20	62.50	0.39	31.25	0.20	31.25	0.20	31.25	0.20	31.25	0.20	31.25	0.20	0.20

MRSA: Methicillin-resistant *Staphylococcus aureus*

CIP: Ciprofloxacin

4.2 Minimum Bactericidal Concentration (MBC)

MBC is the minimal level of an antimicrobial agent needed to kill 99.9% of the starting inoculum after the inoculum is incubated for 24 hours under controlled temperature (Balouiri, Sadiki and Ibnsouda, 2016). As the starting inoculum in this project was 5×10^5 CFU/mL and 10 µL of the final inoculum was used for MBC determination, less than 5 bacterial colonies were expected in an agar plate in order to achieve a 99.9% antibacterial activity by the compounds at a particular concentration. The MBC/MIC ratio is used to classify the antibacterial activity into bacteriostatic or bactericidal activities. A MBC/MIC ratio lower than or equal to 4 is categorized as bactericidal whereas an antimicrobial agent with MBC/MIC ratio higher than 4 is classified as bacteriostatic (Patel, et al., 2023).

4.2.1 NAH Derivative Compounds in Combination with Ciprofloxacin as Adjuvants

Table 4.5 tabulated the MBC of the 7 NAH derivative compounds in combination with ciprofloxacin against the 8 selected bacterial strains whereas Table 4.6 showed the MBC/MIC ratio for the 7 NAH derivative compounds against the 8 bacterial strains. As the ciprofloxacin-adjuvants recorded poor MIC values against *S. aureus* (ATCC 6538), *P. aeruginosa* (ATCC 27853), Methicillin-resistant *S. aureus* (ATCC 33591) and Methicillin-resistant *S. aureus* (ATCC 43300), MBC determination was not proceeded. Due to low MIC values, the MBC determination for ciprofloxacin-adjuvants 1, 3 and 6 was also not conducted for *B. cereus* (ATCC13061). Higher

concentrations of compounds were required to study the antibacterial activity against the bacterial strains mentioned.

In reference to Tables 4.5 and 4.6, Compounds 2, 4, 5 and 7, featuring 2-Cl, H, 2-NO_2 and 2,4-Cl_2 aromatic substitutions respectively, demonstrated MBC values of 7.81 to 15.63 µg/mL and MBC/MIC ratio ranging from 1.00 to 2.00 against *B. cereus* (ATCC13061). Hence, it suggested that Compounds 2, 4, 5 and 7 displayed bactericidal effects when combined with ciprofloxacin. Meanwhile, Compounds 1 to 7 displayed bactericidal effects against *B*. *subtilis* subsp. *spizizenni* (ATCC 6633), *E. coli* (ATCC 25922), and *S.* Typhimurium (ATCC 14028), characterized by the MBC/MIC ratio ranging from 1.00 to 4.00.

Table 4.5: The Minimum Bactericidal Concentration (MBC) of NAH derivative compounds in combination with ciprofloxacin against 8 selected bacterial strains.

Bacterial strains	**MBC of NAH derivative compounds in combination with ciprofloxacin**													
	1		2		3		4		5		6		7	
	2-Br		2-Cl		2-F		H		2-NO_2		2-OCH_3		2,4-Cl_2	
	NAH	CIP	NAH	CIP	NAH	CIP	NAH	CIP	NAH	CIP	NAH	CIP	NAH	CIP
***Bacillus cereus* (ATCC 13061)**	-	-	15.63	0.10	-	-	7.81	0.05	15.63	0.10	-	-	7.81	0.05
***Bacillus subtilis* subsp. *spizizenni* (ATCC 6633)**	7.81	0.05	15.63	0.10	7.81	0.05	7.81	0.05	3.91	0.03	7.81	0.05	3.91	0.03
***Staphylococcus aureus* (ATCC 6538)**	-	-	-	-	-	-	-	-	-	-	-	-	-	-
***Escherichia coli* (ATCC 25922)**	7.81	0.05	1.95	0.02	3.91	0.03	1.95	0.02	3.91	0.03	1.95	0.02	1.95	0.02
***Pseudomonas aeruginosa* (ATCC 27853)**	-	-	-	-	-	-	-	-	-	-	-	-	-	-
***Salmonella* Typhimurium (ATCC 14028)**	1.95	0.02	1.95	0.02	7.81	0.05	1.95	0.02	1.95	0.02	3.91	0.03	1.95	0.02
MRSA (ATCC 33591)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MRSA (ATCC 43300)	-	-	-	-	-	-	-	-	-	-	-	-	-	-

-: No MBC was determined.

Table 4.6: The MBC/MIC ratio of NAH derivative compounds against 8 selected bacterial strains.

Bacterial Strains	**MBC/MIC Ratio of NAH Derivative Compounds in Combination with Ciprofloxacin**													
	1		2		3		4		5		6		7	
	2-Br		2-Cl		2-F		H		2-NO_2		2-OCH_3		2,4-Cl_2	
	NAH	CIP	NAH	CIP	NAH	CIP	NAH	CIP	NAH	CIP	NAH	CIP	NAH	CIP
***Bacillus cereus* (ATCC 13061)**	-	-	2.00	2.00	-	-	1.00	1.00	2.00	2.00	-	-	1.00	1.00
***Bacillus subtilis* subsp. *spizizenni* (ATCC 6633)**	2.00	2.00	4.00	3.33	2.00	2.00	2.00	2.00	1.00	1.00	2.00	2.00	1.00	1.00
***Staphylococcus aureus* (ATCC 6538)**	-	-	-	-	-	-	-	-	-	-	-	-	-	-
***Escherichia coli* (ATCC 25922)**	4.00	2.50	1.00	1.00	2.00	1.50	1.99	2.00	3.99	3.00	1.99	2.00	1.00	1.00
***Pseudomonas aeruginosa* (ATCC 27853)**	-	-	-	-	-	-	-	-	-	-	-	-	-	-
***Salmonella* Typhimurium (ATCC 14028)**	1.00	1.00	1.00	1.00	4.00	2.50	1.00	1.00	1.00	1.00	2.00	1.50	1.00	1.00
MRSA (ATCC 33591)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MRSA (ATCC 43300)	-	-	-	-	-	-	-	-	-	-	-	-	-	-

MRSA: Methicillin-resistant *Staphylococcus aureus*

4.3 Fractional Inhibitory Concentration (FIC) Index

Tables 4.7, 4.8 and 4.9 demonstrated the FIC indices of the NAH derivative compounds 1 to 7 combined with streptomycin, chloramphenicol and ciprofloxacin respectively. The FIC index is used to determine the interactive effect of a compound paired with antimicrobial drugs, assessing whether the combination provides enhanced inhibitory effects or reduced inhibitory effects compared to the compounds used individually (Konaté, et al., 2012). When the combination gives better inhibitory effects with an FIC index less than or equal to 0.5, synergism occurs in the drug combination. There is no difference in antimicrobial effect when the FIC index is in the range of 0.5 to 4.0. Meanwhile, a FIC index of more than 4.0 suggests antagonism in which the drug combination provides poorer inhibitory effects (Odds, 2003).

As noticed in Table 4.7, Compounds 2, 4, 5, 6 and 7 displayed synergetic effects against *B. cereus* (ATCC 13061) when combined with streptomycin, with the FIC index of 0.37. Compounds 2, 3, 4 and 5 showed synergism with the addition of streptomycin against *S. aureus* (ATCC 6538) as characterized by FIC indices ranging from 0.19 to 0.38. Lastly, the FIC index of 0.50 obtained by the 7 compounds against Methicillin-resistant *S. aureus* (ATCC 43300) suggested the presence of synergism between the compounds and streptomycin. No significant interaction between the compounds and streptomycin was noticed against *B. subtilis* subsp. *spizizenni* (ATCC 6633), *E. coli* (ATCC 25922), *S.* Typhimurium (ATCC 14028) and Methicillin-resistant *S. aureus* (ATCC 33591) with FIC indices

ranging from 1.00 to 2.00. Antagonism was detected in compounds 1 to 7 combined with streptomycin against *P. aeruginosa* (ATCC 27853) with a FIC index of 8.25.

Referring to Table 4.8, Compounds 1 to 7 were found to be synergistic with chloramphenicol against *S.* Typhimurium (ATCC 14028) with a FIC index of 0.50. Furthermore, Compounds 1 to 7 did not have significant interactions with chloramphenicol against *B. cereus* (ATCC 13061), *B. subtilis* subsp. *spizizenni* (ATCC 13061), *S. aureus* (ATCC 6538), *E. coli* (ATCC 25922), *S.* Typhimurium (ATCC 14028), Methicillin-resistant *S. aureus* (ATCC 33591) and Methicillin-resistant *S. aureus* (ATCC 43300) with FIC indices ranging from 0.62 to 2.51. Compounds 1 to 7 showed antagonism when combined with chloramphenicol against *P. aeruginosa* (ATCC 27853) as indicated by FIC indices ranging from 4.26 to 8.51.

Based on Table 4.9, synergism was observed in Compounds 2 to 7 against *S. aureus* (ATCC 6538) when combined with ciprofloxacin with FIC indices ranging from 0.25 to 0.38. Compound 1 did not interact significantly with ciprofloxacin against *S. aureus* (ATCC 6538). Moreover, Compounds 1 to 7 exhibited no interaction with ciprofloxacin against *B. cereus* (ATCC 13061), *B. subtilis* subsp. *spizizenni* (ATCC 6633), *P. aeruginosa* (ATCC 27853), *S.* Typhimurium (ATCC 14028), Methicillin-resistant *S. aureus* (ATCC 33591) and Methicillin-resistant *S. aureus* (ATCC 43300) with FIC indices ranging from 0.56 to 2.02. Compounds 4, 5 and 6 demonstrated

no interactive effect while Compounds 1, 2, 3 and 7 displayed antagonistic effects against *E. coli* (ATCC 25922) with FIC indices of 2.01 and 4.02 respectively.

Table 4.7: The Fractional Inhibitory Concentration (FIC) Index of the NAH derivative compounds in combination with streptomycin against selected bacterial strains.

Bacterial Strains	**FIC Index of NAH Derivative Compounds in Combination with Streptomycin**						
	NAH Derivative Compounds						
	1	**2**	**3**	**4**	**5**	**6**	**7**
	2-Br	**2-Cl**	**2-F**	**H**	**2-NO_2**	**2-OCH_3**	**2,4-Cl_2**
***Bacillus cereus* (ATCC 13061)**	0.75	0.37	0.75	0.37	0.37	0.37	0.37
***Bacillus subtilis* subsp. *spizizenni* (ATCC 6633)**	1.03	1.03	1.06	2.06	0.53	1.03	1.03
***Staphylococcus aureus* (ATCC 6538)**	0.63	0.38	0.19	0.31	0.38	0.63	0.63
***Escherichia coli* (ATCC 25922)**	1.50	1.50	1.50	1.50	1.50	1.50	1.50
***Pseudomonas aeruginosa* (ATCC 27853)**	8.25	8.25	8.25	8.25	8.25	8.25	8.25
***Salmonella* Typhimurium (ATCC 14028)**	2.00	2.00	2.00	2.00	2.00	2.00	2.00
MRSA (ATCC 33591)	1.00	1.00	1.00	1.00	1.00	1.00	1.00
MRSA (ATCC 43300)	0.50	0.50	0.50	0.50	0.50	0.50	0.50

MRSA: Methicillin-resistant *Staphylococcus aureus*

Table 4.8: The Fractional Inhibitory Concentration (FIC) Index of the NAH derivative compounds in combination with chloramphenicol against selected bacterial strains.

Bacterial Strains	**FIC Index of NAH Derivative Compounds in Combination with Chloramphenicol**						
	NAH Derivative Compounds						
	1	**2**	**3**	**4**	**5**	**6**	**7**
	2-Br	**2-Cl**	**2-F**	**H**	**2-NO_2**	**2-OCH_3**	**2,4-Cl_2**
***Bacillus cereus* (ATCC 13061)**	0.75	0.75	1.50	0.75	0.75	0.75	0.75
***Bacillus subtilis* subsp. *spizizenni* (ATCC 6633)**	0.75	0.75	2.00	0.75	1.00	0.75	0.75
***Staphylococcus aureus* (ATCC 6538)**	1.25	0.75	0.75	1.25	0.75	1.25	0.62
***Escherichia coli* (ATCC 25922)**	1.50	1.50	1.50	1.50	1.50	1.50	1.50
***Pseudomonas aeruginosa* (ATCC 27853)**	4.26	4.26	8.51	4.26	4.26	4.26	4.26
***Salmonella* Typhimurium (ATCC 14028)**	0.50	0.50	0.50	0.50	0.50	0.50	0.50
MRSA (ATCC 33591)	1.00	1.00	1.00	1.00	1.00	1.00	1.00
MRSA (ATCC 43300)	2.51	2.51	2.51	2.51	2.51	2.51	2.51

MRSA: Methicillin-resistant *Staphylococcus aureus*

Table 4.9: The Fractional Inhibitory Concentration (FIC) Index of the NAH derivative compounds in combination with ciprofloxacin against selected bacterial strains.

Bacterial Strains	**FIC Index of NAH Derivative Compounds in Combination with Ciprofloxacin**						
	NAH Derivative Compounds						
	1	**2**	**3**	**4**	**5**	**6**	**7**
	2-Br	**2-Cl**	**2-F**	**H**	**2-NO_2**	**2-OCH_3**	**2,4-Cl_2**
***Bacillus cereus* (ATCC 13061)**	1.13	0.56	1.13	0.56	0.56	0.56	0.56
***Bacillus subtilis* subsp. *spizizenni* (ATCC 6633)**	1.02	1.02	1.03	1.02	1.03	1.02	1.02
***Staphylococcus aureus* (ATCC 6538)**	0.76	0.25	0.25	0.38	0.25	0.38	0.38
***Escherichia coli* (ATCC 25922)**	4.02	4.02	4.02	2.01	2.01	2.01	4.02
***Pseudomonas aeruginosa* (ATCC 27853)**	1.13	1.13	1.13	1.13	1.13	1.13	1.13
***Salmonella* Typhimurium (ATCC 14028)**	2.02	2.02	2.02	2.02	2.02	2.02	2.02
MRSA (ATCC 33591)	1.13	1.13	1.13	1.13	1.13	1.13	1.13
MRSA (ATCC 43300)	1.13	2.20	1.13	1.13	1.13	1.13	1.13

MRSA: Methicillin-resistant *Staphylococcus aureus*

CHAPTER 5

DISCUSSION

5.1 Relationship between Chemical Structure of NAH Derivative Compounds and Antibacterial Activity

As observed in Table 4.1, most of the compounds were considered inactive against the tested bacterial strains except for Compounds 1, 4, 6 and 7, bearing aromatic substitution of 2-Br, H, 2-OCH_3 and 2,4-Cl_2 respectively, exhibited moderate antibacterial activity against *S. aureus* (ATCC 6538). The exhibition of the antibacterial activity can be explained by the type of functional groups substituted to the aromatic ring of *N*-acylhydrazone derivative compounds. The results of He, et al. (2017) proposed that the electron-withdrawing groups such as the nitro group (NO_2) and halogens possessed a better inhibitory effect than the electron-donating group such as the amino group (NH_2) and methoxy group (OCH_3). Gu, et al. (2012) and Yao, et al. (2021) also showed that halogen groups displayed better antibacterial activity in their research. According to Henary, et al. (2024), electron-withdrawing groups adjust the lipophilicity of the molecule, enhancing its ability to be transported through the cell quickly and efficiently. Besides, He, et al. (2017) declared that the antibacterial activity of NAH derivative compounds can be influenced by the sizes of the substituent group, specifically halogen groups. It exhibited that the bromo group has the highest antibacterial activity due to it being

the largest size, followed by the chloro and fluoro groups. The antibacterial effects of Compound 6 featuring 2-OCH_3 may be explained by its property of being an activating group that produces a resonance effect by donating a lone pair of electrons to the benzene ring. This leads to an increase in the rate of reaction, suggesting moderate antibacterial activity (Ashenhurst, 2017). Yang, et al. (2024) stated that most bactericides applied the hydrophobic interactions with the bacterial membrane to kill the cell. As OCH_3 is a hydrophobic group, it may induce the ability of the compound to inhibit bacterial growth. The findings of He, et al. (2017) also revealed that the position in which the functional groups are substituted to the benzene ring can greatly affect the antimicrobial activity. They stated that the halogen groups substituted at the *meta* position of the aromatic ring demonstrated the highest antibacterial activity, followed by *para* and *ortho* positions.

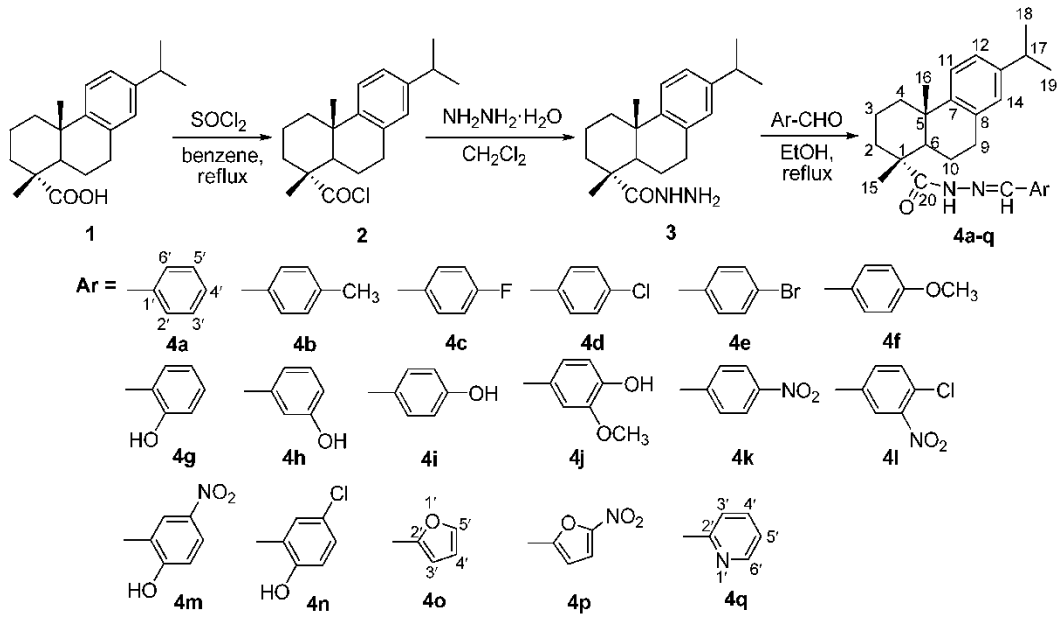


Figure 5.1: The synthesis pathway of *N*-acylhydrazone derivatives from dehydroabietic acid with different aromatic substitutions (Gu, et al., 2012).

Although fluoro and nitro groups were electron-withdrawing groups, it was noticed that Compounds 3 (2-F) and 5 (2-NO_2) exhibited inactive antibacterial properties as compared to chloro and bromo groups. Fang, et al. (2019) stated that the lipophilicity of a drug molecule can be enhanced with chlorine as a substituent in the drug which may ease the drug diffusion through the lipophilic phase of the bacterial cell membrane and bind to the target site. Aside from chlorine substituents, electron-withdrawing groups such as phenyl and bromo substituents will also raise the lipophilicity of the compounds while electron-donating groups are the opposite (Tamaian, et al., 2015). The chlorine substituents induce steric and electronic effects, leading to a stronger attraction between the chlorine-substituted compounds and the protein binding pocket of the bacteria through the local electronic attraction

with the amino acid residue that meets the chlorine atom, thus enhancing the biological activity of the compound (Naumann, 2000; Fang, et al., 2019).

In addition, it was observed that Compound 7 with 2,4-Cl_2 substituents obtained a stronger antibacterial activity than Compound 2 with a 2-Cl substituent. This observation suggested the presence of more chlorine groups in the chemical compound boosted its antibacterial activity. This statement is supported by Niu, et al (2021) who proposed that the number of chlorine atoms in the backbone of the compound influences the antibacterial effect of a compound. Trichlorinated compound turns out to be the most active antibacterial activity among the tested compounds, followed by dichlorinated and monochlorinated compounds. Apart from that, Friedmann, Henika and Mandrell (2003) revealed that the antibacterial activity was the highest in trisubstituted compounds, followed by disubstituted and monosubstituted compounds, hence suggesting that Compound 7 with 2,4-Cl_2 substituents will be more active in antibacterial activity than Compound 2 with the 2-Cl substituent.

5.2 Antibacterial Activity and Potential Antibacterial Mechanism of NAH Derivative Compounds

Moderate antibacterial activity was observed in NAH derivative Compounds 1, 4, 6 and 7 featuring 2-Br, H, 2-OCH_3 and 2,4-Cl_2 respectively against *S. aureus* (ATCC 6538) while exhibiting low antibacterial activity against other bacterial

strains. Hence, it can be assumed that the compounds were species-specific against *S. aureus* (ATCC 6538). suggesting a narrow spectrum activity of NAH derivative compounds. According to Melander, Zurawski and Melander (2018), narrow-spectrum antibiotics are advantageous over broad-spectrum antibiotics in terms of antibiotic resistance. Broad-spectrum antibiotics target a wide range of bacteria regardless of non-pathogenic bacteria and pathogens that cause infection whereas narrow-spectrum antibiotics only target certain groups of bacteria types (Demeke, et al., 2021). The usage of broad-spectrum antibiotics may lead to the risk of antibiotic resistance in such a way that the non-pathogenic bacteria acquire the resistance genes and pass on the genes to harmful bacteria, causing fewer options for antibiotics in clinical treatments. As broad-spectrum antibiotics do not discriminate between beneficial and harmful bacteria, these antibiotics alter the host microbiome, hence damaging the host. The normal microflora in the host can no longer protect the host from the colonization of pathogens due to an imbalance of microflora caused by the broad-spectrum antibiotics (Manshadi, Setoodeh and Zare, 2024). As such, narrow-spectrum antibiotics are preferred.

In contrast, in the studies by Gu, et al. (2012), Compound 4e bearing the functional group of Br displayed moderate antibacterial activity with a MIC value of 31.2 μg/mL against *S. aureus* CGMCC1.2465 and *B. subtilis* CGMCC1.3343 whereas a low antibacterial activity (>100 μg/mL) against *E. coli* CGMCC1.3373 and *P. fluorescens* CGMCC1.1802. In the studies by Yao, et al. (2021), Compound 3e with the same functional group also displayed the active antibacterial activity of MIC

value *B. subtilis* CMCC 63501 and *E. coli* CMCC 25922, showing a broad-spectrum activity. These observations further supported that the position of the functional group at the aromatic ring can affect the antibacterial activity of NAH derivative compounds as mentioned in the previous part because Compound 1 had the Br group at the *ortho* position whereas Compounds 3e and 4e had the Br groups at the para position. Meanwhile, compound 4f bearing the functional group of OCH_3 is moderately active (62.5 µg/mL) against *B. subtilis* CGMCC1.3343 while inactive (>100 µg/mL) against the other 3 bacteria strains. Hence, this supported the idea that Compound 6, bearing the same functional group of OCH3 in this project, had a narrow spectrum of antibacterial activity.

The molecular docking studies demonstrate that the NAH derivative compounds have strong affinities to bind to the active site of subunit GyrB of the DNA gyrase, contributing to the antimicrobial activity (Aarjane, et al., 2020). The compounds interact with the binding site of DNA gyrase through hydrogen bonds and inhibit the functioning of DNA gyrase (Alves, et al., 2014; Baig, et al., 2015; Aarjane, et al., 2020). The studies displayed that NAH derivative compounds had a high potential antibacterial activity with MIC values of 19.61 to 67.62 µg/mL against *S. aureus* whilst a moderate antibacterial activity with MIC values ranging from 38.46 to 74 µg/mL against *E. coli*.

Apart from that, Gu, et al. (2012) proposed the antibacterial mechanism shown by NAH derivative compounds containing nitrophenyl or nitrofuranyl moieties. When the bacteria are subjected to these derivatives, the nitroreductase of the bacteria reduces these derivatives, producing toxic intermediates including reactive oxygen species, nitro radical-anion and hydroxylamine derivatives (Quillardet, et al., 2006; Gu, et al., 2012). Under aerobic conditions and the presence of superoxide dismutase, the radical anions can react with the oxygen to form hydrogen peroxide as the end product. When hydrogen peroxide builds up, highly reactive hydroxyl radicals are produced and attack the biomolecules within bacterial cells, leading to toxic effects that inhibit growth or cause bacterial cell death (Viodé, et al., 1999). Lannes, et al. (2014) also displayed the potential antibacterial activity of the derivatives containing nitrofuranyl structure with both MIC and MBC values ranging from 1 to 16 μg/mL, indicating that these derivatives were able to inhibit bacterial growth and confer bactericidal effect to bacteria.

5.3 Susceptibility of Gram-Positive, Gram-Negative and Resistant Bacteria to Different Antibiotics

In this project, three antibiotics were used as positive controls, namely STR, CHL and CIP. Positive controls are important in quality control to determine whether contamination exists in the reagents used, particularly the MH broth and drugs (The Editors of Microchem Laboratory, 2016). By using different antibiotics, the susceptibility of the selected bacterial strains to the drugs can be determined (Chandrakant, 2023).

According to the clinical breakpoint of STR provided by The European Committee on Antimicrobial Susceptibility Testing (2020), bacteria strains obtaining MIC value of higher than 512 µg/mL display high resistance to STR whereas those lower than or equal to 512 µg/mL have low resistance to STR. Thus, Table 4.2 showed that STR was effective against Gram-positive, Gram-negative and resistant bacteria except Methicillin-resistant *S. aureus* (ATCC 33591) with a MIC value of 800 µg/mL.

The clinical breakpoint of CHL also demonstrated that bacteria strains obtaining MIC value lower than or equal to 8 µg/mL are sensitive to CHL (The European Committee on Antimicrobial Susceptibility Testing, 2020). In contrast, those with MIC values higher than 8 µg/mL are resistant to CHL. As such, CHL showed high effectiveness against most selected bacterial strains except Methicillin-resistant *S. aureus* (ATCC 33591) as it obtained a MIC value of 50 µg/mL from Table 4.3.

The European Committee on Antimicrobial Susceptibility Testing (2020) proposed the clinical breakpoints of CIP for *S. aureus* are lower than or equal to 0.001 µg/mL as sensitive strains whilst more than 1 µg/mL as resistant strains. For *Pseudomonas* spp., the MIC of CIP with values lower than or equal to 0.001 µg/mL is perceived as sensitive whereas that higher than 0.5 is resistant to CIP. For the *Enterobacterales* which include *E. coli* and *S.* Typhimurium, strains with MIC

breakpoints lower or equal to 0.25 μg/mL are considered sensitive to CIP while resistant if MIC value exceeds 0.5 μg/mL. Besides, Reeves, et al. (1984) obtained MIC values ranging from 0.004 to 0.25 μg/mL for *E. coli*. Alhumaid, et al. (2021) also claimed that *E. coli* is highly sensitive to ciprofloxacin in their research. Thus, it can be noted that CIP exhibited a broad spectrum of activity against Gram-positive, Gram-negative and resistant bacteria from Table 4.5.

In summary, STR, CHL and CIP were still effective against Gram-positive, Gram-negative and resistant bacteria except Methicillin-resistant *S. aureus* (ATCC 33591). STR and CHL were not recommended to treat Methicillin-resistant *S. aureus* (ATCC 33591) due to high MIC values whilst CIP may be an alternative to treat the infections caused by this strain. Meanwhile, The European Committee on Antimicrobial Susceptibility Testing (2020) suggested that STR is frequently combined with other antimicrobial agents to enhance its activity and target a wider range of bacteria strains.

5.4 Mechanisms of Antibiotic Resistance of Gram-Positive and Gram-Negative Bacteria to Different Antibiotics

In the previous part, the results of streptomycin-adjuvant combinations proposed that Gram-positive bacteria were easier to inhibit compared to Gram-negative and resistant bacteria. Generally, Gram-negative bacteria possess higher resistance against antibiotics than Gram-positive bacteria due to the structural differences

(Breijyeh, Jubeh and Karaman, 2020). The outer membrane (OM) is the main reason that results in the variation in the penetration of the antimicrobial agents into the bacterial cell (Exner, et al., 2017). The OM is a selectively permeable protective barrier that prevents the entry of certain drugs into bacterial cells (Kapoor, Saigal and Elongavan, 2017). The abusive usage of antibiotics causes the outer membrane proteins such as porins in the OM to be mutated, hindering certain antibiotic molecules from passing through, hence creating intrinsic resistance. In addition, Gram-negative bacteria acquire resistance due to the ability to produce antibiotics-degrading enzymes and the presence of an efflux pump which decreases the effectiveness of antibiotics (Mancuso, et al., 2021).

On the contrary, chloramphenicol resistance is shown through the inactivation by the bacterial enzyme, namely chloramphenicol acetyltransferase (Fernández, et al., 2012). This enzyme acetylates the hydroxyl group at the first and third carbon atoms of the chloramphenicol structure into monoacetylated and diacetylated derivatives, altering its antibacterial function (Ma, et al., 2023). In addition, the generation of resistant genes due to survival after chloramphenicol treatment or chromosomal changes due to mutations causes a reduced uptake of antibiotics, leading to chloramphenicol resistance (Cohen, Opal and Powderly, 2017). The chromosomal mutations are observed in multiple antibiotic resistance (*mar*) locus of *E. coli*. Besides, *cmlA* genes are found in *P. aeruginosa* and are responsible for an efflux system to export chloramphenicol from the cells (Schwarz, et al., 2004).

In general, the bacterial resistance to ciprofloxacin can be described with the plasmids containing the resistant genes to ciprofloxacin through mutations and survival after exposure to ciprofloxacin. When the bacteria are re-treated with ciprofloxacin, the plasmids will encode the genes to produce a protein that protects against ciprofloxacin so that the bacterial enzymes, DNA gyrase and topoisomerase IV are not attacked (Van Hoek, et al., 2011; Sharma, et al., 2017). Another plasmid mediates an efflux pump to remove the hydrophilic fluoroquinolone such as ciprofloxacin from the bacteria (Yamane, et al., 2007; Van Hoek, et al., 2011). Moreover, ciprofloxacin resistance of Gram-negative bacteria, particularly *E. coli* and *S.* Typhimurium is demonstrated through the chromosomal mutations of *gyrA* genes encoding the DNA gyrase and the *par*C genes of topoisomerase IV (Chang, et al., 2021). Additionally, the reduction of OmpF porin expression, a pore protein found on the outer membrane of *E. coli* and *S.* Typhimurium is discovered as another mechanism for ciprofloxacin resistance (Shariati, et al., 2022).

5.5 Mechanism of Antibiotic Resistance of Resistant Bacteria to Different Antibiotics

In the susceptibility of resistant bacteria discussed previously, Methicillin-resistant *S. aureus* (ATCC 33591) was relatively resistant to STR and CHL whilst sensitive to CIP. Methicillin-resistant *S. aureus* (ATCC 43300) was relatively sensitive to all drugs used. According to Vestergaard, Frees and Ingmer (2019), MRSA contains aminoglycoside-modifying enzymes which alter the structure of streptomycin to inactivate its antibacterial activity to MRSA and the enzymes are found in the *S.*

aureus clinical isolates. In addition, mutated MRSA strains can depolarize their membrane potential to influence the uptake of aminoglycoside (Proctor, et al., 2006). Next, the resistance of MRSA to chloramphenicol is contributed by the presence of various chloramphenicol acetyltransferases through the change of a hydroxyl group at C-3 with a fluoride residue, altering the acceptor site of the acetyl group of chloramphenicol and thus, inactivate it (Schwarz, et al., 2004). Besides, MRSA removes chloramphenicol through active efflux aided by an efflux pump, specifically the lincomycin resistance protein of *Staphylococcus aureus* (LmrS) (Floyd, 2010). In resisting ciprofloxacin, a type of fluoroquinolone, the mechanism displayed by MRSA includes the expression of efflux pump systems. MRSA contains three chromosomally encoded efflux pumps, but the NorA protein is the pump responsible for expressing ciprofloxacin from the bacterial cells (Foster, 2017). The resistance of MRSA towards ciprofloxacin is also contributed by the mutational changes of the *gyrA* gene encoding the DNA gyrase and *grlA* gene of topoisomerase IV, causing an increase in the fluoroquinolone resistance (Ince and Hopper, 2001).

5.6 Antibacterial Activity of NAH Derivative Compound in Combination with Ciprofloxacin as Adjuvants

Referring to Table 4.5, the MBC/MIC ratio of the ciprofloxacin-adjuvant combinations against certain selected bacteria ranged from 1.00 – 4.00, indicating the presence of bactericidal activity. The ciprofloxacin kills bacteria through inhibition of the genes encoding DNA gyrase, topoisomerase II and topoisomerase

IV (Serizawa, et al., 2010). Topoisomerase IV encoded by *ParC* and *ParE* genes initiates the DNA replication whereas DNA gyrase encoded with *gyrA* and *gyrB* genes repairs the fractures in the DNA double-strand through resealing. As they are inhibited, DNA replication is slowed or blocked, and breaks of double-strand DNA are created (Rehman, Patrick and Lamont, 2019; Shariati, et al., 2022). Hence, summing up the findings that the NAH derivative compounds bound to the DNA gyrase of *S. aureus* through molecular docking studies, the antibacterial mechanism of ciprofloxacin and the activities displayed by different adjuvants, it can be assumed that NAH derivative compounds fell under Class IB adjuvant – the inhibitors of enzymes involved in cellular processes. DNA gyrase is involved in cellular processes, particularly DNA replication and transcription. DNA gyrase adds negative supercoils to the DNA molecules to facilitate DNA replication (Nöllmann, Crisona and Arimondo, 2007). As the NAH derivative compounds bind to the DNA gyrase, its functions are inhibited and protein cannot be synthesized, leading to cell death which allows the MBC to be determined.

5.7 Synergism, Indifference and Antagonism of Different Adjuvants Combinations

Based on Table 4.7, the FIC index of Compounds 2, 4, 5, 6 and 7 displayed synergisms against *B. cereus* (ATCC 13061). Compounds 2, 3, 4 and 5 showed synergistic inhibitory effects against *S. aureus* (ATCC 6538). All compounds were synergistic with streptomycin against Methicillin-resistant *S. aureus* (ATCC 43300). According to Table 4.8, all compounds were synergistic with chloramphenicol

against *S.* Typhimurium (ATCC 14028). In Table 4.9, Compounds 2 to 7 were observed to be synergistic with ciprofloxacin against *S. aureus* (ATCC 6538).

As noticed in Tables 4.7, 4.8 and 4.9, particular streptomycin-adjuvant, chloramphenicol-adjuvant and ciprofloxacin-adjuvant combinations displayed indifference in the antibacterial activity based on the FIC values ranging from 0.56 to 2.51 respectively. According to Meletiadis, et al. (2010), indifference is a term that states in a combination of two drugs, the added drug is described as inactive when combined with a studied active drug as it does not affect the overall activity while only the effect of active drug is observed. Hence, it proposed that the compounds did not have significant effect in antibacterial activity when combined with the antibiotics.

In accordance with Table 4.7, antagonisms were observed in Compounds 1 to 7 combined with streptomycin against *P. aeruginosa* (ATCC 27853). In Table 4.8, Compounds 1 to 7 demonstrated antagonistic inhibitory effects against *P. aeruginosa* (ATCC 27853) when combined with chloramphenicol. Antagonisms were noticed in Compounds 1, 2, 3 and 7 against *E. coli* (ATCC 25922) in ciprofloxacin-adjuvant combinations under Table 4.9. These results demonstrated that the antibacterial effects of the compounds paired with antibiotics are lesser than those when they are being used separately. This indicates that the compounds hinder the mechanism that attacks the bacterial cells of the antibiotics, which is generally

undesirable as it may increase the risk of resistance of the bacteria against the antimicrobial agents (Tyres and Wright, 2019). However, hyperantagonism is beneficial in conditions where antibiotic combinations can selectively kill resistant strains without killing other bacteria in the population (Torella, Chait and Kishony, 2010; Tyres and Wright, 2019). Furthermore, antagonism occurs when a drug prevents another chemical from acting on the receptor of the bacteria, leading to the blocking of action of the chemical (Bullock and Manias, 2011). Hence, it can be assumed that NAH derivative compounds interfered with the action of the antibiotics and depressed their effectiveness on the bacteria strains.

To summarize, synergism was displayed in all compounds except Compound 1 featuring 2-Br aromatic substitution but was observed to be species-specific when used as adjuvants. As most of the adjuvant combinations exhibited indifference or antagonism in the antibacterial activity against the bacterial strains, it was not encouraged to use the NAH derivative compounds in combination with the antibiotics used as adjuvants in clinical uses as it will increase the antimicrobial resistance of these bacterial strains to the NAH derivative compounds and antibiotics.

5.8 Implications for Animal Health and Food Safety

Although NAH derivative compounds were potential adjuvants, studies on the toxicity effects of these compounds should be conducted before use in clinical

treatments. According to Lannes, et al. (2014), the existing NAH derivative compounds such as nitrofurans were low in cytotoxicity, suggesting that the compounds were less harmful to human cells in the *in vitro* and in-silico experiments. It also showed that the compounds were less likely to cause skin irritation and affect humans' reproductive systems. Based on Figure 5.2, Silva, et al. (2014) demonstrated that Compounds 5a, 5c, 5d, 5g and 5i had low toxicity risk whereas the others showed medium to high theoretical toxicity risk through in-silico evaluation. Apart from that, Mikus, et al. (2023) conducted the MTT assay to evaluate the cytotoxicity of NAH derivative compounds using normal human dermal fibroblast (NHDF) cells. The results exhibited cell viability of over 70%, showing that the compounds were low in cytotoxicity potential.

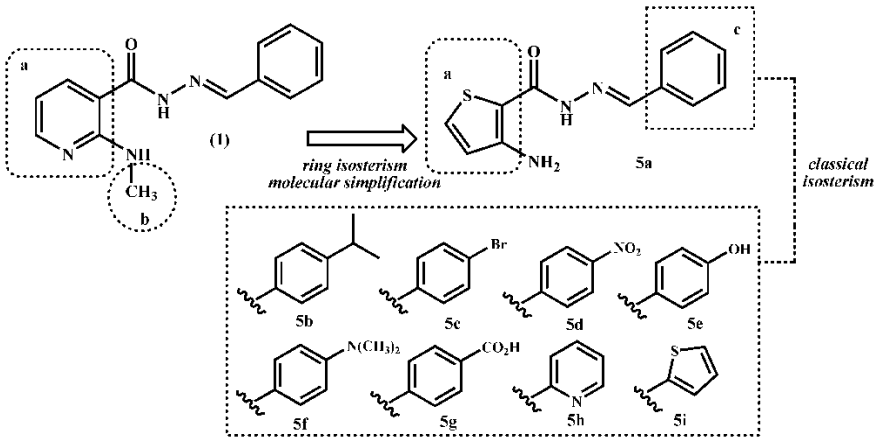


Figure 5.2: The chemical structure of title NAH derivative compounds (Silva, et al., 2014).

On the other hand, Quillardet, et al., (2006) proposed the potential of NAH derivatives compounds containing nitrophenyl and nitrofuranyl moieties to induce toxicity and mutagenic effects on mammalian cells. This is due to the occurrence

of enzymatic nitroreduction of nitrofurans in the animal tissues which produces metabolic intermediates that attack the macromolecules in animal tissues, leading to mutagenic effects (McCalla, 1983; Quillardet, et al., 2006). As such, this suggested that mutagenic effects may potentially occur and harm humans. As it may affect animals, this may indirectly cause safety issues in human food, particularly livestock when these compounds are used on them. Nevertheless, Gu, et al. (2012) proposed that a prodrug approach can be implemented on these nitro compound-containing NAH derivative compounds to reduce these side effects. This can be achieved by the modification of the functional groups of these compounds through esterification to improve the physicochemical properties, and biological activity as well as reduce genotoxicity (Chung, Bosquesi and dos Santos, 2011; Gu, et al., 2012).

Plant viruses, particularly tobacco mosaic viruses (TMV) and pests such as insects have been a challenge in the agricultural industries. Some studies showed that NAH derivative compounds had potential as agents in treating diseases induced by plant viruses and pests. According to Ni, et al. (2023), chloroinconazide containing acylhydrazone moiety displayed excellent antiviral activity against TMV. Their studies also showed that the matrine derivatives with acylhydrazone moiety exhibited better anti-TMV activity than the commercialized virucide Ribavirin. Besides, Liu, et al. (2014) claimed that excellent antiviral efficacy against TMV was displayed by the tetrahydro-β-carboline derivatives containing acylhydrazone moiety. On the other hand, some studies showed that NAH derivative compounds

possessed insecticidal activity. Apart from showing moderate to good in-vivo anti-TMV activities, some echinopsine derivatives containing acylhydrazone moieties revealed insecticidal activities against different insects including cotton bollworm, corn borer, oriental armyworm and fall armyworm (Cui, et al., 2022). Strong insecticidal activities were exhibited by the acylhydrazone derivatives studied by Sun and Zhou (2015) against the larvae of *H. armigera*, *P. xyllostella* and *P. rapae* at 10 μg/mL for 3 days. In a nutshell, these indicated that NAH derivative compounds had the potential to be used in the agricultural sector in treating pests and virus-related diseases in improving crop quality.

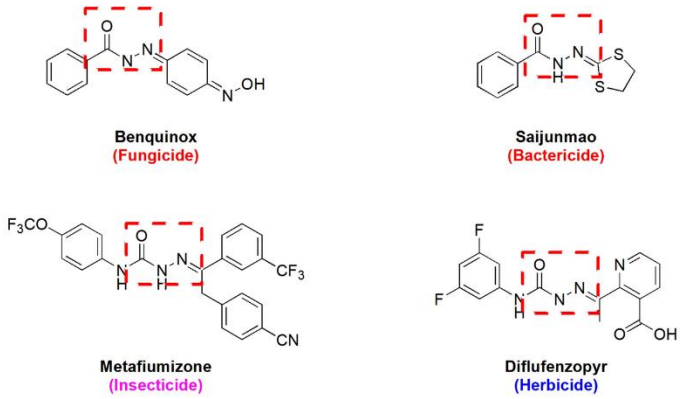


Figure 5.3: The chemical structure of bioactive drugs containing acylhydrazone moieties (Cui, et al., 2022).

5.9 Limitations and Future Recommendations of Study

A limitation of this project is that the mechanism of action (MOA) of the NAH derivative compounds remains unclear. The MOA is important because it helps drug development by reducing the failure risk during clinical trials. According to

Hudson and Lockless (2022), both biochemical and genetic approaches can be used to elucidate the MOA of the NAH derivative compounds. For example, affinity chromatography elucidates the MOA of an antimicrobial by analyzing the interactions of the immobilized antimicrobial and its targets. After the proteins bind to the antimicrobial, the mixture is washed out and eluted to obtain target molecules for further analysis using mass spectrometry (Hudson and Lockless, 2022).

Compounds 2, 4, 5 and 7 demonstrated bactericidal effects against *B. cereus* (ATCC13061) and all compounds exhibited bactericidal effects against *B. subtilis* subsp. *spizizenni* (ATCC 6633), *E. coli* (ATCC 25922), and *S.* Typhimurium (ATCC 14028) upon the combination with CIP, hence, a time-kill curve can be performed. This test can help to reveal the dependency of an antimicrobial or a combination of drugs on concentration or time. In other words, the impact of concentration or time on the effectiveness of the antimicrobials to kill bacteria can be understood (Gajic, et al., 2022). Besides, the time-kill test can be used to determine the synergism and antagonism between 2 antimicrobial agents to validate the results of the FIC index of the combinations used (Pfaller, Sheehan and Rex, 2004). This time-kill curve can also provide information on the bactericidal or bacteriostatic effects of the antimicrobials over time. As some studies proposed that NAH derivative compounds may have toxicity effects on animal cells including humans, a dose-response curve can be studied to obtain information on the potency, efficiency and toxicity of the NAH derivative compounds by changing concentration (Foerster, et al., 2016).

Moreover, more categories of antibiotics such as macrolides and tetracycline can be used in future studies apart from aminoglycosides, chloramphenicol and fluoroquinolones used in this project. This can help to study the synergism of the NAH derivative compounds with other drugs. As some NAH derivative compounds showed synergism with ciprofloxacin, it may be assumed that these compounds enhance the activity of ciprofloxacin such as by acting as an inhibitor to the efflux pump systems that express ciprofloxacin in the bacteria due to unclear mechanism of action of these compounds. As such, other fluoroquinolones such as moxifloxacin can be used to validate the assumption above if moxifloxacin showed synergism with NAH derivative compounds in the same manner (Vestergaard, Frees and Ingmer, 2019).

CHAPTER 6

CONCLUSION

The *in vitro* antibacterial activity of NAH derivative compounds used individually was successfully determined. The comparison of the effectiveness of *in vitro* antibacterial activity of NAH derivative compounds individually and in combination with STR, CHL and CIP respectively as adjuvants against selected Gram-negative, Gram-positive and Methicillin-resistant bacteria strains was also achieved. The results showed that Compounds 1 (2-Br), 4 (H), 6 (2-OCH_3) and 7 (2,4-Cl_2) exhibited moderately active antibacterial activity against *S. aureus* (ATCC 6538) with a MIC value of 62.5 µg/mL as well as inactive antibacterial activity against remaining 7 selected bacterial strains with MIC values of 125 to 250 µg/mL, suggesting that these compounds had narrow-spectrum activity. Compounds 2 (2-Cl), 3 (2-F) and 5 (2-NO_2) demonstrated inactive antibacterial activity with MIC values of 125 to 250 µg/mL against all selected bacterial strains. This was explained by the substitution of different functional groups, which electron-withdrawing groups such as halogen groups boosted the antibacterial activity of the compounds by adjusting the lipophilicity of the compounds and enhancing penetration of the compounds into the bacterial cell membrane. The size of the substituents, particularly halogen groups on the aromatic ring showed that the larger the size, the higher the antibacterial activity exhibited by the compounds, in which the bromo

group had the highest antibacterial activity due to the largest size. The amount of chlorine groups present on the aromatic ring increases the antibacterial activity, showing that Compound 7 (2,4-Cl_2) had a higher antibacterial activity than Compound 2 (2-Cl). The potential antibacterial mechanism of Compounds 1, 4, 6 and 7 was found in the molecular docking studies, where the compounds bind to the DNA gyrase to inhibit its function, achieving antibacterial activities.

In addition, all antibiotic-NAH adjuvant combinations demonstrated enhanced antibacterial activity against certain selected bacterial strains. The streptomycin-NAH adjuvant combinations exhibited better antibacterial activity against all bacterial strains except MRSA (ATCC 33591) and *Salmonella* Typhimurium (ATCC 14028). The chloramphenicol-NAH adjuvant combinations exhibited better antibacterial activity against all bacterial strains except MRSA (ATCC 33591) and MRSA (ATCC 43300). The ciprofloxacin-NAH adjuvant combinations showed active to moderately active antibacterial activity against all selected bacterial strains. Hence, this indicated MRSA strains exhibited resistance to streptomycin and chloramphenicol due to the presence of aminoglycoside-modifying enzymes and chloramphenicol acetyltransferase. Moreover, the ciprofloxacin-NAH adjuvants exhibited bactericidal activity with MBC/MIC ratios less than and equal to 4. The findings of molecular docking studies of NAH derivative compounds led to an assumption that the compounds acted as inhibitors of DNA gyrase in DNA replication, enhancing the activity of ciprofloxacin which also targeted DNA gyrase.

Moving on, synergism was observed in all antibiotic-NAH adjuvant combinations based on the FIC index calculated, but neither of the adjuvant combinations was recommended for further studies. Both streptomycin-NAH adjuvant and chloramphenicol-NAH adjuvant combinations did not obtain MBC values, indicating that the combinations could not kill bacteria. For ciprofloxacin-NAH adjuvant combinations, most of the combinations exhibited insignificant interactions, suggesting that the bactericidal effect from the MBC/MIC ratio was contributed by ciprofloxacin itself.

Further studies on the mechanism of action of NAH derivative compounds can be conducted using affinity chromatography to enhance the understanding of the antibacterial mechanism exhibited by the compounds. As MBC was obtained in ciprofloxacin-NAH adjuvant combinations, a time-kill curve can be conducted to analyse the impact of concentration or time on the effectiveness of the adjuvants and the bactericidal activity over time. Next, adding more categories of antibiotics can help in the studies of drug interaction. Additional members of the antibiotic from the same class can be incorporated into the studies to validate the mechanism of action of the NAH derivative compounds as adjuvants.

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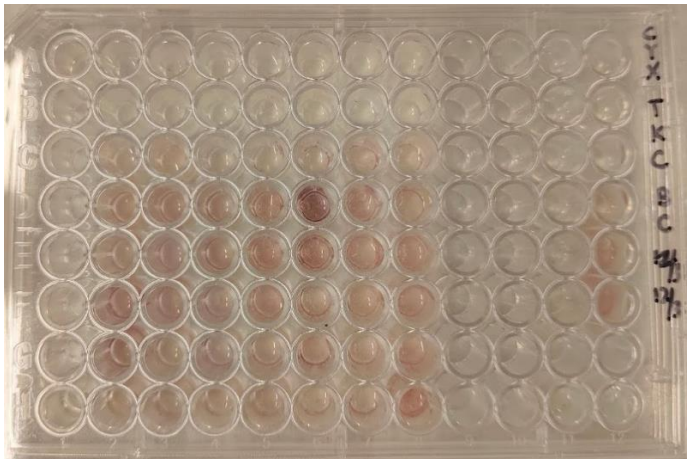
APPENDICES

APPENDIX A

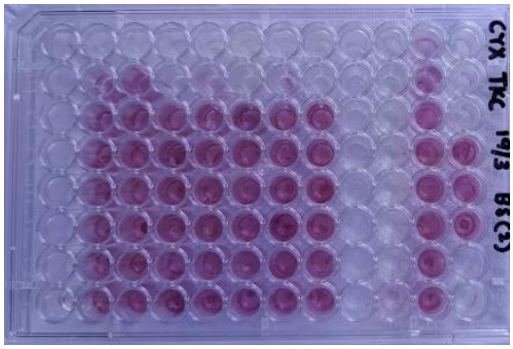
(A)



(B)

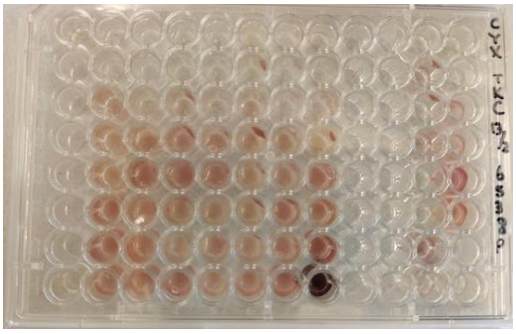
Figure A.1 (A) and (B): The duplicates of MIC of NAH derivative compounds against *B. cereus* (ATCC 13061).

(A)



(B)

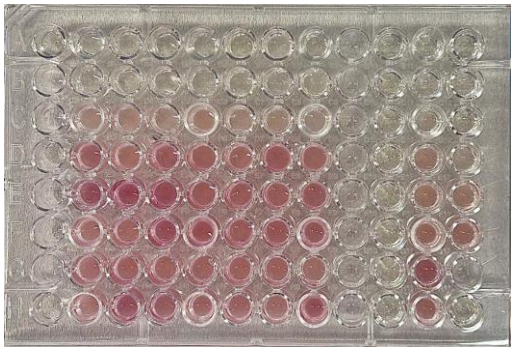
Figure A.2 (A) and (B): The duplicates of MIC of NAH derivative compounds against *B. subtilis* subsp. *spizizenni* (ATCC 6633).



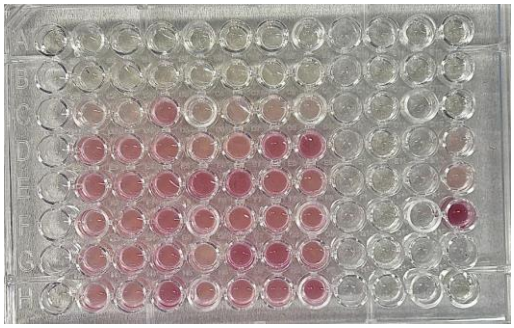
(A)

(B)

Figure A.3 (A) and (B): The duplicates of MIC of NAH derivative compounds against *S. aureus* (ATCC 6538).

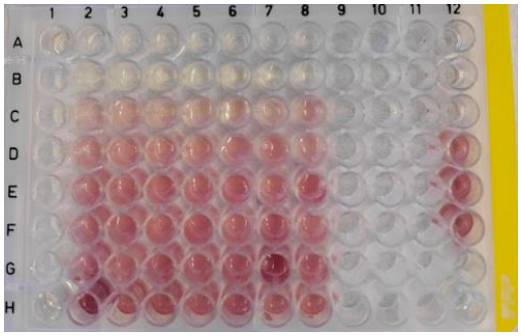


(A)

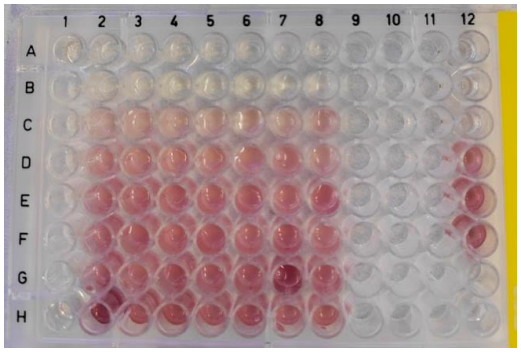


(B)

Figure A.4 (A) and (B): The duplicates of MIC of NAH derivative compounds against *E. coli* (ATCC 25922).

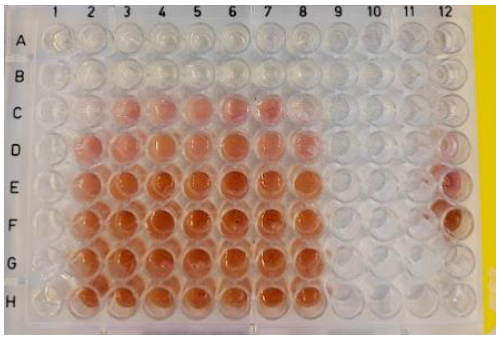


(A)

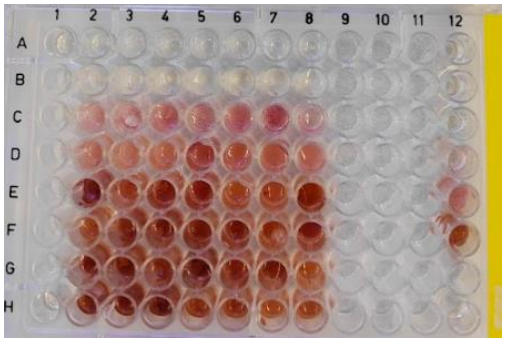


(B)

Figure A.5 (A) and (B): The duplicates of MIC of NAH derivative compounds against *S.* Typhimurium (ATCC 14028).

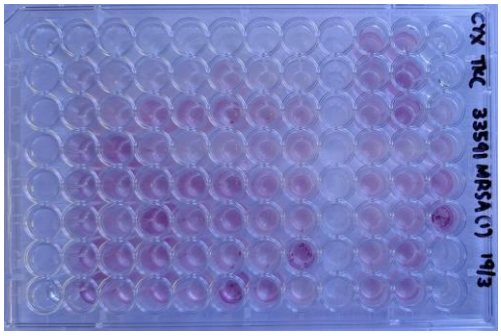


(A)

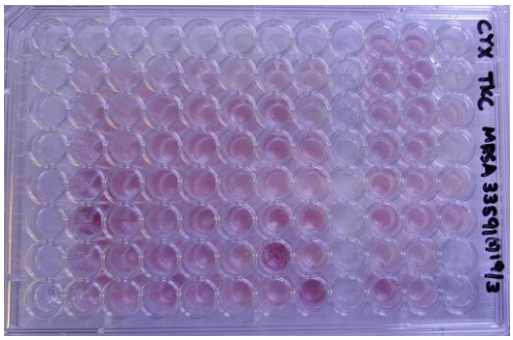


(B)

Figure A.6 (A) and (B): The duplicates of MIC of NAH derivative compounds against *P. aeruginosa* (ATCC 27853).

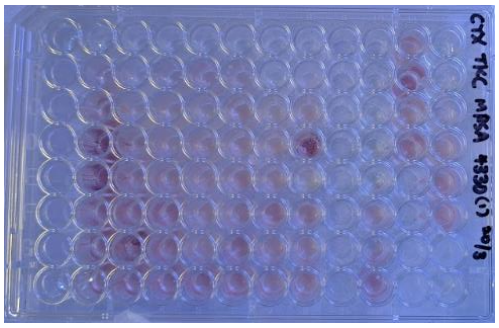


(A)

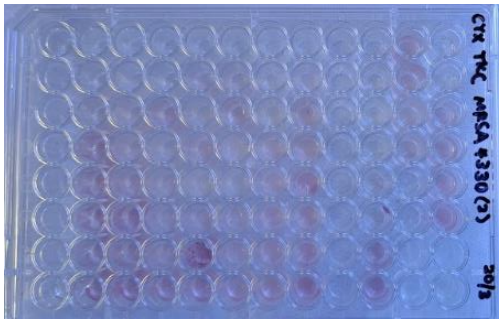


(B)

Figure A.7 (A) and (B): The duplicates of MIC of NAH derivative compounds against MRSA (ATCC 33591).



(A)



(B)

Figure A.8 (A) and (B): The duplicates of MIC of NAH derivative compounds against MRSA (ATCC 43300).

APPENDIX B

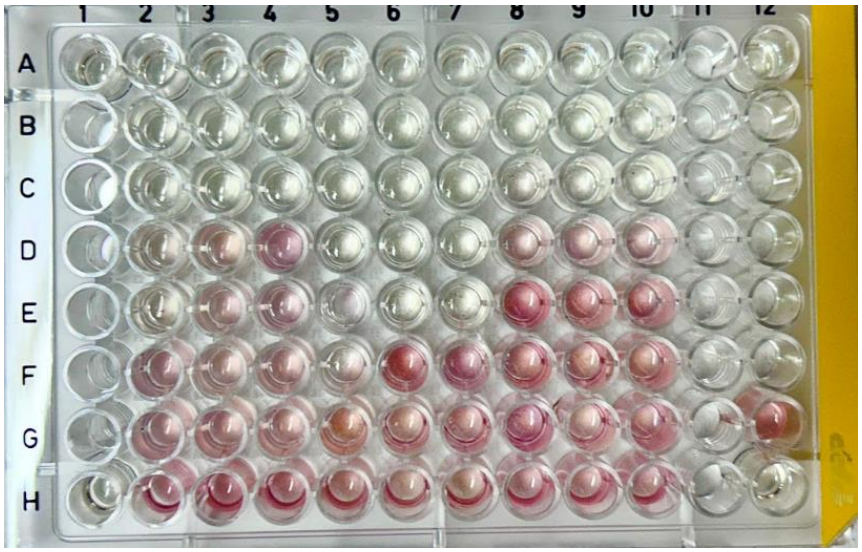


Figure B.1: The MIC of streptomycin (Columns 2 – 4), ciprofloxacin (Columns 5 – 7) and chloramphenicol (Columns 8 – 10) against *B. cereus* (ATCC 13061) in triplicates.

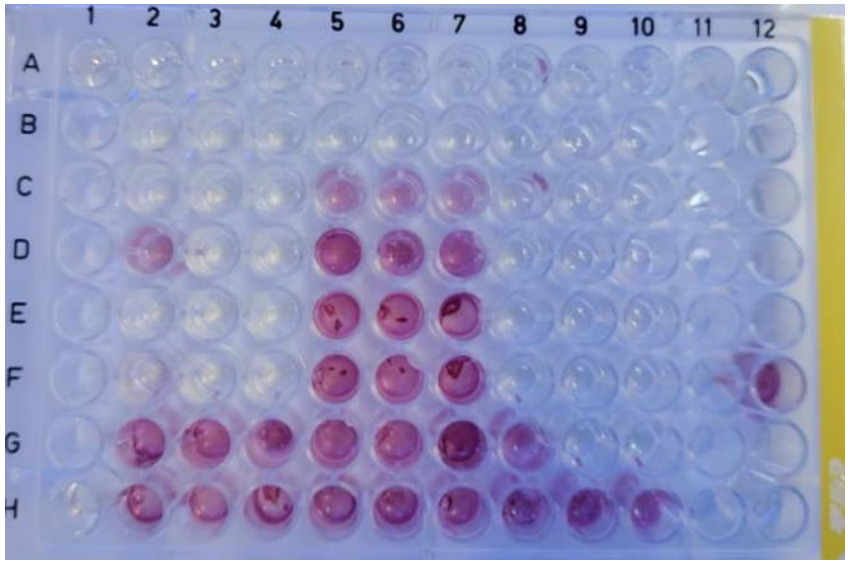


Figure B.2: The MIC for streptomycin (Columns 2 – 4), chloramphenicol (Columns 5 – 7) and ciprofloxacin (Columns 8 – 10) against *B. subtilis* subsp. *spizizenni* (ATCC 6633) in triplicates.

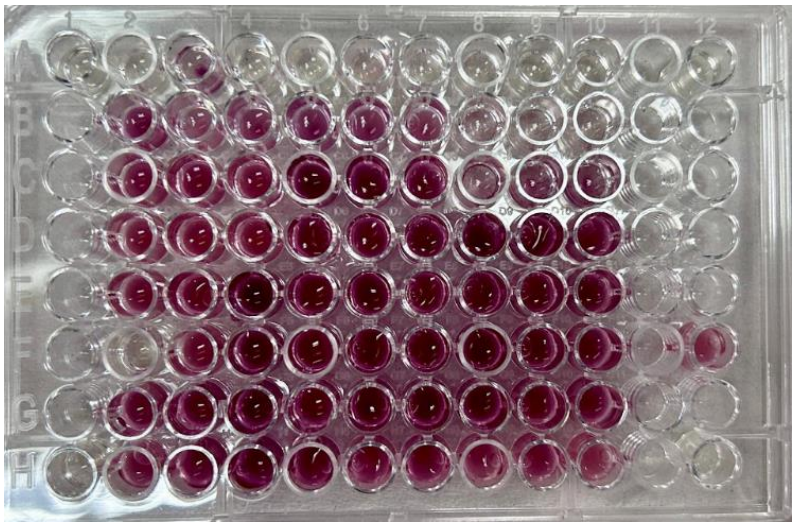
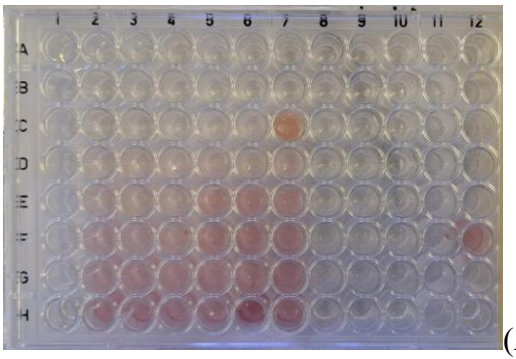


Figure B.3: The MIC for streptomycin (Columns 2 – 4), chloramphenicol (Columns 5 – 7) and ciprofloxacin (Columns 8 – 10) against *S. aureus* (ATCC 6538) in triplicates.

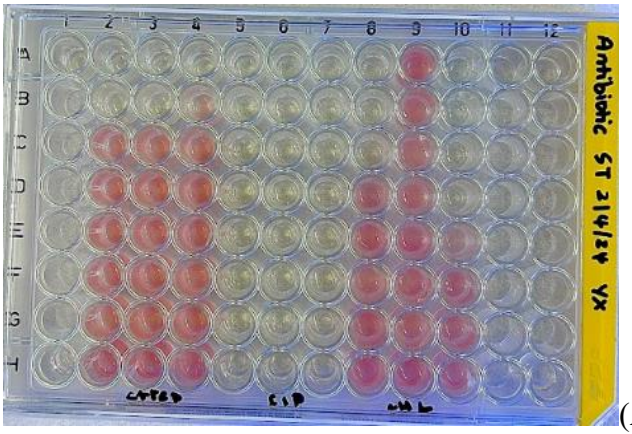


(A)

Figure B.4 (A): The MIC for streptomycin (Columns 2 – 4) and chloramphenicol (Columns 5 – 7) against *E. coli* (ATCC 25922) in triplicates.

(B)

Figure B.4 (B): The MIC for ciprofloxacin (Columns 2 – 7) against *E. coli* (ATCC 25922) in triplicates.



(A)

Figure B.5 (A): The MIC for streptomycin (Columns 2 – 4) against *S.* Typhimurium (ATCC 14028) in triplicates.

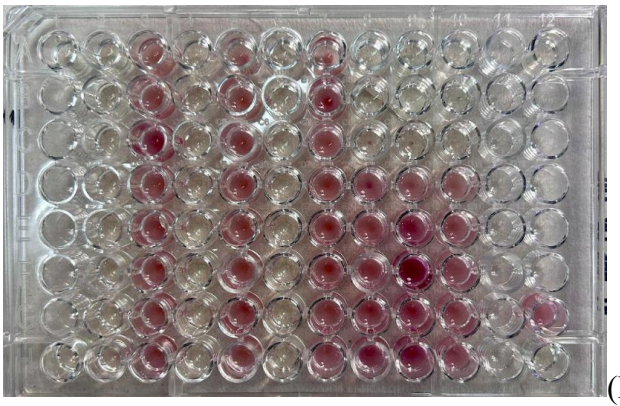
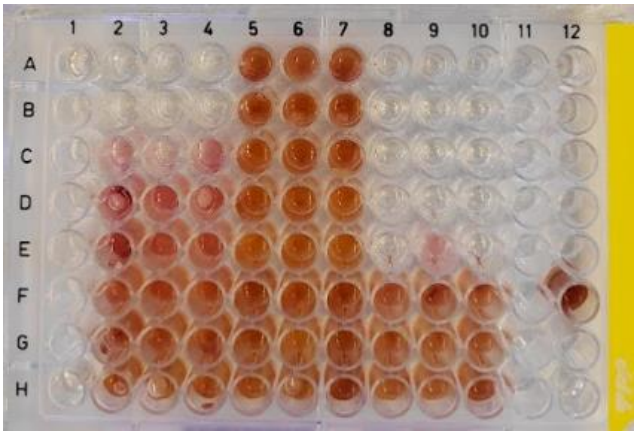

(B)

Figure B.5 (B): The MIC for ciprofloxacin (Columns 2 – 7) and chloramphenicol (Columns 8 – 10) against *S.* Typhimurium (ATCC 14028) in triplicates.



(A)

Figure B.6 (A): The MIC for streptomycin (Columns 2 – 4) and ciprofloxacin (Columns 8 – 10) against *P. aeruginosa* (ATCC 27853) in triplicates.

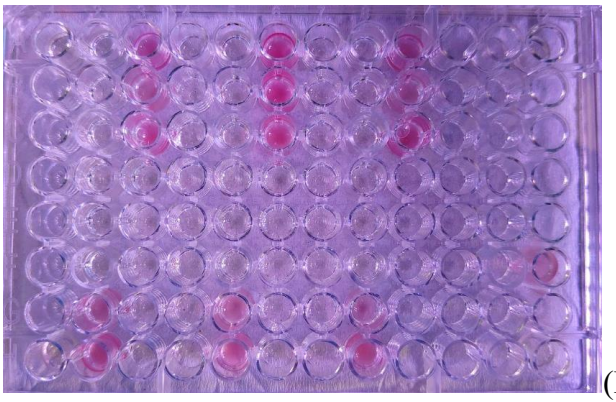
 (B)

Figure B.6 (B): The MIC for chloramphenicol at 200 µg/mL (Columns 2 – 3, Columns 5 – 6 and Columns 8 – 9) against *P. aeruginosa* (ATCC 27853) in triplicates.

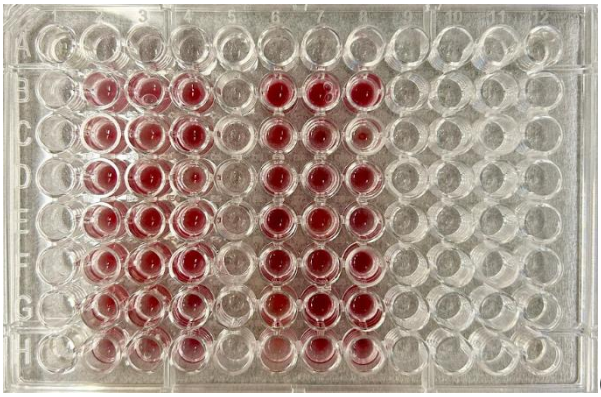
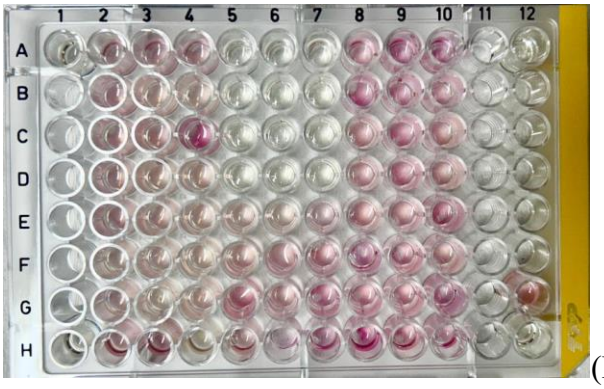
 (A)

Figure B.7 (A): The MIC for streptomycin at 3200 µg/mL (Columns 2 – 4) and chloramphenicol at 200 µg/mL (Columns 6 – 8) against MRSA (ATCC 33591) in triplicates.

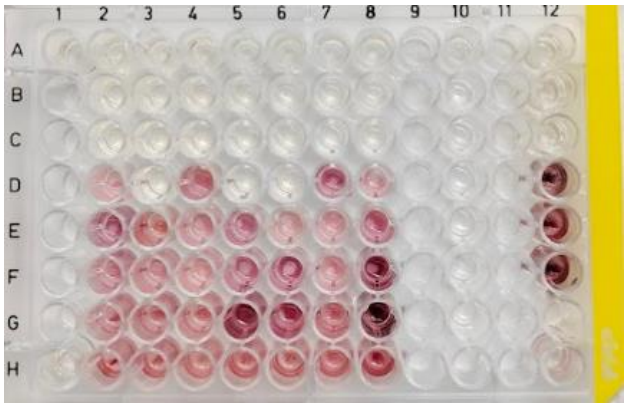


(B)

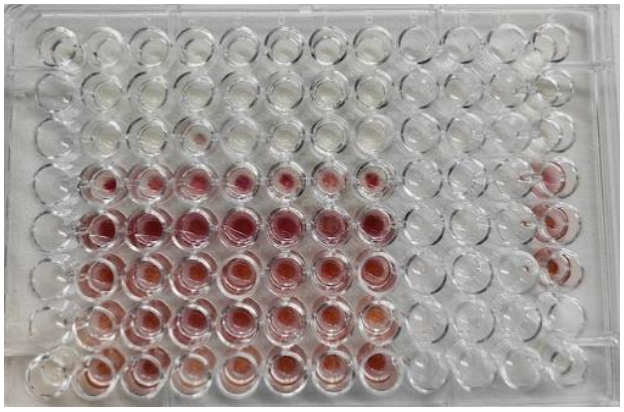
Figure B.7 (B): The MIC for ciprofloxacin (Columns 5 – 7) against MRSA (ATCC 33591) in triplicates.

Figure B.8: The MIC for streptomycin (Columns 2 – 4), chloramphenicol (Columns 5 – 7) and ciprofloxacin (Columns 8 – 10) against MRSA (ATCC 43300) in triplicates.

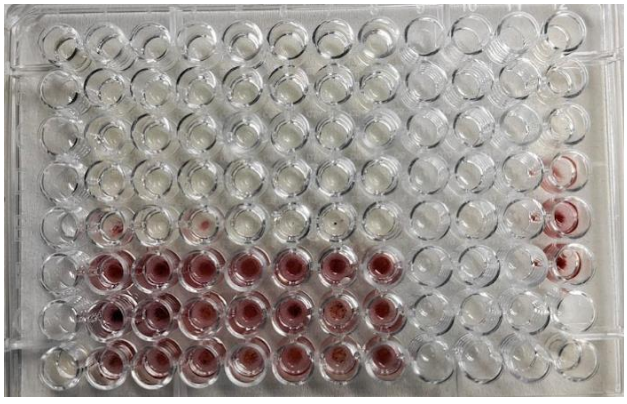
APPENDIX C



(A)

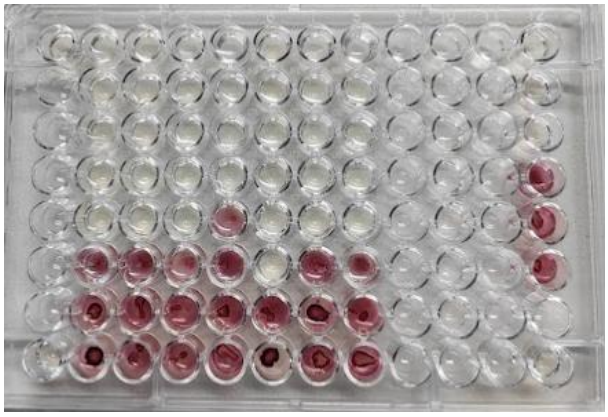


(B)

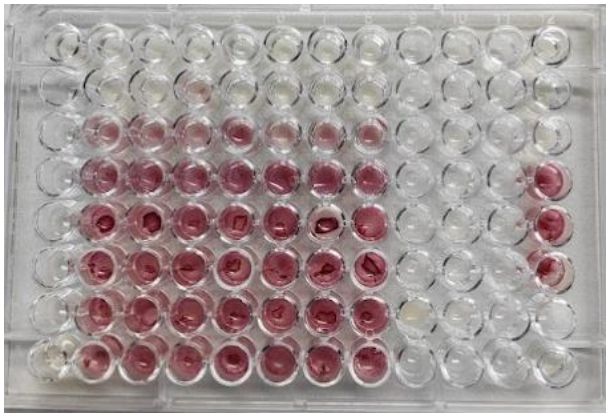


(C)

Figures C.1, C.2 and C.3: The MIC of NAH derivative compounds in combination with streptomycin **(A)**, chloramphenicol **(B)** and ciprofloxacin **(C)** as adjuvants against *B. cereus* (ATCC 13061).



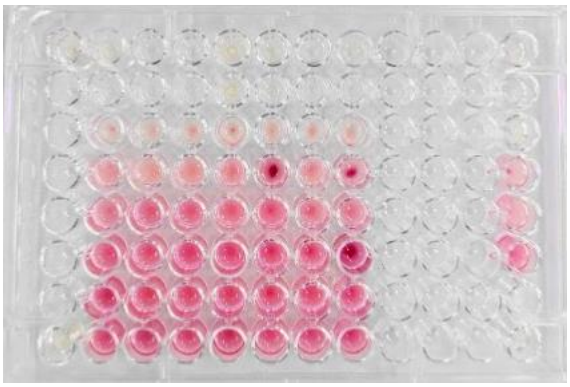
(A)



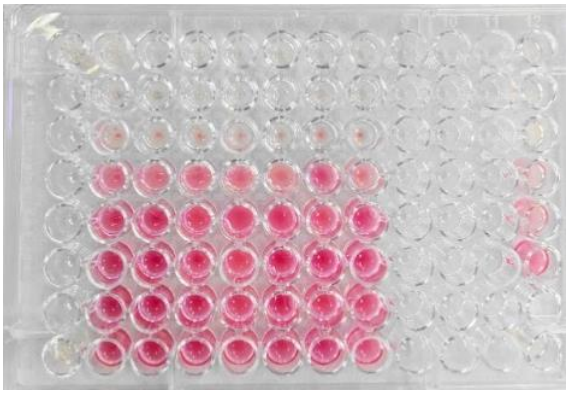
(B)

(C)

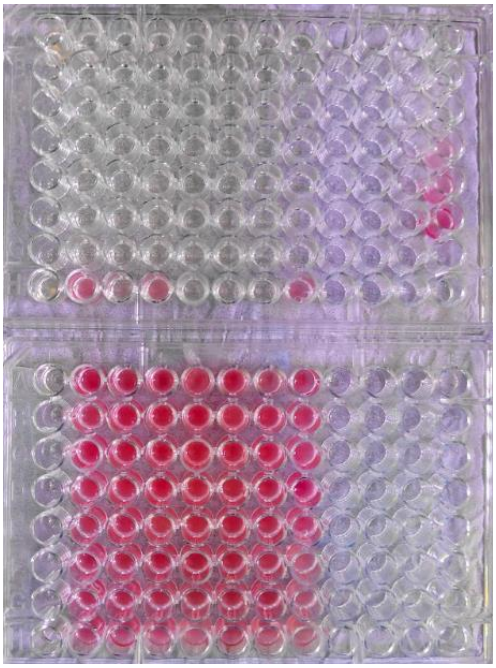
Figures C.4, C.5 and C.6: The MIC of NAH derivative compounds in combination with streptomycin **(A)**, chloramphenicol **(B)** and ciprofloxacin **(C)** as adjuvants against *B. subtilis* subsp. *spizizenni* (ATCC 6633).



(A)

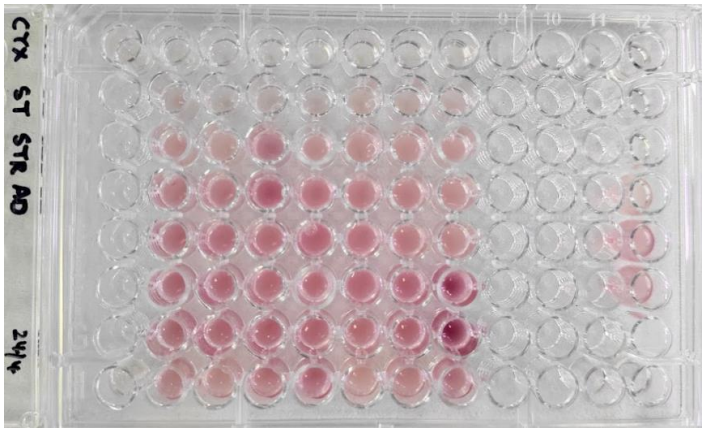


(B)

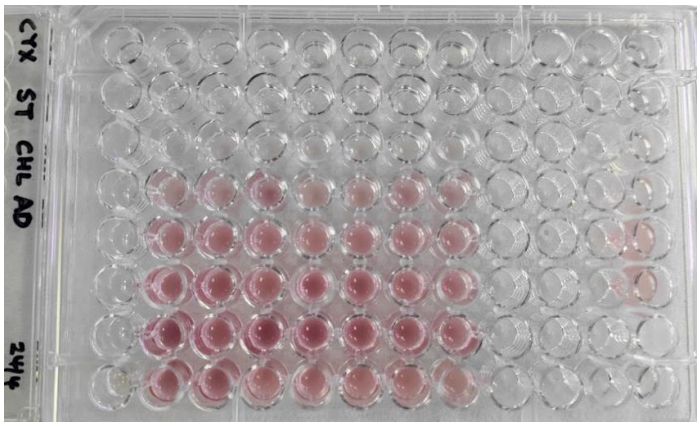


(C)

Figures C.7, C.8 and C.9: The MIC of NAH derivative compounds in combination with streptomycin **(A)**, chloramphenicol **(B)** and ciprofloxacin **(C)** as adjuvants against *E. coli* (ATCC 25922).



(A)



(B)

(C)

Figures C.10, C.11 and C.12: The MIC of NAH derivative compounds in combination with streptomycin **(A)**, chloramphenicol **(B)** and ciprofloxacin **(C)** as adjuvants against *S.* Typhimurium (ATCC 14028).

APPENDIX D

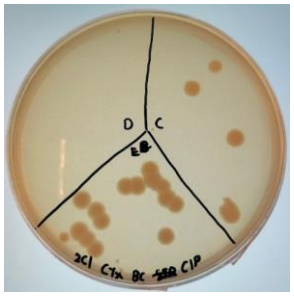


Figure D.1: The MBC plate for NAH derivative compound 2 (2-Cl) in combination with ciprofloxacin as adjuvant against *B. cereus* (ATCC 13061).

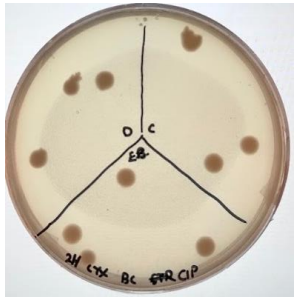


Figure D.2: The MBC plate for NAH derivative compound 4 (H) in combination with ciprofloxacin as adjuvant against *B. cereus* (ATCC 13061).

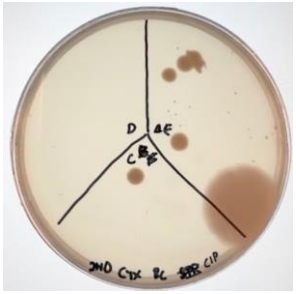


Figure D.3: The MBC plate for NAH derivative compound 5 (2-NO_2) in combination with ciprofloxacin as adjuvant against *B. cereus* (ATCC 13061).

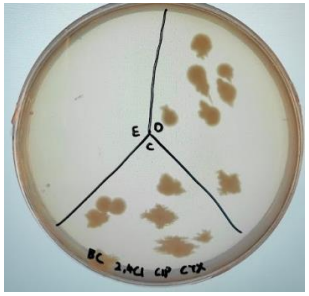


Figure D.4: The MBC plate for NAH derivative compound 7 (2,4-Cl_2) in combination with ciprofloxacin as adjuvant against *B. cereus* (ATCC 13061).

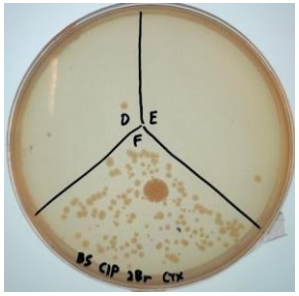


Figure D.5: The MBC plate for NAH derivative compound 1 (2-Br) in combination with ciprofloxacin as adjuvant against *B. subtilis* subsp. *spizizenni* (ATCC 6633).

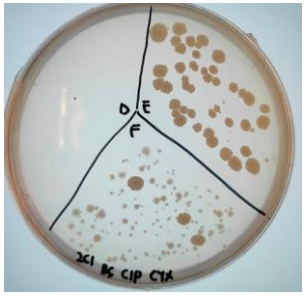


Figure D.5: The MBC plate for NAH derivative compound 2 (2-Cl) in combination with ciprofloxacin as adjuvant against *B. subtilis* subsp. *spizizenni* (ATCC 6633).

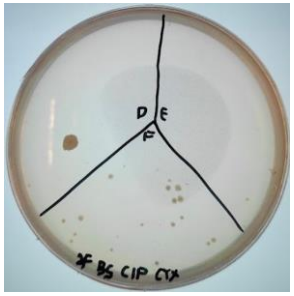


Figure D.6: The MBC plate for NAH derivative compound 3 (2-F) in combination with ciprofloxacin as adjuvant against *B. subtilis* subsp. *spizizenni* (ATCC 6633).

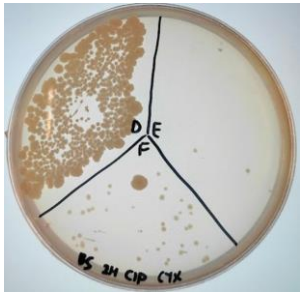


Figure D.7: The MBC plate for NAH derivative compound 4 (H) in combination with ciprofloxacin as adjuvant against *B. subtilis* subsp. *spizizenni* (ATCC 6633).

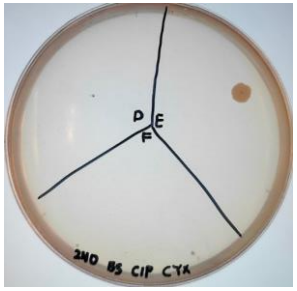


Figure D.8: The MBC plate for NAH derivative compound 5 (2-NO_2) in combination with ciprofloxacin as adjuvant against *B. subtilis* subsp. *spizizenni* (ATCC 6633).

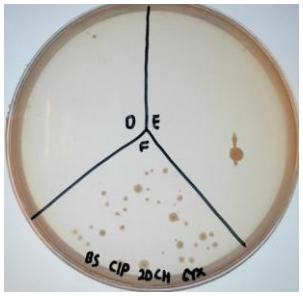


Figure D.9: The MBC plate for NAH derivative compound 6 (2-OCH_3) in combination with ciprofloxacin as adjuvant against *B. subtilis* subsp. *spizizenni* (ATCC 6633).

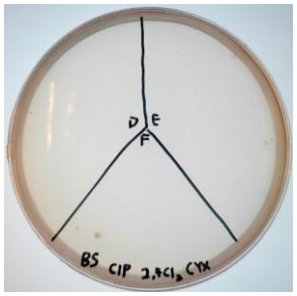


Figure D.10: The MBC plate for NAH derivative compound 7 (2,4-Cl_2) in combination with ciprofloxacin as adjuvant against *B. subtilis* subsp. *spizizenni* (ATCC 6633).

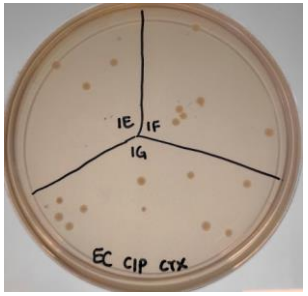


Figure D.11: The MBC plate for NAH derivative compound 1 (2-Br) in combination with ciprofloxacin as adjuvant against *E. coli* (ATCC 25922).

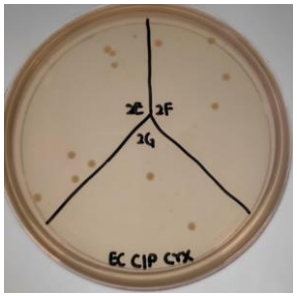


Figure D.12: The MBC plate for NAH derivative compound 2 (2-Cl) in combination with ciprofloxacin as adjuvant against *E. coli* (ATCC 25922).

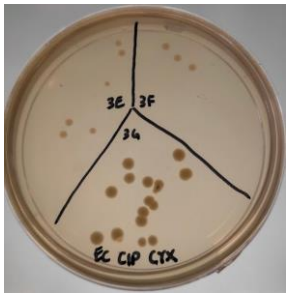


Figure D.13: The MBC plate for NAH derivative compound 3 (2-F) in combination with ciprofloxacin as adjuvant against *E. coli* (ATCC 25922).

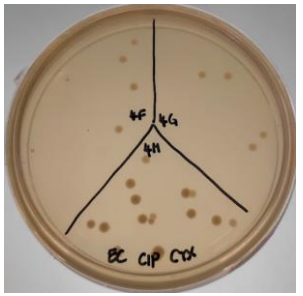


Figure D.14: The MBC plate for NAH derivative compound 4 (H) in combination with ciprofloxacin as adjuvant against *E. coli* (ATCC 25922).

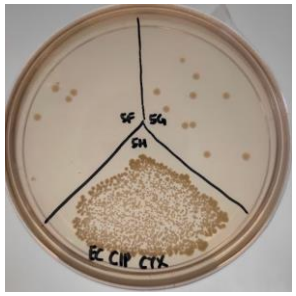


Figure D.15: The MBC plate for NAH derivative compound 5 (2-NO_2) in combination with ciprofloxacin as adjuvant against *E. coli* (ATCC 25922).

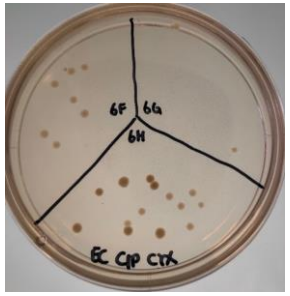


Figure D.16: The MBC plate for NAH derivative compound 6 (2-OCH_3) in combination with ciprofloxacin as adjuvant against *E. coli* (ATCC 25922).

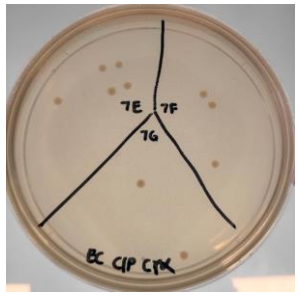


Figure D.17: The MBC plate for NAH derivative compound 7 (2,4-Cl_2) in combination with ciprofloxacin as adjuvant against *E. coli* (ATCC 25922).

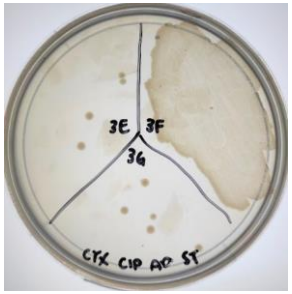


Figure D.18: The MBC plate for NAH derivative compound 1 (2-Br) in combination with ciprofloxacin as adjuvant against *S.* Typhimurium (ATCC 14028).

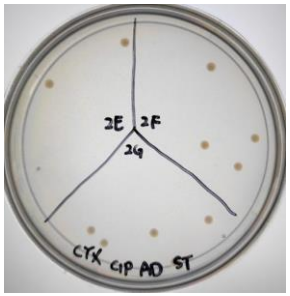


Figure D.19: The MBC plate for NAH derivative compound 2 (2-Cl) in combination with ciprofloxacin as adjuvant against *S.* Typhimurium (ATCC 14028).

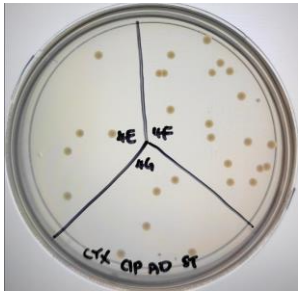


Figure D.20: The MBC plate for NAH derivative compound 3 (2-F) in combination with ciprofloxacin as adjuvant against *S.* Typhimurium (ATCC 14028).

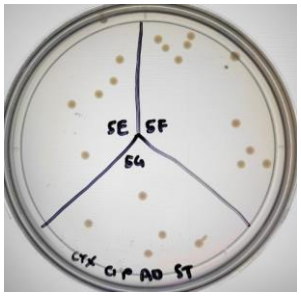


Figure D.21: The MBC plate for NAH derivative compound 4 (H) in combination with ciprofloxacin as adjuvant against *S.* Typhimurium (ATCC 14028).

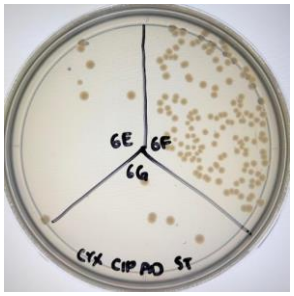


Figure D.22: The MBC plate for NAH derivative compound 5 (2-NO_2) in combination with ciprofloxacin as adjuvant against *S.* Typhimurium (ATCC 14028).

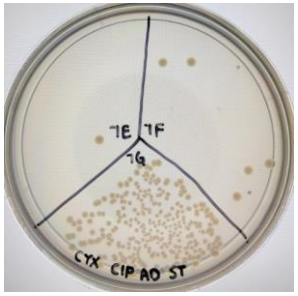


Figure D.23: The MBC plate for NAH derivative compound 6 (2-OCH_3) in combination with ciprofloxacin as adjuvant against *S.* Typhimurium (ATCC 14028).

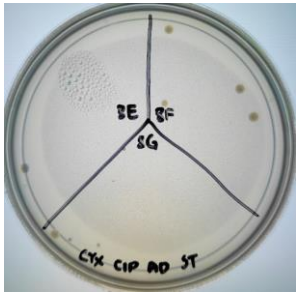


Figure D.24: The MBC plate for NAH derivative compound 7 (2,4-Cl_2) in combination with ciprofloxacin as adjuvant against *S.* Typhimurium (ATCC 14028).

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FACULTY OF SCIENCE

Full Name(s) of Candidate(s)	Chan Yao Xiang
ID Number(s)	20ADB03747
Programme / Course	Bachelor of Science (Honours) Food Science
Title of Final Year Project	Antibacterial Activity of 5-Chloro Substituted Phenyl *N*-Acylhydrazones with Aromatic Substitution at Ortho- and Para-Directors as Potent Adjuvants

Similarity	**Supervisor's Comments (Compulsory if parameters of originality exceeds the limits approved by UTAR)**
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KahCheng

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