ASSESSING THE COMBINATIONS OF 'OLD' POLYMYXIN DRUG AND MEDICINAL PLANT EXTRACTS IN COMBATING GRAM-NEGATIVE BACTERIA TOWARDS DEVELOPMENT OF HALAL AND TOYYIB ANTIBIOTICS

BY

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A thesis submitted in fulfilment of the requirement for the degree of Master of Science (Halal Industry Science)

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ABSTRACT

A rapid increase of bacterial resistance particularly Gram-negative bacteria is a major threat to human health as many standard antibiotics have become less effective in treating infectious diseases. With the fairly dry discovery and development pipeline of novel drugs, it is very unlikely to obtain new drugs in the near future. Polymyxins, an 'old' antibiotic class, has been revived as the last-line therapeutic arsenal for infections caused by multidrug-resistant (MDR) Gram-negative bacteria. Nevertheless, its application in clinical practice was restrained due to a number of nephrotoxicity and neurotoxicity cases reported. Plant-based therapy has been considered safe, effective and has been promisingly integrated with modern medicine. Combination therapy is a potential approach in the fight against Gram-negative bacteria or 'superbugs' which comply with the toyyiban viewpoint in Halal pharmaceutical. Toyyib is an important aspect to ensure that the medicines must be non-hazardous, non-intoxicating, and safe for clinical practice according to the prescribed dosage. This study sought to investigate the effect of local medicinal plant extracts in combination with polymyxin B against Gram-negative bacteria. Selected plants such as *Annona muricata*, *Andrographis paniculate*, *Piper sarmentosum*, *Clinacanthus nutans* and *Aquilaria malaccensis* which are known for their antimicrobial property were extracted by soxhlet method. The crude ethanolic extracts were screened for their antibacterial activity and assessed for their killing activity in combination with polymyxin B against *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*. The selected extract was analyzed for their chemical constituents using Gas chromatography-mass spectrometry (GC-MS). The study revealed that, throughout ten plants that have been tested, only five plants possessed an antibacterial activity, with the highest was *A. malaccensis* leaves against *A. baumannii* ATCC 19606 and *K. pneumoniae* ATCC 10031. The combination of *A. malaccensis* extract (64 mg/mL) and polymyxin B (1 mg/L) was found to be able to inhibit the bacteria growth and exhibit a better bacterial killing compared to polymyxin B alone. *A. malaccensis* extract at 64 mg/mL also showed a bactericidal effect with a reduction of ≥ 3 $\log_{10}$ CFU/mL below the initial inoculum at 24 h against both *A. baumannii* and *K. pneumoniae* isolates. GC-MS analysis identified phytol, 9,12-octadecadienal, oleic acid, n-hexadecanoic acid, squalene which likely correlated to the antibacterial activity observed from the extract. The results highlighted the potential of polymyxin B and *A. malaccensis* extract combination to be further leveraged for the treatment against MDR Gram-negative bacteria. In addition, *A. malaccensis* extract is importantly a subject of potential antibiotic to be explored and developed.

خلاصة البحث

تعتبر الزيادة السريعة في المقاومة البكتيرية وخاصةً البكتيريا سالبة الجرام تهديدًا كبيرًا لصحة الإنسان حيث أصبحت العديد من المضادات الحيوية الاعتيادية أقل فعالية في علاج الأمراض المعدية. ويعدّ العلاج المركب نهجاً محتملاً في مكافحة البكتيريا سالبة الجرام المقاومة للأدوية المتعددة أو (آفة خارقة). سعت هذه الدراسة إلى تقصّي تأثير مستخلصات نباتية طبية محلية مختارة ودمجها مع polymyxin B ضد البكتيريا سالبة الجرام. تم استخلاص نباتات محلية مختارة مثل *Annona muricata* و *Andrographis paniculate* و *Piper sarmentosum* و *Clinacanthus nutans* و *Aquilaria malaccensis* المعروفة بخصائصها المضادة للميكروبات بواسطة جهاز سوكسليت للاستخلاص. وتمّ فحص المستخلصات الإيثانولية الخام لمعرفة نشاطها المضاد للميكروبات وتقييم نشاطها للقضاء على البكتيريا بمفردها وبالاقتران مع polymyxin B ضدّ كلٍّ من *Acinetobacter baumannii* و *Pseudomonas aeruginosa* و *Klebsiella pneumoniae* باستخدام مكوناته الكيميائية من حيث تحليل المستخلص المختار . كشفت الدراسة أنه من بين عشر نباتات تمّ فحصها، تمتلك خمس نباتات منها فقط نشاطًا مضادًا للبكتيريا، وكان أعلاها أوراق *A. malaccensis* ضدّ كلٍّ من *A. baumannii* ATCC 19606 و *K. pneumoniae* ATCC 10031 (64 ملجم/ *A. malaccensis*. وقد وُجِد أن مزيجاً من مستخلص (1 ملجم / لتر) كان قادراً على قمح المقاومة البكتيرية وأظهر إبادةً بكتيرية أفضل مقارنة بـ polymyxin B (مل و عند 64 ملجم/ *A. malaccensis* منفردًا. بالإضافة إلى ذلك فقد أظهر مستخلص polymyxin B بالبوليميكسين بـ دون اللقاح الأولي عند 24 ساعة 3 $\log_{10}$ CFU/mL مل تأثيراً مبيداً للجراثيم مع اختزال بقيمة مساوية أو أكبر من وجود فيتول ، 9،12-GC-MS. وأكّد تحليل *K. pneumoniea* و *A. baumannii* ضدّ كلٍّ من عزلات ، و سكوالين والذي من المحتمل أن تكون مرتبطة n-hexadecanoic acid ، حمض الأوليك ، octadecadienal و polymyxin B بالنشاط المضاد للبكتيريا الذي لوحظ من المستخلص. سلطت النتائج الضوء على قدرة مزيج من *A. malaccensis* وإضافة إلى ذلك يعتبر مستخلص MDR في العلاج ضد البكتيريا سالبة الجرام *A. malaccensis* مستخلص موضوعًا مهمًا لإمكانية استكشاف وتطوير لمضاد حيوي.*malaccensis*

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Science (Halal Industry Science)

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DECLARATION

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In the name of Allah, Most Precious Most Merciful.

At this moment of submission of my thesis entitled “Assessing the Combinations of ‘Old’ Polymyxin Drug and Medicinal Plant Extracts in Combating Gram-Negative Bacteria towards Development of Halal and Toyyib Antibiotics”, I would like to praise The Almighty God for strength and blessing and to give me a chance to complete this thesis.

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LIST OF ABBREVIATIONS

AKI	Acute kidney injury
AME	*Aquilaria malaccensis* extract
ATCC	American Type Culture Collection
CAMHB	Cation-adjusted Mueller-Hinton broth
CDC	Centers for Disease Control and Prevention
CFU	Colony forming unit
CRE	*Enterobacteriaceae*
CTRL	Growth control
Dab	Diaminobutyric acid
DMSO	Dimethylsulfoxide
GC-MS	Gas chromatography-mass spectrometry
h	Hour
IZ	Inhibition zone
LPS	Lipopolysaccharide
MDR	Multidrug-resistant
MBC	Minimum bactericidal concentration
MIC	Minimum inhibitory concentration
mg	Milligram
mL	Mililiter
PCE	Plant crude extract
PK/PD	Pharmacokinetics/pharmacodynamics
PmB	Polymyxin B
Pos	Position
RT	Retention time
Sol.	Solution

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

The rapid emergence of Multidrug-resistant (MDR) Gram-negative bacteria and the dry discovery and development pipeline of novel antibiotics is a major healthcare challenge worldwide (Ventola, 2015). With very little hope and limited capacity to obtain new drugs in the near future, the optimization of currently available drugs is strategically employed against bacterial resistance.

Polymyxin, an old class of cationic antibiotic is an important last-line weapon for MDR Gram-negative bacteria. However, polymyxin-induced toxicity has become a major concern. With dose-limiting nephrotoxicity, pharmacokinetics/pharmacodynamics (PK/PD) data demonstrate that polymyxin monotherapy is unable to achieve *in vivo* efficacious plasma concentration which may lead to treatment failure and occurrence of resistance (Bergen et al., 2013).

Plant-based therapy has been considered safe, effective and has been promisingly integrated with modern medicine (Zhang, Onakpoya, Posadzki, & Eddouks, 2015). Abundant medicinal plants have been used from time immemorial to treat ailments and to preserve human health (Yadav & Agarwala, 2011). Maximum therapeutic efficacy of a plant extract is assumed due to the collective synergy contributed by its phytochemical constituents. Due to the diversity of bioactive compounds with potential biological activity possessed by plants, it is considered as a good option for the development of new alternative and effective drugs (Zaidan et al., 2005).

A drug combination therapy is a strategy used either to combine two different antibiotics or a combination of antibiotics with a plant extract producing a synergistic effect (Chanda & Rakholiya, 2011). Notably, a combination between plant extracts and antibiotics has been shown to significantly induce synergistic killing, including against

Gram-negative bacteria (Stefanović, 2018). Therefore, polymyxin combination therapy with potential plant extracts was explored as an alternative to overcome the limitations in polymyxin monotherapy.

The concept of halal pharmaceutical is not limited to the use of haram (forbidden) materials in medicinal products but also concerned about the quality and safety elements of the product which is known as toyyib (Halim et al., 2014). Toyyib is an important aspect of halal pharmaceuticals which is to ensure that the medicines must be non-hazardous, non-intoxicating, and safe for clinical practice according to prescribed dosage (Malaysian Standard, 2012). A previous clinical study showed that the significant advantage of employing the combinatory approach is that it can significantly minimize toxicity and reduce the risk of side effects by allowing the use of lower concentrations of each antibiotic (Rigatto et al., 2015). In this research, combination therapy of polymyxin and plant extract was adopted to enhance the efficacy and reduce the toxicity of the drugs which comply with the toyyiban viewpoint in Halal pharmaceutical.

This study reports the finding of antibacterial activity of selected local medicinal plant extracts alone and the combination of polymyxin with selected plant extract against Gram-negative bacteria. The antibacterial susceptibility test was done by disc diffusion method, minimum inhibitory concentration and minimum bactericidal concentration was measured by broth microdilution method while static time-kill analysis was performed to evaluate the combined effect of polymyxin-plant extract against the bacterial isolates.

1.2 STATEMENT OF THE PROBLEM

The rising incidence of bacterial infections due to Multidrug-resistant (MDR) pathogens is one of the greatest threats to human health in modern medicine globally. Worryingly, a concern is associated with the rapid emergence of MDR bacteria that are resilient to almost all antibiotics used for the treatment of infectious diseases. Notably, many research reported on the increase in the prevalence of carbapenems-resistance in the last few decades including *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and other carbapenem-

resistant *Enterobacteriaceae* (CRE) that contributed to unacceptably high mortality rates. With the fairly dry discovery and development pipeline of novel drugs, it is very unlikely to obtain new drugs in the near future. Therefore, thorough investigation and optimization of the existing antibiotics and bioactive compounds with antimicrobial properties is highly needed. Polymyxins, an 'old' antibiotic class, has been revived as the last-line therapeutic arsenal for infections caused by MDR Gram-negative bacteria. Nevertheless, its application in clinical practice was restrained due to a number of nephrotoxicity and neurotoxicity cases reported. In addition, with the limited pharmacokinetics/pharmacodynamics (PK/PD) profiles of polymyxins, polymyxin monotherapy is unlikely to produce efficacious plasma concentration which may lead to treatment failure with the potential emergence of heteroresistant sub-population. Plant derived-bioactive compounds are considered safe, low risk of side effects, and effective alternatives as antimicrobial agents. To optimize and highlight the significant importance of local medicinal plants, the combination of bioactive compounds with polymyxins is targeted to produce synergistic killing against Gram-negative bacteria.

1.3 RESEARCH OBJECTIVES

The central objective of this study was to examine the killing activity of the selected local medicinal plant extracts when in combination with polymyxins against Gram-negative bacteria.

Specific objectives:

1. To extract bioactive phytochemical compounds from selected local medicinal plants using Soxhlet extraction method.
2. To evaluate the effect of *in vitro* antibacterial activity of selected crude plant extracts against Gram-negative bacteria (i.e. *A. baumannii*, *Pseudomonas aeruginosa*, and *K. pneumoniae*) via disc diffusion method, minimum inhibitory concentration, and minimum bactericidal concentration.

3. To investigate *in vitro* antibacterial activity of polymyxin B in combination with selected plant extracts against Gram-negative bacteria (i.e. *A. baumannii*, *P. aeruginosa*, and *K. pneumoniae*) via time-kill analysis.
4. To identify chemical compounds of selected plant extracts via Gas Chromatography Mass Spectrometry analysis.

1.4 RESEARCH QUESTION

i. Does the crude plant extract alone inhibit and kill Gram-negative bacteria (i.e. *A. baumannii*, *P. aeruginosa*, and *K. pneumoniae*).?
ii. What are the effects of crude plant extract and polymyxin B combination against Gram-negative bacteria (i.e. *A. baumannii*, *P. aeruginosa*, and *K. pneumoniae*)?
iii. What are the chemical compounds of selected plant extract?

1.5 RESEARCH HYPOTHESIS

That combination of polymyxins and crude plant extracts exhibit antibacterial effects against Gram-negative bacteria.

1.6 SIGNIFICANCE OF THE STUDY

As bacterial resistance is spreading, the development of new and effective antimicrobial agents has to be expedited. A latter approach, combination therapy may lead to new ways of overcoming these resistance mechanisms. However, extensive research on antibacterial activity of polymyxin has been concerned only on monotherapy or a combination between different standard antibiotics. This study focused on the combination therapy of polymyxin incorporating local medicinal plant extract as a means of therapy which has been barely studied previously. The finding of this study will contribute to the development of new

classes of antimicrobial substances which may lead to new ways of overcoming the emergence of MDR, particularly Gram-negative bacteria. Furthermore, this study also contributed significantly to the progress and evolution of existing medicinal plant research in Malaysia for better utilization in the future especially in health care system.

1.7 SCOPE OF STUDY

The study focused on the antibacterial activity of polymyxin B in combination with selected local medicinal plants. A number of 10 plant extracts which are known for their antimicrobial property such as *A. muricata* (durian belanda), *A. paniculata* (hempedu bumi), *P. sarmentosum* (kadok), *M. citrifolia* (mengkudu), *F. deltoidei* (mas cotek), *M. koenigii* (daun kari), *C. caudatus* (ulam raja), *L. pumila* (kacip fatimah), *C. nutans* (belalai gajah) and *A. malaccensis* (gaharu) were chosen as the subject for this study. The leaves from the plants were extracted using the Soxhlet method with ethanol as a solvent. The screening of antibacterial activity of the crude ethanolic plant extracts was evaluated using the disc diffusion method against several laboratory strains of Gram-negative bacteria namely; *A. baumannii* ATCC 19606, *P. aeruginosa* ATCC 27853, and *K. pneumoniae* ATCC 10031 and ATCC 700603. Only the plant extracts with a most promising result from the disc diffusion test were further tested for *in vitro* antibacterial activity of polymyxin-plant combination therapy and identification of chemical compounds.

CHAPTER TWO

LITERATURE REVIEW

2.1 BAD BUGS, NO DRUGS

Bacteria will inevitably evolve and develop resistance towards antibiotics as their natural response. Multidrug-resistant (MDR) bacteria are defined as a microorganism that emerges resistant to several different classes of antibiotics by eliminating or reducing the effectiveness of drugs used in treating or preventing infections (Centre for Disease Control and Prevention [CDC], 2013). These pathogens have a variety of mechanisms to escape the action of many antibiotics used in medical practice. Some of the molecular and biochemical mechanisms of resistance include drug efflux mechanism (Li, Plésiat, & Nikaido, 2015), target modification and permeability alterations (Ruppé, Woerther, & Barbier, 2015), degradation of enzymes, and the chromosomal mutation of the drug (Naas, Dortet, & Iorga, 2016). The inverse relationship of the rapid emergence of MDR bacteria and the dry discovery and development pipelines of a new antibiotic is a significant global medical challenge (Schäberle & Hack, 2014). Worryingly, the threat is projected to persist and rise by 2050 which would potentially lead to a mortality rate of 10 million people every year (O'Neill, 2014). With today's global economic climate and challenging regulatory requirements thus requires a comprehensive effort to fully optimize the available antibacterial compounds or drugs to fight against MDR bacterial infections.

2.1.1 Gram-negative bacteria

Infectious diseases caused by bacteria are an extensive global health problem. In particular, Gram-negative bacteria has created a major bacterial resistance issue as they can become resistant to almost all drugs used for the treatment of infectious disease. Systemic infections from Gram-negative pathogen has contributed to the significant increase in global morbidity and mortality rates due to the lack of efficacious treatment regimens (CDC,

2013). Gram-negative bacteria contain a double membrane wall, external and internal. The external membrane expresses a potent immune response inducer, lipopolysaccharide (LPS), which excludes large or hydrophobic molecules. This feature slows the access of antibiotics that may cross it, hence augmenting the effectiveness of the internal line of defense. The internal layer of these bacteria consists of a cytoplasmic membrane efflux pump which will pump out back the antibiotics that enter the membrane. The combination of membrane impermeability and efflux clearance justify why Gram-negative bacteria are naturally more resilient (Livermore, 2012).

A number of Gram-negative bacteria that have been identified as a major threat for human infection and raised a major concern by the WHO are *A. baumannii*, *P. aeruginosa*, and *K. pneumoniae* (World Health Organization, 2017). Notably, many studies reported on the increase in the prevalence of carbapenem-resistance in the last few decades including, *A. baumannii*, *K. pneumoniae*, and other carbapenem-resistant *Enterobacteriaceae* (CRE) that contributed to unacceptably high mortality rates of up to 50% (Gupta et al., 2011). These bacteria also have been classified as *ESKAPE* organisms, which are recognized as one of the most emerging multi-drug resistant pathogens since they are effectively "escape" the actions of antibacterial drugs (Boucher et al., 2009).

2.1.2 *Acinetobacter baumannii*

A. baumannii is one of the species of Acinetobacter genus from Moraxellaceae family, non-motile, strictly aerobic, non-fastidious, and non-lactose fermenting (Bergogne-Bérézin & Towner, 1996). These organisms can be found throughout the environment and can be observed as short, plump, form smooth and greyish white colonies, and difficult to destain (Peleg, Seifert, & Paterson, 2008). The bacteria are known as one of the dangerous pathogens among the Gram-negative bacteria as it's responsible for opportunistic infections on urinary tract, bloodstream or pneumonia, skin, and other soft tissues (CDC, 2013). MDR *A. baumannii* is rising due to several virulence factors of antibiotic resistance mechanisms including evasion of the host immune response, able to adapt and survive in selective

environmental pressure and having an innate resistance mechanism that can facilitate colonization of patients (Lee et al., 2017).

This pathogenic bacteria have evolved to acquire resistance against broad-spectrum antibiotics, making it one of the alarming organisms in recent antibiotic era (Chen, Kuo, Chang, Cheng, & Yu, 2017). National Antibiotic Resistance Surveillance Report published in 2018 by Institute for Medical Research Malaysia revealed the data on antibiotic susceptibility testing (Institute for Medical Research [IMR], 2018). The analysis was performed on several bacteria strains including *A. baumannii* isolated from patients admitted to different hospitals throughout Malaysia. The report showed an increment in resistance rate for almost all antibiotics tested against *A. baumannii* samples for the last two years of observation (Figure 2.1). Although last resort treatments are available for this dangerous pathogen such as polymyxin, these antibiotics may cause severe side effects, including neurotoxicity or nephrotoxicity. In addition, *A. baumannii* is beginning to develop resistance to colistin (polymyxin E) in settings worldwide (Maraki, Mantadakis, Mavromanolaki, Kofteridis, & Samonis, 2016). Thus, the optimization of last line therapy may be a potential alternative in combating the infections of multi-drug resistant *A. baumannii.*

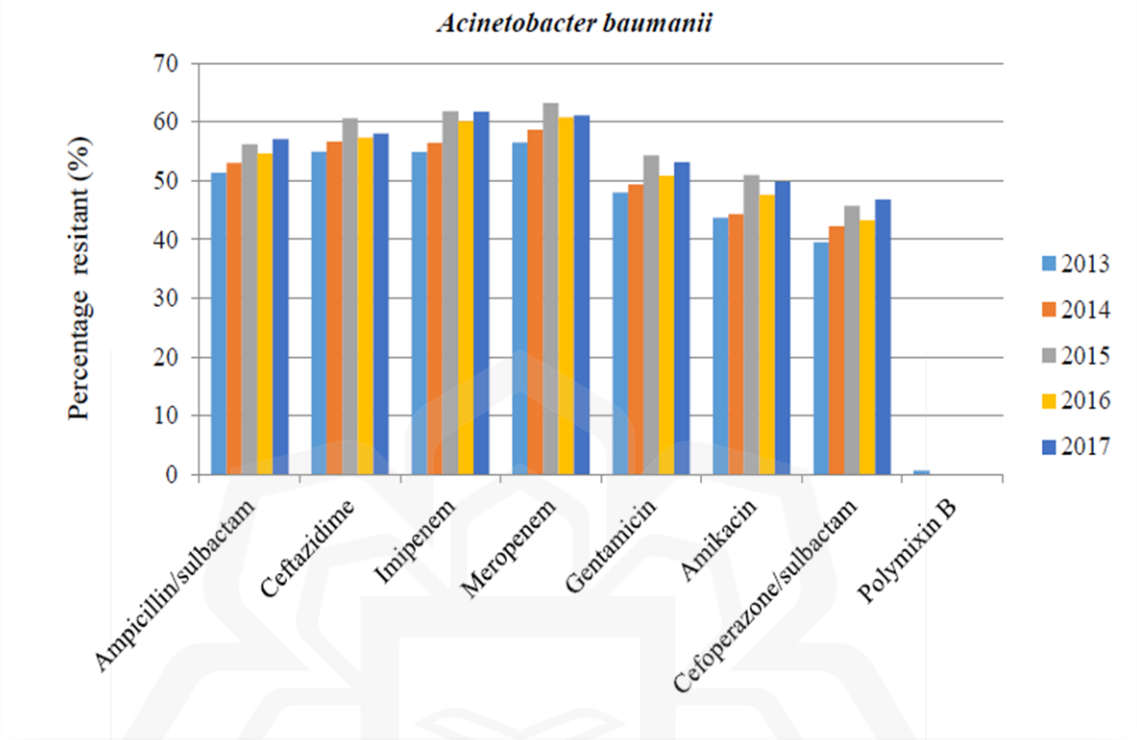

Figure 2.1 Percentage resistance for *A. baumannii* tested with several antibiotics
Source: (IMR, 2018).

2.1.3 Pseudomonas aeruginosa

P. aeruginosa is undoubtedly one of the most alarming pathogens of Gram-negative bacterium which belongs to Pseudomonadaceae family and known with a rod-structured, aerobic, and encapsulated bacterium. As a ubiquitous species, *P. aeruginosa* can be found abundantly in nature such as from plants, humans, and animal sources (Lister, Wolter, & Hanson, 2009).

This pathogen has gained a substantive concern worldwide not only due to its ability to cause a lot of healthcare-associated infections to humans, but also because of its high resistance to antibiotics. Centers for Disease Control and Prevention (CDC) had categorized *P. aeruginosa* in "serious" level of antibiotic resistance threats (CDC, 2013). The high antibiotic resistance of this microorganism comes from their low permeability of outer membrane which eventually limits the uptake of drug and substrate molecules (Pang, Raudonis, Glick, Lin, & Cheng, 2019). Another factor for the low antibiotic susceptibility of *P. aeruginosa* is due to the secondary resistance mechanism such as multidrug efflux pumps which involve chromosomally encoded antibiotic resistance protein (Druge et al., 2019).

A previous study of *P. aeruginosa* shows a potential trend in antibiotic resistance where it was found to be resistant towards almost all antibiotics including fluoroquinolones, aminoglycosides, beta-lactams, macrolides, sulphonamides, and tetracycline (Khan, Stapleton, Summers, Rice, & Willcox, 2020). Data by Yayan et al. in 2015 also revealed the significant increment in antibiotic-resistant of *P. aeruginosa* in patients with pneumonia due to *P. aeruginosa* from 2004 to 2014 (Figure 2.2). This pathogen showed a high resistance rate towards all antibiotics tested including against combination of two antibiotics, piperacillin and tazobactam (Yayan, Ghebremedhin, & Rasche, 2015). Therefore, the development of new antibiotics form is needed to keep up with resistant bacteria.

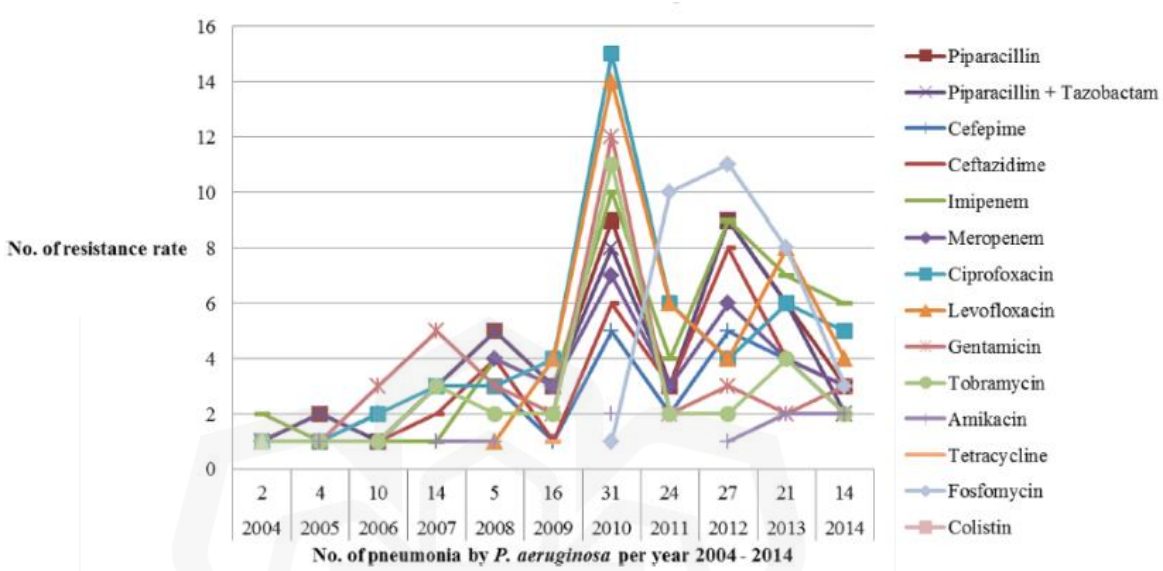

Figure 2.2 Number of antibiotic resistance rate over time for pneumonia due to *P. aeruginosa* from 2004 to 2014
Source: (Yayan et al., 2015)