ANTIMICROBIAL AND ANTIOXIDANT ACTIVITIES OF ENDOPHYTIC FUNGI ISOLATED FROM *Curcuma mangga*, WITH EMPHASIZE ON *Ceratobasidium ramicola* IBRLCM127

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by/oleh

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
BSLT	Brine Shrimp Lethality Test
BUT	Butanol
CCHF	Crimean-Congo haemorrhagic fever
CDC	Centers for Disease Control and Prevention
CFU	Colony Forming Unit
CLSI	Clinical and Laboratory Standard Institute
CYA	Czapek Yeast Extract
DCM	Dichloromethane
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	Diphenyl-pierylhydrazyl
EA	Ethyl acetate
EC_{50}	50% effective concentration
EID	Emerging infectious disease
EM	Electron Microscopy
GCMS	Gas Chromatography-Mass Spectrometry
HAI	Hospital-acquired infection
HMDS	Hexamathyldisilazine
IBRL	Industrial Biotechnology Research Laboratory
INT	p-iodonitrotetrazolium violet salt
ITS	Internal Transcribed Spacer
LC_{50}	50% lethal concentration
LM	Light Microscope
MBC	Minimum Bactericidal Concentration
MEA	Malt Extract Agar
MERS	Middle East Respiratory Syndrome

MHA	Mueller Hinton Agar
MHB	Mueller Hinton Broth
MIC	Minimum Inhibitory Concentration
MLR	Mixed Lymphocyte Reaction
MRSA	Methicillin-resistant *Staphylococcus aureus*
NA	Nutrient Agar
NCBI	National Centre of Biotechnology Information
NIST	National Institute of Standard Technology
OD	Optical Density
P. I	Polarity index
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
R^2	Linear Regression coefficient
R_f	Retentation factor
ROS	Reactive Oxygen Species
rRNA	ribosomal Ribonucleic Acid
RT	Retention Time
SARS	Severe Acute Respiratory Syndrome
SD	Standard Deviation
SDA	Sabouraud Dextrose Agar
SEM	Scanning Electron Microscope
SPSS	Statistical Package for the Social Sciences
TEM	Transmission Electron Microscope
TLC	Thin Layer Chromatography
TP	Thymocyte Proliferation
TPC	Total Phenolic Content
UV	Ultraviolet
WHO	World Health Organization
YES	Yeast Extract Sucrose

AKTIVITI ANTIMIKROB DAN ANTIOKSIDA KULAT ENDOFIT YANG DIPENCILKAN DARIPADA C*urcuma mangga,* DENGAN PENEKANAN TERHADAP C*eratobasidium ramicola* IBRLCM127

ABSTRAK

Kulat endofit yang dipencilkan daripada tumbuhan herba telah diakui sebagai salah satu sumber berpotensi untuk pembangunan ubat berasaskan produk semula jadi yang mungkin dapat menyelesaikan masalah global berkaitan kerintangan bakteria terhadap antibiotik yang sering digunakan. Kajian ini bertujuan untuk mengkaji aktiviti antimikrob dan antioksida kulat endofit yang dipencilkan daripada pelbagai bahagian tumbuhan ubatan *Curcuma mangga*. Sebanyak 125 kulat endofit diperolehi, termasuklah 90 daripada daun, 23 daripada batang dan 12 daripada rizom. Keseluruhannya, 118 daripada 125 pencilan menunjukkan aktiviti antimikrob yang signifikan terhadap sekurang-kurangnya satu mikroorganisma melalui ujian penyebaran palam agar. Pencilan IBRLCM127 telah menunjukkan aktiviti antimikrob yang paling signifikan dan ia telah dikenalpasti sebagai *Ceratobasidium ramicola* IBRLCM127 melalui morfologi makro dan mikronya serta urutan molekulnya. Penambahbaikan keadaan kultur melibatkan pra-kultur, penambahan tumbuhan perumah, bilangan plag dan kelajuan pergolakan telah meningkatkan aktiviti antibakteria oleh pencilan kulat ini secara signifikan dengan peningkatan sebanyak 11.72%. Ekstrak etil asetat daripada *C. ramicola* IBRLCM127 menunjukkan aktiviti antimikrob yang signifikan terhadap beberapa mikroorganisma ujian dengan nilai kepekatan perencatan minimum (MIC) dan kepekatan kematian minimum (MLC) dalam julat di antara 0.25 hingga 2.50 mg/mL dan 0.50 hingga 5.00 mg/mL. Aktiviti

antimikrob pencilan ini bergantung kepada kepekatan ekstrak terhadap bakteria MRSA ATCC33591, *Yersenia enterocolitica* dan *Candida albicans* IBRL berdasarkan kajian keluk masa-bunuh. Pendedahan mikrob tepilih kepada ekstrak etil asetat daripada *C. ramicola* IBRLCM127 menunjukkan kerosakan strukktur sel yang signifikan yang mana ia mengganggu sintesis dinding sel dan kebolehtelapan membran sel melalui pemerhatian mikroskop elektron pengimbas (SEM) dan mikroskop elektron transmisi (TEM). Berdasarkan pengasaian-bio pemisahan ekstrak berpandu, 5H-Pirrolo(3,2-d)pirimidin-4-amina adalah sebatian utama yang berkemungkinan menyumbang kepada aktiviti antimikrob bagi fraksi ini. Sementara itu, asai ketoksikan menggunakan ujian maut bagi udang air garam menunjukkan bahawa etil asetat tidak toksik pada ketoksikan akut (1627.18 µg/mL) tetapi toksik pada ketoksikan kronik (111.16 µg/mL). Kajian antioksida bagi ekstrak juga menunjukkan ia mempunyai kandungan yang sederhana tinggi bagi aktiviti antioksida (324.14 µg/mL) dan kandungan keseluruhan fenol (165.33 µg/mL). Secara ringkasnya, *C. mangga* berperanan sebagai gedung bagi kulat endofit dengan aktiviti antimikrob yang hebat yang boleh dibangunkan sebagai agen antimikrob semulajadi.

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ABSTRACT

Endophytic fungi isolated from medicinal herbs have been acknowledged as one of the potential sources for the development of natural product-based drug that might be able to solve the global problem of bacterial resistant to the commonly used antibiotics. This research was aimed to study the antimicrobial and antioxidant activity of fungal endophytes isolated from various parts of medicinal herb, *Curcuma mangga*. A total of 125 fungal endophytes were obtained, including 90 from leaves, 23 from stems and 12 from rhizomes. Overall, 118 out of 125 isolates demonstrated significant antimicrobial activity against at least on one test microorganisms via agar plug diffusion assay. Isolate IBRLCM127 showed the most significant antimicrobial activity and was identified as *Ceratobasidium ramicola* IBRLCM127 according to macro-, micro-morphology and molecular identification. Improvement of cultural conditions involving pre-culture age, host plant addition, number of agar plug and agitation speed had significantly increased antibacterial activity of the fungal isolate with 11.72% of increment. The ethyl acetate extract of *C. ramicola* IBRLCM127 demonstrated a significant antimicrobial activity against several test microorganisms with the minimal inhibitory concentration (MIC) and minimal lethality concentration (MLC) values ranged from 0.25 to 2.50 mg/mL and 0.50 to 5.00 mg/mL, respectively. The antimicrobial activity of this isolate was concentration-dependent against MRSA ATCC33591, *Yersenia enterocolitica* and *Candida albicans* IBRL according to the

time-kill curve study. The exposure of the selected microbial cells to the ethyl acetate extract of *C. ramicola* IBRLCM127 disclosed the significant structural damages of the cells which mainly affected the cell wall synthesis and cell membrane permeability, based on scanning electron microscopy (SEM) and transmission electron microscopy (TEM) observations. According to the bio-assay guided separation of extract, 5H-Pyrrolo(3,2-d) pyrimidin-4-amine was the major compound that may contribute to the antimicrobial activity of the fraction. Meanwhile, the toxicity assay using brine shrimp lethality test indicated that the ethyl acetate extract was not toxic at acute toxicity (1627.18 µg/mL) but toxic at chronic toxicity (111.16 µg/mL). The antioxidant study revealed that the extract possessed moderate antioxidant activity (324.414 µg/mL) and total phenolic content (165.33 µg GAE/mg). In short, *C. mangga* serves as a great warehouse of endophytic fungi with remarkable antimicrobial activity that can be further developed as natural antimicrobial agent.

CHAPTER 1

INTRODUCTION

1.1 Introduction

Antibiotic resistance is becoming increasingly problematic and has been regarded as one of a global concern. World health problems related to drug-resistant bacteria are increasing with an alarming rate, of which required an immediate action to tackle this increasing variety of infections. As of now, many bacterial strains are developing resistant to the antibiotics that used to treat them, causing the increment of new cases of life-threatening infections. Generally, antibiotic is used to treat bacterial infection and it is powerless against viral infection. Thus, the misuse of antibiotics to treat wrong illness or frequent usage of them might eliminate beneficial bacteria that populate human body, which in turn threatening the delicate balance of human health. Additionally, the nature of bacteria itself is prone to evolve and mutate causing them to be easily adaptable to the antibiotics. As a result, bacteria will spread, multiply and even giving rise to more new strains of antibiotic resistant bacteria. Therefore, an intensive search for novel and effective antibiotics to combat this every increasing global problem is needed. A novel antibiotic scaffold can serve as an effective drug that can replace the old drugs that already lost their effectiveness or the microbes has become resistant to them.

1.2 Problem statements

In recent years there has been widely acknowledged that the increase usage of antibacterial and antifungal agents has resulted in better control of microbe caused diseases and amazingly some of the diseases were even successfully annihilated.

However, this spectacular achievement has also led to the development of multidrug resistant strains and has been considered as a serious public health concern globally that needs to be addressed in the 21st century (Cars *et al*., 2011). In the past few years, *Enterococcus faecium, Staphylococcus aureus, Streptococcus pneumoniae, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Escherichia coli* and *Candida albicans* have been described as major classes of pathogens that developed resistance towards commercialized antibiotics and drugs (Newman *et al*., 2008; Hogberg *et al*., 2010). The phenomenon of multidrug-resistant infections resulted to the failure of standard treatment in microbial response that subsequently caused the prolonged ailment, increasing in health care spending and mortality rate (Tanwar *et al*., 2014). Hence, there is a need in seeking the novel antibiotics to replace the older antibiotics of which the bacteria have developed resistance to them.

Most of the available antibiotics in the market today derive from terrestrial soil actinomycetes with *Streptomyces* as a main producer (Weber *et al*., 2003). These antibiotics were first discovered in 1940s and constantly evolved till 1970s which is this period was regarded as a "golden era" of antibiotic discovery (Fernebro, 2011). For the past 70 years, the usage of antibiotics to treat myriad of infectious diseases has successfully saved many lives. However, the widespread use of these antibiotics in a longer term and in uncontrollable manner caused the infectious pathogens that supposedly to be killed by the designated antibiotics, finally become adapted to them making the antibiotics no longer effective. The resistance develops towards these synthetic antibiotics and drugs has become a catalytic factor to search for new antimicrobial compounds from various natural sources. Natural sources are the best sources to replace the synthetic antibiotics owing to the fact that the continuous usage of synthetic antibiotics has been reported previously in some of the health cases to

bring adverse side effect to the host such as immune-suppression, hypersensitivity reaction, loss of symbiotic intestinal microorganism, diarrhea and allergies (Wang *et al*., 2006).

Therefore, research and development of antibiotics and drugs derived from natural sources are garnered a significant interest from the scientists due to the lower occurrence of adverse reactions in plant derived antimicrobial compounds compared to the synthetic pharmaceuticals (Cheesman *et al*., 2017). In future, antimicrobial compounds derived from natural sources such as medicinal plant and its endophytes are anticipated to play a significant role in combating the advent of multidrug resistance pathogens and newly emerging infectious diseases. Plant endophytic fungi have been proven to produce a number of bioactive metabolites with unique and diverse structures including alkaloids, flavonoids, quinones, benzopyranones, phenolic acids, steroids, tetralones, terpenoids and xanthones (Tan & Zou, 2001; Schulz & Boyle 2005). The naturally produced bioactive metabolites by these endophytic fungi are less toxic as it does not kill the eukaryotic host plant system and these compounds offer a promising source of drugs with less adverse effect to human cell (Alvin *et al*., 2014). Due to the long period of co-evolution between endophytic fungi and their host plant, endophytic fungi are capable to produce the similar bioactive metabolites as their host plant. Owing to this fact, the endophytic fungi isolated from local medicinal herb, *Curcuma mangga* could become a potential source of natural antimicrobial compounds which are less toxic and more effective compared to the synthetic antibiotics in treating numerous diseases and infections.

1.3 Rationale of the study

Nowadays, people are more favouring natural products and traditional medicines over synthetic drugs because they believe herb remedies possess amazing benefit and free from undesirable adverse reaction. For example, death or hospitalization caused by the herb medications are rarely reported and hard to find in the United States and even the National Poison Control Centres of the United States also does not have a category in their database for adverse effect to herbs (Nasri, 2013). Contradictorily, there were about 8% of hospital admission in the United States are caused by the adverse reactions to synthetic drugs with approximately 100,000 people die each year due to these toxicities (Philomena, 2011). Owing to this fact, over 25% of the drugs prescribed worldwide derive from plants due to their long-term safety and efficacy (Rates 2001). Since time immemorial, natural products derived from plants have been used by human in traditional medicine, food preservatives and also as a spice. Additionally, medicinal plants are well-acknowledged as a repository of numerous types of bioactive metabolites with therapeutic potentials such as anticancer, antioxidant, antimicrobial, and anti-inflammatory (Raina *et al*., 2014). Each of this medicinal plant is identified according to its therapeutic properties due to the presence of active compounds. The advancement in biological and chemical means in natural product discovery make the modification and extraction of this source into potent drug becomes easy. An important example of plant derived drug is aspirin, the world's best medicinal agent that is produced from plant genera *Salixx* spp. and *Populus* spp. Another breakthrough of natural product discovery is the serendipitous discovery of antibiotic penicillin from the fungus Penicillium notatum. The first billion-dollar anticancer drug taxol is also isolated from the bark of Pacific yew tree, *Taxus brevifolia* and it has made a significant impact in medicine (Littleton, 2007). Since then, many

valuable drugs have been produced from medicinal plants such as analgesics (morphine), antitussives (codeine), antihypertensives (reserpine), cardiotonics (digoxin), antineoplastics (vinblastine and taxol), and antimalarials (quinine and artemisinin) (Kumar *et al*., 2015). Based on the fact that many very important life-saving drugs have been provided by natural products particularly medicinal plants, many researchers and pharmaceutical companies become interested in investing their efforts and funds to develop good-quality herbal drugs. Unfortunately, there are some limitations to have a sufficient plant supply such as arduous sampling task due to the climate change and isolated geographical region as well as the issues with endangered plant species (Handa, 1991). Besides, abundant of plant source could only yield a relatively low amount of desired compounds. Therefore, there is a surge need to find a novel source of natural products that are renewable and inexhaustible supply to replace this non-renewable source.

Endophytic fungi which live symbiotically in the internal tissues of their host plant could be a great choice in replacing their host plant as natural product source. Endophytic fungi benefit their host by producing secondary metabolites to assist their host in defending mechanism against predators and pathogens as well as adapted to harsh environmental conditions such as hyper salinity, acidic, and drought (Rodriguez, 2012). For example, a wild plant *Euphorbia indica* L. that grows in desert area of Pakistan is found to be colonized by endophytic fungus, *Aspergillus japonicus* which can modulate host plants growth under heat stress and act as thermal stress alleviator in arid regions where the mean for summer temperature exceeding 40^0 C (Ismail, 2018). Interestingly, endophytic fungi also possess capability to mimic their host plants in producing secondary metabolites with pharmaceutical properties (Venieraki *et al.*, 2017) and they might have advantage over their host as they could be mass-

propagated in the laboratory and their secondary metabolite production could be manipulated. Based on the aforementioned advantages, endophytic fungi isolated from medicinal plant, *Curcuma mangga* were selected to be the main focus in the current study in order to investigate their therapeutic properties.

1.4 Knowledge gap

Plant endophytes have been acknowledged not only as a potential source of bioactive compounds that can combat against various infections caused by the resistant bacterial species, but also can meet the emergent demand of exploring highly effective and low in toxicity and environmental impacted antibiotics. The ideal endophyte research should go beyond the simple isolation and metabolite characterization in order to study any possible interaction exist between endophytes and their host plant or other fungi and insects. Only by this way we can fully understand their evolution and harness them for a various pharmaceutical and industrial applications. Despite the apparent progress in the endophyte study, there are still significant gap remains in this field particularly from an interdisciplinary perspective. Biochemically, most of the biosynthetic pathway and enzymes involved in endophytes study remains unidentified. The metabolic biosynthesis of endophytes and its host plant are left unexplored as none of the research tries to invest their effort to investigate this matter. Besides that, very little is known about the intracellular location of biosynthesis of endophytic antimicrobial compound and their mode of secretion. Though numerous studies reporting the capability of endophytes to produce the secondary metabolites that are chemically similar to their host plants, but how and why this process occurred remain poorly investigated. Even though countless effort to quantify endophytic fungal

metabolites by using a broad array of spectrometric methods and NMR, there is no conclusive proof to distinguish the source of these metabolites. The methods employed cannot conclusively distinguish the compounds emerging *de-novo* synthesis in the fungal endophytes to that carried over from their host plant. Demonstrating the *de-novo* synthesis of the fungal secondary metabolites is the most direct method to prove the fungi possess independent pathway to produce secondary metabolite similar to their host plant. This could be done by using radio-tracer and pulse-chase methods to examine the existence of any incorporation of the tracer into the metabolite under investigated. Unfortunately, very few studies were employed these methods and all of them were only focusing for taxol biosynthesis. Another method to prove the capability of fungal endophytes in synthesizing secondary metabolites is the use of insertional mutagenesis tools coupled with complementation research. Regrettably, none of study has adopted this approach so far.

Genetically, up to now, very few genes encoding the relevant biosynthetic enzymes have been successfully isolated causing to the low number of researches that have been conducted to investigate the regulation of these gene expression at the molecular level. With respect to understanding the activities of these endophytes, there is very limited information available about structure-function relationship, specifically factors that trigger the endophyte to shift from mutualism to parasitism, along with associated changes in the production of secondary metabolites. Basically, most of the studies on antimicrobial activities of endophytes are conducted to only a few model species including human pathogens, but very limited studies have been conducted on virus. Besides that, a better understanding of the host plant contextual ecology is of important since identifying pathogens that populate the host may give clues to the specific anti-pathogenic targets of the investigated endophyte.

1.5 Objectives of research

The aims of the present study are listed as follow:

1. To isolate and identify the potential of endophytic fungi from *C. mangga* and to screen their antimicrobial activity on various pathogenic microorganisms.

2. To improve fermentation parameters for maximum production of antimicrobial compounds by *C. ramicola* IBRLCM127.

3. To investigate the antibacterial and antifungal inhibition mechanisms of the extract produced by *C. ramicola* IBRLCM127.

4. To partially purify and characterize the extract of the *C. ramicola* IBRLCM127 with prominent antimicrobial activity

5. To determine the antioxidant activity and toxicity of the crude ethyl acetate extract of *C. ramicola* IBRLCM127.

CHAPTER 2

LITERATURE REVIEW

2.1 The need to search for new antibiotics

Since the antibiotic discovery about eight decades ago, the development in infection treatments undergo a major improvement in which once considered deadly contagious diseases can be transformed into mere cases of healthy problems (Cars *et al*., 2008). For example, the antibiotics are essentially needed not only to treat ordinary infections but also gravely infections such as tuberculosis, pneumoniae and meningitis (Herbst *et al*., 2009). They are not only able to save a patient's life but also helping in extending the life spans by preventing further infections of bacteria (Piddock, 2012). Currently, expectancy of human life has been increasing amazingly due to the better health care and improved medical treatment (Oeppen & Vaupel, 2002). This amazing achievement was true as in 1920, the American were estimated to live only 56.4 years old but now the average life span in the U.S. turns to be nearly 80 years old (CRSR, 2005). In medical field and surgery, antibiotic also plays a significant role in preventing or treating the infections caused by post-chemotherapy treatment or those who are suffering from chronic diseases like diabetes, rheumatoid arthritis, end-stage of renal disease and also those who undergo surgeries for organ transplants, cardiac surgery and joint replacements (Wright, 2014; Rossolini *et al*., 2014).

The successful use of antibiotics in medicine is compromised by the potential antimicrobial resistance development to that antimicrobial compound from the very first time it was used (Davies & Davies, 2010). However, the misconception in the late 1960s and early 1970s that infectious diseases in which once creating dilemmas to clinicians to be treated had been fully solved resulted in the infectious diseases to

become the third leading cause of mortality in the United States (Pinner *et al.,* 1996) and second leading cause of mortality globally (WHO, 2002a). This unpredicted calamity occurred mainly due to the increasing amounts of antibiotics usage for over the last 70 years that finally ignited the emergence of rapid appearance of bacterial resistant strains in which the antibiotics are no longer susceptible to them (D'Costa *et al*., 2011). Antibiotic resistance can be described as an ability of certain pathogenic microorganisms to survive the toxic effect of antibiotics. The excessive prescription and incorrectly prescribed antibiotics have been identified as reasons that promoting more pathogenic bacteria to become resistant towards the available arsenal of antibiotics in the market (Ventola, 2015). About 30% to 50% of the studied cases involved in the hospitals were found to be mistaken regarding the selection of agent, indication of treatment or the period of antibiotic therapy (Luyt *et al*., 2014). Besides that, there was also a close relationship between the usage of antibiotic and the bacterial resistance development (Fridkin *et al*., 2005; Tong *et al*., 2008).

Basically, all living organisms need to maintain their fitness all the time to survive in their environment. In other words, living organisms regardless of their size need to strive for successfully adapted to their environmental conditions including microorganisms. The adaptation to the environmental conditions includes adjusting to the climate conditions, availability of nutrients, water, oxygen and to the presence of possibly hazardous or even lethal external agents such as antibiotics. Therefore, bacteria have shown an amazing capability to endure and adapt to their harsh environment including the development of various resistance mechanisms towards readily available and even newly introduced antibiotics. As a result of this adaptation, the phenomenon called antibiotic resistance occurred in many bacteria strains in which they have become resistant to the antibiotics used and, in many cases, multi-resistant

to different antibiotics caused these drugs are becoming ineffective to treat serious infections caused by the pathogenic microorganisms (Alanis, 2005). These bacteria are not only showing an amazing resistance to the used of antibacterial drugs but they also possess the capability to remain alive and viable in the hospital environment. Therefore, the majority of the vulnerable patients especially those in the intensive care unit or receiving steroids, the immunosuppressed or debilitated patient are prone to this bacterial infection called nosocomial infections (Kollef, 2001; Picazo, 2004; Nser, 2005). The common multidrug resistant bacteria that caused nosocomial infections are *Clostridium difficile, Enterococcus faecium, Acinetobacter baumannii, Enterobacter* spp.*, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa, Salmonella* spp.*, Serratia spp., Staphylococcus aureus, Citrobacter freundii, Streptococcus pneumonia* and *Burkholderia cepacia* (Davies & Davies, 2010). The term "superbug" was used to describe these multidrug-resistant bacteria which mean microorganisms with improved morbidity and mortality due to multiple mutations that lead to great resistance towards antibiotic classes specifically recommended for their treatment, thus reduce the remedial options and effective prevention for these microbes. Consequently, the periods of hospital care were extended and becoming costlier. This was the burden that needs to bear by the patient in the 21st century as antibiotic resistance has emerged as one of the primary public health problems worldwide. Therefore, to tackle the emergence of resistant pathogen, the discovery of new antibiotic candidate should become the main goal for researchers and other medical community.

2.1.1 Emergence of new pathogens and diseases

Since ancient times, the occurrences of emerging pathogens or infectious diseases have brought a significant effect and burden on human health and economic stability. An emergence of new pathogen refers to the novel etiological agent of an infectious disease in which an occurrence is increasing after its appearance either in existing population resulted from long-term changes in its epidemiology or in a new host population (Woolhouse & Gaunt, 2007). These pathogens can persist or remain in host population if only each infected host also infect one or more other susceptible hosts. The pathogen persistence requires an adequate supply of susceptible hosts which can be generated via birth, immigration, or immunity loss (Cleaveland *et al.*, 2007). For over the past 30 years, at least 30 new pathogens infecting human across the globe have emerged despite the advancement in biomedical field (Nii-Trebi, 2017). Most of these pathogens are zoonotic wherein their origins are closely related to socioeconomic, ecological factors and environmental conditions. A list of human pathogens that have been discovered from 1980 to 2005 was shown in Figure 2.1.

The World Health Organization (WHO) had introduced the term "emerging infectious disease" (EID) which represented the disease that either appeared and infected a population for the first time or already existed earlier with the increasing number of cases within a population or widespread to the other geographical regions (WHO, 2005). After a few years, WHO also included the re-emergence of infectious disease that used to infect a certain area in the past but declining with time or under controlled as EID (WHO, 2014). A recent publication of a list of top emerging diseases that can potentially cause major epidemics by WHO includes Crimean-Congo haemorrhagic fever (CCHF), Ebola and Marburg, Lassa fever, MERS and SARS, Nipah, and Rift Valley fever, chikungunya, severe fever with thrombocytopenia

syndrome and Zika (WHO, 2018). This proposed list will be reviewed annually or when the outbreaks occur.

Human bocavirus	2005
Human coronavirus HKU1	2005
Human T-lymphotropic Virus 3	2005
Human T-lymphotropic Virus 4	2005
Human coronavirus NL63	2004
SARS coronavirus	2003
Cryptosporidium hominis	2002
Baboon cytomegalovirus	2001
Human metapneumovirus	2001
Cryptosporidium felis	2001
Whitewater Arroyo virus	2000
Brachiola algerae	1999
Ehrlichia ewingii	1999
Nipah virus	1999
TT virus	1999
Brachiola vesicularum	1998
Menangle virus	1998
Trachipleistophora anthropophthera	1998
Bartonella clarridgeiae	1997
Laguna Negra virus	1997
Andes virus	1996
Australian bat lyssavirus	1996
BSE agent	1996
Ehrlichia canis	1996
Juquitiba virus	1996
Metorchis conjunctus	1996
Trachipleistophora hominis	1996
Usutu virus	1996
Bayou virus	1995
Black creek canal virus	1995
Cote d'Ivoire Ebola virus	1995

Hepatitis G virus	1995
New York virus	1995
Anaplasma phagocytophila	1994
Hendra virus	1994
Human herpesvirus 7	1994
Human herpesvirus 8	1994
Sabia virus	1994
Bartonella elizabethae	1993
Encephalitozoon intestinalis	1993
Gymnophalloides seoi	1993
Sin Nombre virus	1993
Bartonella henselae	1992
Dobrava-Belgrade virus	1992
Ehrlichia chaffeensis	1991
Encephalitozoon hellem	1991
Guanarito virus	1991
Nosema ocularum	1991
Banna virus	1990
Gan gan virus	1990
Reston Ebola virus	1990
Semliki Forest virus	1990
Trubanaman virus	1990
Vittaforma corneae	1990
Corynebacterium amycolatum	1989
European bat lyssavirus 1	1989
Hepatitis C virus	1989
Barmah Forest virus	1988
Picobirnavirus	1988
Dhori virus	1987
Sealpox virus	1987
Suid herpesvirus 1	1987

Cyclospora cayetanensis	1986
European bat lyssavirus 2	1986
Human herpesvirus 6	1986
Human immuno-deficiency virus 2	1986
Kasokero virus	1986
Kokobera virus	1986
Rotavirus C	1986
Borna disease virus	1985
Enterocytozoon bieneusi	1985
Pleistophora romneafiei	1985
Human torovirus	1984
Rotavirus B	1984
Scedosporium prolificans	1984
Candiru virus	1983
Capnocytophaga canimorsus	1983
Helicobacter pylori	1983
Hepatitis E virus	1983
Human adenovirus F	1983
Human immuno-deficiency virus 1	1983
Borrelia burgdorferi	1982
Human T-lymphotropic Virus 2	1982
Seoul virus	1982
Microsporidian africanum	1981
Human T-lymphotropic Virus 1	1980
Puumala virus	1980

Figure 2.1: Discoveries of human pathogens (Woolhouse & Gaunt, 2007)

Current infamous emerging disease that possesses ability to switch its genetic information is influenza or commonly known as flu, wherein this disease emerges mainly due to the human and environmental factors. Major changes of the influenza virus lead to the pandemics due to the unprepared human immune system in recognizing and defending towards new virus variant. There is high possibility of this

influenza to undergo major genetic alteration and pass down to human particularly when human have a close contact with livestock such as ducks, chickens, and pigs. This livestock is natural host for influenza virus and at the same time serves as a mixing vessel for the creation of new version of influenza virus which do not exist previously. For example, avian H5N1 influenza or also called as bird flu was first reported to affect humans in 1997 during poultry outbreak occurred in Hong Kong. The virus of H5N1 emerged from epizootics of wild birds that triggered the virus transmission in domestic poultry which caused the precipitation of dead-end viral transmission to the poultry-exposed human (Moren & Fauci, 2013). The virus of H5N1 is considered deadliest as more than half of the cases have been reported to be fatal. However, H5N1 virus do not acquire the ability to be transmitted efficiently among human. Since the re-emergence of H5N1 in 2003, this disease has been reported to infect poultry in Asia till Europe and Africa with 16 countries including Bangladesh, Thailand, Indonesia and Myanmar confirmed 846 cases of human infections up to January 2016 (Mukherjee, 2017). In contrast to the virus H5N1, H1N1 virus possesses the ability to be passed down easily between human, thus making this virus spreads faster throughout the world resulted from human activity especially air travel. The first case of H1N1 was reported in Mexico in March 2009, followed by consecutive reports in USA and India thereafter (CDC, 2009). In India, the first positive case reported in May 2009 was derived from airplane passenger travelling back to India from infected country, USA (John & Moorthy, 2009). Soon after that, the virus H1N1 became endemic in India which then spread throughout all the big cities of India (Mukherjee *et al*., 2010). The next 6 months witnessed the spreading of H1N1 virus to almost all the countries with the number of death cases exceeding 18, 000 cases, wherein 67% of these cases contributed solely by USA (WHO, 2009). The highest number of H1N1

cases were reported during its first outbreak in 2009 (27236 cases), followed by 2010 (20604 cases) and 2012 (5054 cases). Meanwhile, the highest fatal cases were reported in 2011 (1763 death cases), followed by 2009 (981 death cases) and 2012 (405 death cases) (Kawanpure *et al*., 2014). Up to February 2015, the accumulated cases of H1N1 including death in the current year were reported to be exceeding the previous number of cases with 33000 cases of H1N1 and 2000 deaths. Fortunately, despite its rapid transmittance between human, H1N1 is less deadly compared to H5N1.

Chikungunya literally can be defined as disease which cause the joints to bend up in pain, of which this disease is infected by the chikungunya virus (CHIKV) associated with tiger mosquito. This virus was first reported in 1952 during an outbreak of dengue-like fever in Tanzania. This disease affects peripheral joints such as knees, ankles, wrists as well as small joints of hand causing a severe pain and fever that may continue for weeks or years (Suhrbier *et al*., 2012). Additional symptoms of this disease are arthritis, skin rash, tenosynovitis, and muscle pain especially at the lower back and leg. In chronic cases, there are also severe neurologic and cardiac infection that lead to death have been reported particularly among the newborn and old folk (Vega-Rua *et al.,* 2014). Additional reports also indicate that the transmission of virus from mother to infant also cause high morbidity rate of CHIKV (Chaves *et al*., 2012). The chikungunya symptoms can be sometimes mistaken with dengue and zika infections (Ng *et al.,* 2018), thus making it hard to diagnosis particularly in the area with frequent cases of dengue and zika (Cabral-Castro *et al*., 2016; Calvo *et al.*, 2016).

Another serious and recently emerge disease is Zika infection which cause a birth defect called as microcephaly. Zika infection is caused by the mosquito-borne flavivirus known as Zika virus. This virus was first reported associated with monkey in 1947 in Uganda before it first affected human in 1952 in Tanzania and Uganda.

During the period of 1960s until 1980s, the outbreak of Zika was reported all over Africa and Asia with the first largest outbreak occurred in Island of Yap, Micronesia in 2007 (Mukherjee, 2017). In 2015, Brazil had reported the association of Zika virus with both Guillain-Barre syndrome and microcephaly (WHO, 2016).

Another severe respiratory infection which spread rapidly through air travelling is SARS and MERS. The coronavirus of SARS was first emerged in China in 2002 and spread quickly thereafter to over 30 countries across Asia, USA and Europe with 8439 cases were reported including 812 deaths were recorded within a year (WHO, 2002b). At that time, SARS outbreak had caused global catastrophe that alarming everyone to take safety measures towards this EID (WHO, 2005). Meanwhile, MERS that caused by MERS-coronavirus was originated from Arabian Peninsula and emerged in 2012. Due to the airplane travelling, this disease had spread to other countries including Asia, Africa, Europe and North America.

2.1.2 Antibiotic resistance

Over recent years, we have been witnessed multiple advancements involving the early identification and treatment of contagious diseases mainly due to the introduction of antibiotic. The word 'antibiotic' was first introduced by Selman Waksman in 1941 to describe any fine molecule produced by a microbe that has a potential to inhibit the other microbial growth (Clardy *et al.,* 2009). This word was derived from Greek word anti (against) and biotikos (concerning life). Historically, the first classes of antibiotic namely sulfonamides and penicillin had been introduced in 1937 (Davies & Davies, 2010). Since then, there were drastic declinations in the morbidity and mortality cases associated with infectious diseases. As time passes,

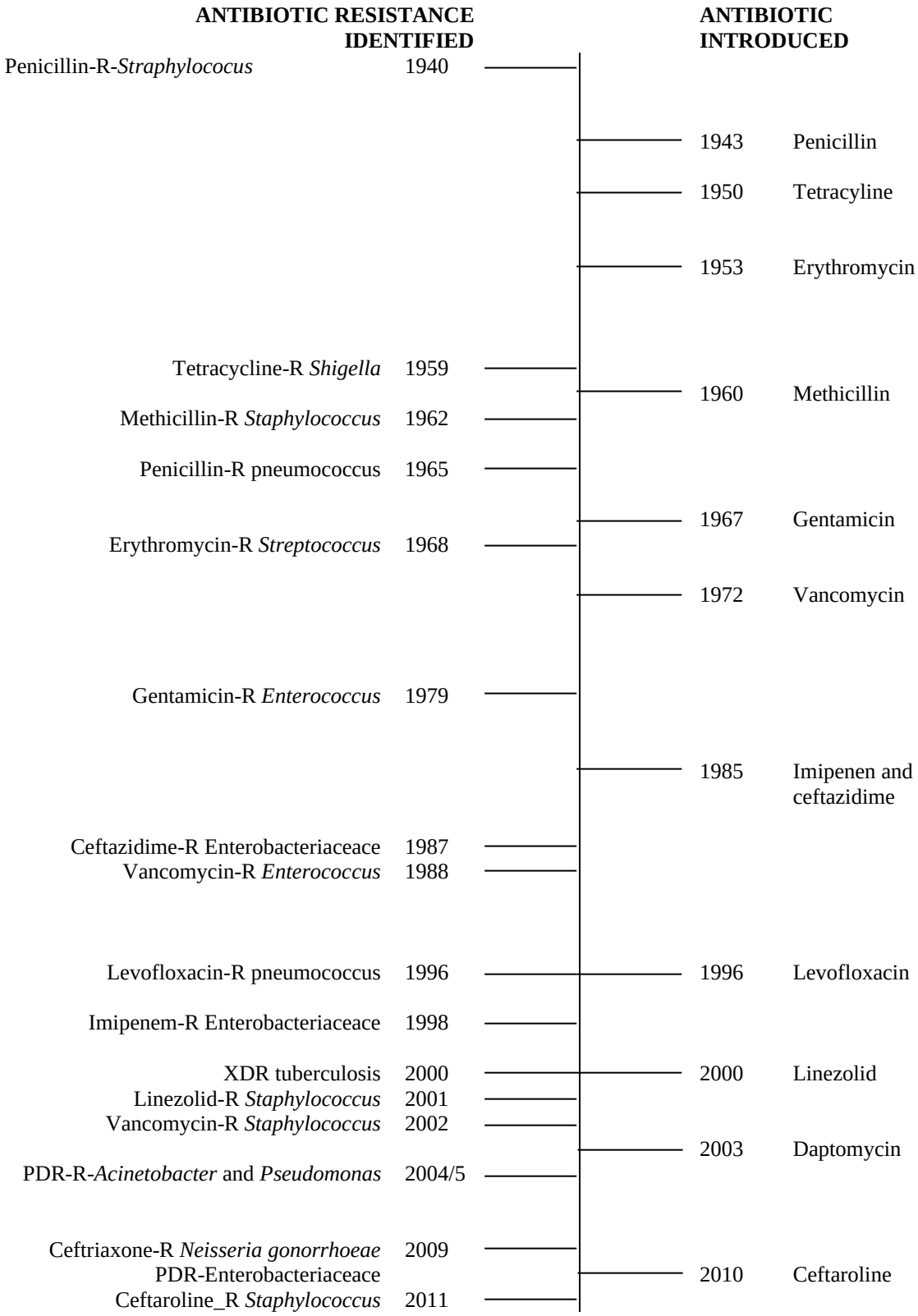
more antibiotics were successfully discovered, the manufacturing processes were becoming easier and more new and effective formulation had been developed making the usage of antibiotics widespread. At the 'golden age' of antibiotic discovery between the 1940s and 1960s, antibiotic was regarded as a miracle drug that becoming the key achievement in the medical field and was used to treat various infections even the most common and trivial one. Antibiotics represent a unique class of medicines with special characteristics like usage complication and restriction, long-term effectiveness and most importantly it does not target biochemical process of human but those of another life form of microbe such as bacteria (Hogberg *et al*., 2010).

Since the antibiotic discovery about eight decades ago, the revolutionary in infection treatments undergo a major improvement in which once considered deadly contagious diseases can be transformed into mere cases of healthy problems (Cars *et al*., 2008). For example, antibiotics are essentially needed to treat gravely infections such as tuberculosis, pneumonia and meningitis (Finch, 2007; Herbst *et al*., 2009). They are not only able to save a patient's life but also helping in extending the life spans by preventing further infections of bacteria (Piddock, 2012; Rossoline *et al*., 2014). This amazing achievement was true as in 1920, U.S. people were estimated to live only 56.4 years old but now the average U.S. life span turns to be nearly 80 years old (CRSR, 2005). In medical field and surgery, antibiotic also plays a significant role in preventing or treating the infections caused by post-chemotherapy treatment or those who are suffering from chronic diseases like diabetes, rheumatoid arthritis, end-stage of renal disease and those who undergo surgeries for organ transplants, cardiac surgery and joint replacements (Gould & Bal, 2013; Wright, 2014; Rossolini *et al*., 2014).

The antibiotic resistance development of microorganisms is closely related to the degree of DNA simplicity present in those microorganisms that becoming resistant and the easiness of DNA acquiring from other microorganisms (Alanis, 2005). The two main elements in antibiotic resistance development are the presence of antibiotic compounds with the capability to inhibit the majority of the bacteria in a colony and heterogeneous colony of bacteria with at least one of it carries the genetic determinant that has the ability to express resistance towards antibiotic compounds (Levy & Marshall, 2004). Once these two elements are fulfilled, the bacteria that susceptible to antibiotic in the colony will die and only the resistant strains will survive. The bacteria that survived are said to have the genetic determinants that will be used by the bacterial cell to codify the type and resistance intensity. Selection of the survival bacterial strains will lead to the selection of these genes that can be further spread and propagated to other bacteria (Levy & Marshall, 2004). The occurrence of antibiotic resistance can be natural or acquired with two ways of transmission either vertically or horizontally. Natural antibiotic resistance normally occurred when there are spontaneous gene mutations taking place in the scarcity of selective pressure due to the presence of antibiotic compounds and the chances of this natural form of antibiotic resistance to occur is less common compared to the acquired form. Most of the times, the bacterial resistance to antibiotics is caused by micro-ecological pressure that acts as a powerful stimulus to evoke a bacterial adaptation response and it is called as acquired form of antibiotic resistance. Basically, susceptible bacteria develop antibiotic resistance via genetic mutation or receive antibiotic resistance genes from other bacteria. These genes that responsible to codify the resistance are situated in DNA specialized fragments called as transposons and it functions to ease the movement of resistance genes from one plasmid to the other plasmid (Sefton, 2002).

In some transposons, there might be a special and complicated DNA fragment known as ‘integron’, referring to the site with the capability to integrate multiple antibiotic resistance genes that will confer multiple antibiotic resistances to the bacteria. Once a genetic mutation occurs, it will cause the bacterial DNA changes and the transferring of genetic materials among bacteria via a few means particularly the most common means such as transformation, conjugation and transduction (Alanis, 2005).

Historically, the first case of antibiotic resistance was reported in the late 1930s and in the 1940s involving bacterial strains of *Staphylococcus aureus*. The findings from the microbiologists’ work revealed that several bacterial strains had developed resistance to the introduced antibiotic. The occurrence of antibiotic resistance had been warned previously by Sir Alexander Fleming in his interview with The New York Times in 1945. He strictly warned that the misuse of penicillin would lead to the resistant selection of “mutant forms” of *Staphylococcus aureus* that hold potential of serious infections to the host or other people that were in contact with the host. His warning was neglected by the medical practitioner on that time and no wonder within one year of widespread use of this antibiotic, a significant number of *Staphylococcus aureus* strains had developed resistance to penicillin. It only took a couple of years for 50% of the strains to be no longer susceptible to this newly introduced antibiotic (Levy, 2002). Since then, resistance has been reported to almost all antibiotics that have been developed (Figure 2.2) (CDC, 2013; Ventola, 2015).



PDR = pan-drug-resistant; R = resistant; XDR = extensively drug-resistant

Figure 2.2: Development of antibiotic resistance: A timeline of key events (Ventola, 2015).

2.2 Natural products as antimicrobial agents

Since ancient ages and in folklore, natural products with therapeutic values have been used widely as remedies to relieve a vast array of illnesses and infected diseases. The knowledge of healing potential possessed by natural products that has been passed down from one generation to another generation in every part of the world throughout the history has significantly intensified the development of various traditional systems of medication. Natural products often refer to any chemical compounds or substances that can be found naturally or produced by living organisms that possess antimicrobial or pharmacological activity (Koehn & Carter, 2005). Basically, living organisms produce a vast array of primary and secondary metabolites in which these two metabolites are believed to have a different function. Primary metabolites produced by the living organisms have pivotal function in growth, development and reproduction (Kabera *et al*., 2014) whereas secondary metabolites play a significant role in defense mechanisms against environmental harm (Stamp, 2003) or interspecies competition (Samuni-Blank *et al.*, 2012).

The secondary metabolites generally are regarded as biologically active substances of intermediate or final products of secondary plant metabolism. These organic compounds function in controlling unique traits of the plant like fragrance and colour of fruit and flower, vegetables and spices characteristics flavor and also contribute to the biological and pharmacological activities of a plant. Owing to these facts, secondary metabolites produced by the plant are undoubtedly closely related to their medicinal properties (Hartman, 2008).

Nature endows us an abundance of botanical diversity with various types of plants grow wildly all over the different parts of the world. Hence, since ancient times

man has relied on plants not only to cater for their basic necessity as a source of food but also for medicinal purposes. Seeking healing power in plants is primeval thought. Medicinal plants usage in curing maladies is once considered to be as old as the man civilization (Onyeagba *et al*., 2004). For example, the Indians, Chinese and North African ancient civilization had recorded the natural resources usage for treating multiple ailments (Phillipson, 2001). The earliest written document dated 4000-year-old was found in a form of Sumerian clay tablet that documented the medicine used to treat various maladies (Kong *et al*., 2003). For instance, the usage of mandrake to relieve the pain, plant endive roots to cure gall bladder disorders, raw garlic to treat circulatory disorders and turmeric has special properties in blood clotting. Besides that, it was also documented 60,000 years ago, Neanderthals that were used to live in Iraq employing plant likes hollyback as a source of medicine. Other plants that have been used continuously as a habitual practice in traditional treatment are likes cranberry and bearberry juice to cure the infections of urinary tract and lemon balm, tea tree and garlic are reported to possess antimicrobial properties (Heinrich *et al*, 2004). There are also the records dated 2900-2600 BC that documented about 1 000 plant derivatives likes Cedrus species oil (cedar), *Cupressus sempervirens* (cypress), *Commiphora myrrha* (myrrh), *Papaver somniferum* (poppy) and *Glycyrrhiza glabra* (liquorice) (Borchardt, 2002). *Abutilon indicum* that has been grown substantially in Bangladesh, India, Pakistan and Srilanka is used broadly in treating fever, flu, mumps, diabetes, hernia, tuberculosis, diarrhea and infections of worm (Namita & Mukesh, 2012). Iranian use *Zataria multiflora* in curing sore throat, jaundice, asthma, premenstrual pain and as a flavoring agent (Mahboubi *et al*., 2008). Malaysian medicinal plant, *Curcuma mangga* is used widely for the treatment of stomachache, backache, asthma, wounds, sprains, fever, bronchitis, gastric ulcer, womb healing, skin diseases and

general debility (Rukmana, 2004; Abas *et al*., 2005; Wahab *et al*., 2011). This medicinal plant has been reported to produce several natural bioactive compounds such as curcuminoids, halimane, labdane and diterpenes (Anand *et al*., 2007; Hatcher *et al*., 2008; Kita *et al*., 2009; Silva et *al*., 2011) with broad spectrum of biological activities including antitumour, antifungal, insecticidal, antioxidant and anti-inflammatory properties (Abas *et al*., 2005; Tewtrakul *et al*., 2007; Chen *et al*., 2008; Liu & Nair, 2011). Apart of becoming the oldest renown practice for traditional therapy (Ahmad & Wajid, 2013), the role of medicinal plants as a part of human culture is undeniable fact as the plants itself possess the ability to treat various illness (Adnan *et al*., 2014).

The chemical study of natural products was first started by Sertuner in 1803 who was successfully isolated Morphine from *Papaver somniferum* or generally known as opium poppy It was then followed by the ensuing conversion into heroin in 1874 as described by Wright (Siddiqui *et al*., 2014). The first valuable alkaloids isolated was Emetine in 1817 from Ipecacuanha (Der Marderosian & Beutler, 2002) and this success gives rise to another alkaloid isolation likes Quinine (*Colchicum officinalis*), Strychnine (*Strychnos nux vomica*) (Caventou & Pelletier, 1820), Atropine (*Atropa belladonna*) (Allen & Hatfield, 2004), Papaverine (*Papaver somniferum*) (Merck, 1848) and many more. Aspirin as a well renowned drug from natural product was proposed by Maclagan (1876) from his work on *Spirae ulmaria* or Salix extract to produce salicin. The first antiviral agent that were Spongothymidine and Spongouridine had been reported by Bergmann & Feenly (1950) from sponge. However, the discovery of the first antibiotic derived from natural product that was Penicillin from the fungus *Penicillium notatum* by Alexander Fleming in 1928 was regarded as a golden discovery in a medicinal world (Aminov, 2010). Between 1970

to 2006, there was a total of 24 structurally unique natural products had been discovered (Ganesan, 2008) while 13 drugs-origin natural products had been approved from 2005-2007 (Butler, 2008). There were also reported that 75% of drugs for infectious maladies and 60% of cancer drugs are natural product derivatives (Newman & Cragg, 2003). Therefore, it was estimated that 80% of the drugs available in the market were natural origin in the early 19th century (Rios & Recio, 2005).

Nowadays, as synthetic drugs seem to possess fewer therapeutic effects and sometimes lead to the unacceptable side effects, the revival of interest in natural-products-based drugs that is believed to cause a lower incidence of bad health effects have been triggered (Mohanty & Cock, 2009). It was estimated that 40% of the developed anti-infectious drugs approved by United State Food and Drug Administration (FDA) were found to be natural origin for over the past 30 years (Newman & Cragg, 2012). Further investigation of structural properties between natural products and synthetic drug compounds reveals that natural products possess a divergent and broader chemical space as compared to synthetic drugs and its derivatives (Feher & Schmidt, 2003; Grabowski & Schneider, 2007; Ganesan, 2008; Rosen *et al*., 2009). Besides that, 83% of the core ring scaffold that were found in natural products absent in commercially synthetic drug molecules and screening libraries (Hert *et al*., 2009). According to Hert *et al*. (2009), compounds that are regarded to be the successful drugs are the one that possess the characteristic of 'metabolite-likeness'. Harvey *et al*. (2015) characterized natural products as natural metabolites, thus it can be concluded that the compounds of natural products unsurpassed by any synthetic libraries available. This means that the mode of reactions for natural product compounds are biologically active and prone to become substrates for more than one transporter systems that can carry the compounds to the intracellular