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BIOLOGICAL ACTIVITIVIES OF ACANTHOPHORA SPICIFERA (VAHL) BORGESEN FROM PULAU GEDUNG, PENANG

by

NURUL AILI BINTI ZAKARIA

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(*In the name of Allah, The Most Merciful and The most Gracious*)

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Nurul Aili Binti Zakaria

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LIST OF ABBREVIATIONS

AOA	Antioxidant activity
BHT	Butylatedhydroxy toluene
CC	Column Chromatography
CFU	Colony Forming Unit
DMSO	Dimethyl sulfoxide
DPPH	Diphenylpicryl hydrazil
EARSS	European Antimicrobial Resistance Surveillance System
ECDC	European Center for Disease Prevention and Control
EC_{50}	50% efficient concentration
ESI	Electron spray ionization
ESCMID	European Society of Clinical Microbiology and Infectious Diseases
EUCAST	European Committee for Antimicrobial Susceptibility Testing
GAE	Gallic acid equivalent
HMDS	Hexamethyldisilazine
IDSA	Infectious Diseases Society of America
INT	Tetrazolium salt
L-ASC	L-Ascorbic acid
LC-MS	Liquid chromatography mass spectrometry
LC_{50}	50% lethal concentration
MBC	Minimum Bactericidal Concentration
MIC	Minimum Inhibitory Concentration
MOA	Mechanism of Antibiosis
NA	Nutrient Agar
NIAID	National Institute of Allergy and Infectious Disease
PDA	Potato Dextrose Agar
TLC	Thin Layer Chromatography
TPC	Total phenolic content
WHO	World Health Organization

AKTIVITI BIOLOGI ACANTHOPHORA SPICIFERA (VAHL) BORGESEN DARI PULAU GEDUNG, PULAU PINANG

ABSTRAK

Lapan ekstrak daripada *Acanthophora spicifera* yang diperolehi dari dua kaedah pengekstarakan menggunakan pelarut yang bebeza kekutuban telah digunakan. Ekstrak tersebut di uji untuk menentukan aktiviti antimikrob, antioksidan, ketoksikan *in-vitro* dan juga kandungan sebatian fenolik. Kebanyakan ekstrak menunjukkan aktiviti antibakteria ke atas bakteria kajian. Tiada aktiviti antikulat ditunjukkan terhadap kulat kajian. Ekstrak kasar metanol mempamerkan aktiviti antibakteria yang menarik terhadap bakteria Gram-negatif, *Pseudomonas aeruginosa* ATCC 27853 dengan zon perencatan sebanyak 11.0 mm, nilai MIC 250 μg mL^{-1} dan nilai MBC 500 μg mL^{-1}. *A. spicifera* juga menunjukkan aktiviti antioksidan. Ekstrak kloroform menunjukkan aktiviti antioksidan tertinggi dengan 50% penyingkiran radikal bebas sebanyak 789.0 μg mL^{-1}, manakala piawai yang digunakan iaitu quercetin sebanyak 3.361 μg mL^{-1}. Manakala untuk kandungan total fenolik, ekstrak etil asetat menunjukkan kandungan fenolik tertinggi sebanyak (40.58 ± 1.16 μg GAE mg^{-1} ekstrak kering). Kesemua ekstrak yang diuji juga menunjukkan korelasi linear positif di antara aktiviti antioksidan dan kandungan fenolik. Ekstrak kasar methanol juga mempamerkan aktiviti ketoksikan yang signifikan terhadap anak udang, *Artemia salina* dengan nilai kepekatan maut 50% akut pada 635.47 μg mL^{-1}dan kronik pada 275.72 μg mL^{-1}. Ekstrak ini kemudiannya, difraksi menggunakan kromatografi turus gel silika dengan menggunakan campuran pelarut heksana dan etil asetat sebagai fasa bergerak dan

menghasilkan 15 fraksi (af1-af15) keseluruhannya. Hanya tiga fraksi (af14, af15, af7) didapati aktif terhadap *P. aeruginosa* ATCC 27853 dengan nilai MIC di antara 125-250 μg mL^{-1}. Oleh kerana kekurangan hasil fraksi af15 dan af7, hanya fraksi aktif af14 sahaja yang dilakukan pemfraksian lanjut. Hasil sub-fraksi (aff2) daripada pemfraksian fraksi af14 yang aktif terhadap *P. aeruginosa* ATCC 27853 dengan nilai MIC 75 μg mL^{-1} kemudiannya dianálisis dengan LC-MS dan GC-MS. Hasil análisis GC-MS mendapati kehadiran sebatian hidrokarbon, asid lemak, ester asid aromatik dikarbosilat, sebatian benzena dan lain- lain. Walau bagaimanapun, sebatian aktif yang terpisah dari aff2 tidak dapat dikenalpasti berdasarkan analisis LC-MS.

BIOLOGICAL ACTIVITIES OF ACANTHOPHORA SPICIFERA (VAHL) BORGESEN FROM PULAU GEDUNG, PENANG

ABSTRACT

A total of eight extract of *Acanthophora spicifera* extracted using two different methods using solvents of different polarity, were used throughout this study. These extracts were assayed to determine the antimicrobial activities, antioxidant activities, *in-vitro* cytotoxicity and total phenolic contents. Most of the extracts exhibited antibacterial activity against the tested bacteria. Meanwhile, no antifungal activity was observed on tested fungi. Among the extract, crude methanol extract demonstrated promising antibacterial activity against Gram-negative *Pseudomonas aeruginosa* ATCC 27853 with inhibition zone of 11.0 mm, MIC values of 250 μg mL^{-1} and MBC value of 500 μg mL^{-1}. *A. spicifera* also exhibited antioxidant properties. Chloroform extract was found to exhibit the highest DPPH free radical scavenging activity with LC_{50} of 789 μg mL^{-1}, while standard quercetin used is 3.361 μg mL^{-1}. Meanwhile, for total phenolic contents, ethyl acetate extract gave the highest phenol contents (40.58 ± 1.16 μg GAE mg^{-1} dry extract). All extracts also showed good positive linear correlation between the antioxidant activities and total phenolic content, proving the role of phenolics as the main compounds for antioxidants. Crude methanol extract also exhibited significant toxicity effects on brine shrimp, *Artemia salina* with LC_{50} value obtained for acute and chronic was 635.47 μg mL^{-1} and 275.72 μg mL^{-1}, respectively. The crude methanol extract was later fractionated using silica gel column chromatography using a mixture of hexane and ethyl acetate as the mobile phase, yielding a total of

15 fractions (af1-af15). Only three fractions (af14, af15 and af7) were found active against *P. aeruginosa* ATCC 27853 with the MICs ranging from 125-250 μg ml^{-1}. However, due to inadequate amount of fractions collected (af15 and af7), only active fraction of af14 was further fractionated. Sub-fraction (aff2) of the active fraction of af14 which active against *P. aeruginosa* ATCC 27853 with the MIC of 75 μg mL^{-1} was analyze using LC-MS and GC-MS. The GC-MS analysis has identified the presence of hydrocarbons, fatty acids, aromatic dicarboxylate acid ester, benzene compounds and others. However, the active compounds were not being able to be identified based on mass spectral data from LC-MS analysis.

CHAPTER 1

INTRODUCTION

1.1 *Acanthophora spicifera* (Vahl) Borgesen

1.1.1 Morphological description

Figure 1.1 *Acanthophora spicifera* (Vahl) Borgesen

Acanthophora spicifera (Vahl) Borgesen is a common tropical and subtropical red alga (Kilar and McLachlan, 1986). It is an erect marine plant with growing height up to approximately 25-40 cm tall. It is also known as spiny seaweed due to the presence of numerous spines on its branches. The spines are arranged in radial order at all the branches except the main axes. The branches (2-3 mm in wide) are solid with a cylindrical and sharp tip ends. This algal has a wide spread distribution as it can grow either in a scattered clump as free floating algae or attached to other

substrates using the holdfast, such as on reefs and intertidal habitat area. *A. spicifera* also have a high tolerance against strong wave and water motion in which influences the size and morphology (colours and length of thalli). It can appear to be as shades of red, yellow, orange and brown. In intertidal and high water motion areas, the colours are normally darker, while lighter in shallow areas with low water motion (University of Hawaii, 2001). This alga belongs to the largest Rhodophyta family, Rhodomelaceace (Table 1.1).

Table 1.1 Taxonomy of *A. spicifera*

Kingdom	Plantae
Subkingdom	Biliphyta
Phylum	Rhodophyta
Subphylum	Eurhodophytina
Class	Florideophyceae
Order	Ceramiales
Family	Rhodomelaceae
Genus	*Acanthophora*
Species	***Acanthophora spicifera***

(**Source:** Jong *et al*., 1999)

1.1.2 Utilization and therapeutic values

In Fiji, *A. spicifera* is widely used in diets (Payri *et al*., 2000; South, 1993) as it generally exhibited non-starch polysaccharides, minerals and vitamins (Devi *et al*., 2009). While, in some part of Philippines (Trono, 1999), and Indonesia (Chorus and Bartram, 1999), *A. spicifera* are collected and eaten either as fresh raw vegetables or blanched in salads. In India, *Acanthophora* sp. is used as flavors in preparation of porridge (Dhargalkar and Pereira, 2005). Trono (1999) stated that, young shoots of *A. spicifera* serve as food thickening and flavouring agent in cooking, mainly in

soups. It was also reported that *A. spicifera* contain carrageenan, which is a suitable ingredient to be used in foods and non-foods products (Devi *et al*., 2009).

As for this local alga, little is known due to the limited research performed. According to Burkhill (1966), the consumption of *A. spicifera* as foods has been recorded in Philippine and Java (Indonesia) but not in Malaya. Also, none of the previous research outlined the usage of this local red alga for traditional medicine. Despite the lack of information regarding the utilization of this algae as foods and traditional medicine, various studies conducted in other countries have recognized the therapeutic values of *A. spicifera* for its antibiotic properties against various microorganisms (Gupta *et al*., 1991; Tuney *et al*., 2006), antiviral (Duarte *et al*., 2004) and as an anti-fertility agent (Wahidulla *et al*., 1986). *A. spicifera* is also used as feeding material for abalone (Phang, 2006).

1.1.3 Phytochemical constituents and bioactivity

Although study of *A. spicifera* on its chemical constituents is still a few in Malaysia, various previous study have reported the chemical constituents of *A. spicifera* studied in other countries. This bioactive compound was also evaluated using bioassays to investigate the biological properties. Some of the previous studies conducted on the phytochemical constituents and bioactivity of *A. spicifera* is shown in Table 1.2

Table 1.2 Phytochemical constituents and bioactivity of *A. spicifera*

Bioactive compounds	Bioactivity	References
Sterols: 6-hydroxycholest-4-ene-3-one, cholest-4-ene-3, 6-dione, 5 alpha-cholestane-3, 6-dione	Cytotoxicity against human cancer cell line	Han *et al*. (2009)
Flavonoids: kaempferol 3-*O*-α-*L*-fucopyranoside and quercetin 3-*O*-α-*L*-fucopyranoside	Antibacterial activity, antioxidant activity	Zheng *et al*. (2010)
Sulfated agaran	Antiviral activity	Duarte *et al.* (2004)

1.2 The needs of drugs from natural products

The uses of medicinal plants and herbs for therapeutic purposes have long started before the discovery of antibiotics by ancient peoples. The ancient peoples relies on their immune system and also organic and inorganic natural remedies namely, honey, molds, wine, vinegar, copper and others to treat infections and wounds (McKenna, 1998). The uses of the herbs and plants for medicinal purposes were widely practiced especially in rural area, where 65-80% of the world's population relies on it to treat ailments such as open wounds and others (WHO, 2002). Unfortunately, not all infections can be cured using the traditional medicines, thus the needs of antibiotics take place.

The rise of antibiotic resistance bacteria also added to the crucial needs of drugs from nature. According to Sorum (2006), one of the major outbreaks of the emergences of resistance strains was marked by *Shigella* sp. during the epidemics outbreak of bacillary dysentery in Japan. In 1950s, plasmid that contained the resistant gene, known as R-plasmid was found residing in the dysentery agent,

Shigella sp. which was reported to be resistant to sulfonamide antibiotics. Sulfonamides are the most common antibiotics used to treat dysentery during that time. The capability of *Shigella* sp. to transfer the R-plasmid to other, made the treatment with sulfonamides failed and hence resulted in increasing the resistant strains. Later, the resistance of *Shigella* sp was widespread in Japan whereby, they were found resistant against four types of antibiotics, namely sulfonamides, chloramphenicol, tetracycline and streptomycin. As a consequent, the choices of effective treatments become narrow. This rapid increased of the resistant strains may overhead the drug discovery itself. Thus, it has alarmed researchers in continuous searching for alternative and novel antibiotics especially form natural products for the better future.

Moreover, the health concerns regarding toxicity, carcinogenicity and potential hazards of synthetic antibiotics and antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) to living organisms, have triggered the search for new compounds from natural derivatives as a replacement which is naturally safer than the chemically synthesized compounds (Safer and al-Nughamish, 1999).

1.3 Potential drugs from natural products

Natural products from plants, microorganisms and algae are undoubtedly the most important sources in drug discoveries. United States Food and Drug Administration (FDA) reported in Annual Reports of Medical Chemistry from 1983-1994, that 60%-75% drugs in infectious diseases and cancer were derived from natural origin (Newman *et al.*, 2003). Among the natural products, algae have become the interest of current trends in drugs research as they exhibited numerous active compounds with promising biological activities. This remarkable active compounds secreted by the algae are known as secondary metabolites as they are not involved in the basic or primary functions of life (Cimino and Ghiselin, 2001). Marine algae secreted wide varieties of secondary metabolites such as peptides, terpenes, indoles, polyketides, volatile halogenated hydrocarbons and others (Butter and Sandy, 2009). Various studies have been conducted over the past decades to validate the biological properties of these secondary metabolites such as antimicrobial, antioxidant, antifouling, antiproliferative, antitumor and cytotoxic activity of the algae crude extracts (Blunt *et al.*, 2009).

Algae also produced halogenated compounds that play various roles such as, a defense mechanism against grazer and pathogens, against microorganism infections, for competing for space, mate recognition and prey detection (Goodwin *et al.*, 1997; Duarte *et al.*, 1999; Paul and Ritson-Williams, 2008; Hay, 2009). Studies on the importance of these secondary metabolites as a defense mechanism might be useful in searching for new drugs against pathogenic microorganism. Besides, it might become a platform to the development of potential novel drugs.

1.3.1 Seaweeds as source of drugs

Seaweeds have been known as one of the richest and most promising sources of bioactive secondary metabolites from sea which represent about 9% of compounds found from the sea (Baslow, 1969; Smith, 1977). These bioactive compounds with various structures and novel mechanisms of action exhibited wide range of biological functions namely, antimicrobial, antioxidant, antifouling, anti-inflammation and others. Thus, the search for new and natural alternative drugs with the algae as the focused of studies have been the interest among researchers worldwide. Several example of drugs derived from algae was shown in Table 1.3

An example of an interesting drugs discovered from algae is Phycocyanin (Pc) (Figure 1.2). Phycocyanin is a phycobiliprotein that is found as major pigment in *Spirulina* sp. (blue green algae) which have been reported to exhibit various pharmacological properties such as antioxidant activity, anti-inflamatory, neuroprotective and hepaprotective effects (Romay *et al*., 2003). Finding by Romay *et al.* (2003) reported that, Pc was effective in scavenging alkoxyl, peroxyl and hydroxyl radicals and also able to react with other oxidants like peroxinitrate. Pc also reduces inflammatory effects by reducing edema and prostaglandin level in the inflamed tissues. Due to its nutritional and therapeutical values, Pc is used as dietary supplements.

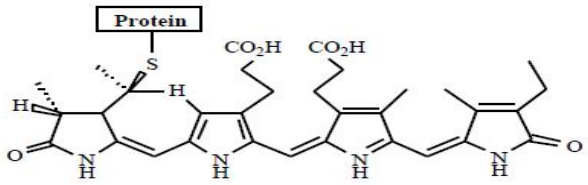
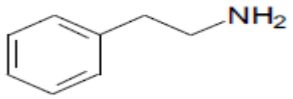
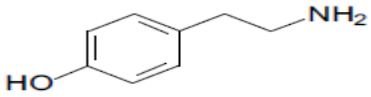
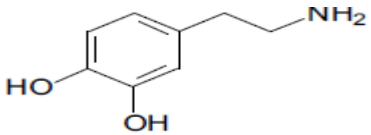
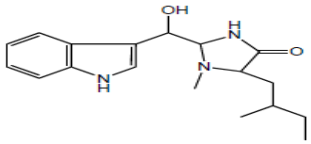
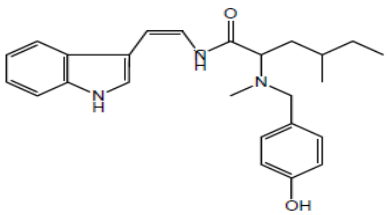


Figure 1.2 Chemical structure of phycocyanin bilin chromophore

Table 1.3 Drugs derived from algae

Drug	Structure	Pharmacological function	Derivatives
Phenethylamine		Antidepressants, Stimulants, hallucinoges	*Gracilaria bursa-pastoris, Ceramium rubrum*
Tyramine		Stimulants for central nervous system	*Laminaria sacclarina, Chondrus crispus*
Dopamine		Hormon, neurotransmitter, tread cardiovascular and kidney disorder	*Monostroma fuscum*
Martensine A		Antibacterials	*Martensia fragilis*
Fragilamide		Antioxidants	*Martensia fragilis*

(**Source:** Guven *et al*., 2010)

1.4 Selection of *Acanthophora spicifera*

In Malaysia, there is a few study conducted on the nutritional value compositions and biological properties of *Acanthophora spicifera* namely proximate composition analysis, antimicrobial activity, antioxidant activity and *in-vitro* toxicity on *Artemia salina*. Studies conducted in Malaysia are more focusing on the ecological study (Phang, 1984; Phang, 1998; Mijan Uddin *et al*., 2007). Whereby, other biological studies of *A. spicifera* have been reported from other countries like Brazil (Ballantine *et al*., 1987; Zubia *et al*., 2007), and India (Wahidulla *et al*., 1986; Ganesan *et al*., 2008). Therefore, the initiative is taken to study the biological properties of *A. spicifera* from Malaysian water. Findings of this study will add new information on the proximate compositions, antimicrobial activity, antioxidant activity, phenolic content and *in-vitro* toxicity on *A. salina* of the algae.

1.5 Objectives of study

The aims of the present study are as follows:

1. to extract bioactive compounds from *Acanthophora spicifera* with two different extraction methods; Soxhlet and maceration followed by solvent partition using various solvents, and also to screen antimicrobial and antioxidant activities, as well as total phenolic contents of the extracts.
2. to study the *in-vitro* toxicity on *Artemia salina*, as well as the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the extracts.
3. to study the structural and morphological degeneration of the selected bacterial strain treated with the active extract using spectroscopy methods: Scanning Electron Microscope (SEM) and Transmission Electron Microscope (TEM).
4. to fractionate the active extract using bioassay guided fractionation.
5. to identify the active compound using LC-MS and GC-MS.

CHAPTER 2

LITERATURE REVIEW

2.1 Fading Miracle: The Needs for New Antibiotics

2.1.1 Declining of antibiotics in pipeline

Despite the glory of the antibiotic discovery over the past 60 years, rapid outgrowth of drug resistance pathogens to most commercialized antibiotics have slowly overshadowed the success. The on going raised of the resistance has faded the effectiveness of antibiotics which gave difficulty in treating infectious diseases (Levy, 1992; Walsh and Amyes, 2004). Tenover (2006) also reported that, there is a rapid increased in the number of resistant bacteria and diversity of mechanism of resistance throughout the years which demanded the needs for new antibiotics especially for clinical fields.

Unfortunately, the development of new and effective antibiotics is declining. This scenario can be seen as reported by FDA database, where there was a decremented of 56% (from 16 to 7) of new antibacterial agent over the past 20 years (1998-2002 compared to 1983-1987). Since 1998, only 2 out of 9 new classes of antibacterial drugs (Linezoid, approved in 2000 and Daptomycin approved in 2003) possessed novel mechanism of actions (ECDC, 2009). Besides, in 2004, only 5 (1.6%) of the drugs developed by 15 major pharmaceutical companies were antibiotics in which none of them were novel class and ineffective against Gram-negative infections (Spellberg *et al*., 2004). Thus, continuous search for effective and novel antibiotics

is essential to compliment and to keep pace with the drying up of antibiotics in the current pharmaceutical industry.

2.1.2 Antibiotic resistance

The term “antibiotic resistance” refers to the ability of microorganism to evolve or withstand the effect of antibiotics (Levy, 1997). Antibiotic resistance was not a new phenomenon, because various studies have reported the spread of the resistance. According to Martinez (2009) antibiotic resistance is an evolutionary process resulted from natural selection which is a good example of Darwinian struggle for live theory. The theory stated that, bacteria can adapt and develop resistant towards the existing antibiotics over time in order to survive in the presence of environmental stress (such as excessive used and dumping of the antibiotics), where the strong bacteria continue to grow and spread the resistant genes (Wright, 2007). These resistance genes widespread were the results of mutations during the replication process or through acquisition of DNA plasmid from other bacteria (Dzidic and Bedekovic, 2003). These genes can be acquired by transferring of DNA plasmid to others via various mean such as conjugation, transformation and transduction process (McManus, 1997). The DNA plasmids hold the key to resistance as it encodes the genes or traits responsible for mechanism of resistance. The resistance in bacteria can evolved naturally over times due to certain factors such as, their naturally high growth rate (fast replication time), ability to acquire resistance from other bacteria (Overbye and Barrett, 2009), alteration of target site for the antibiotics and pumping out the antibiotics from the cells (Mendonco- Filho, 2006).

In certain case, multi-drug resistance can occur. This situation occurs as bacteria become resistance to more than one class of antibiotic introduced, which resulted from the transfer of the resistance genes between bacteria (ECDC, 2009). Hence, the risen of these mutants bacteria with the enhance ability to survive will continuously be emerging (IDSA, 2004; Cars, 2008). Some examples of the frequent antibiotic-resistance Gram-positive bacteria are methicillin-resistance *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Staphylococcus aureus* (Woodford, 2005). *Pseudomonas aeruginosa* is an example of antibiotic-resistance Gram-negative bacteria which was found to be resistant to most antibiotics such as β-lactams and carbapenem (Slama, 2008).

According to Dzidic and Bedekovic (2003), multiple drugs resistance has become a global concern especially in treating nosocomial related infections. It becomes the major concern in patients with weak immune systems such as HIV/AIDS patients, critical care unit patients and also severely ill patients. Beside, European Antimicrobial Resistance Surveillance System (EARSS) (2007) also reported that, it was estimated in 2007 that approximately 25 000 patients died from infections resulted from the frequent antibiotic-resistant bacteria in Norway, Iceland and Europe.

2.1.3 The emergence of diseases and infections

Relationship between emergence of new diseases and antibiotic resistance bacteria can be viewed as liner and in dependable manner, wherein the increased number of resistance bacteria towards antibiotics has resulted in the emergence of new diseases

and pathogens (Levy, 1997). Thus, the fight between human and pathogens have never been resolved. Today, increasing infections caused by the resistant pathogens have become one of the major public threats. According to WHO (2004), infectious diseases are the second major caused of death worldwide following cardiovascular diseases which accounted for 26% of 57 million deaths worldwide in 2002. The word "emergence" can be defined as newly appeared or already existed disease and infections in population and which are gradually increasing (Morse and Schluederberg, 1990; Morse, 1993). Some examples of current leading killing infectious diseases worldwide are HIV/AIDS, malaria and tuberculosis. Whereby, new infections currently reported were the Severe Acute Respiratory Syndrome (SARS), West Nile virus and influenza viruses (NIAID, 2007).

There are numerous factors that contributed to their emergence such as, biotic and abiotic factors. Biotic factors that might involves are the microbial adaptation and evolution to acquire the resistant genes via gene transfer (D' Agata *et al*., 2008) and also immune response of the host cell while, misuse and uncontrolled usage of antibiotics in the treatment of human infections are the abiotic factors that leads to the emergence of the resistance strains and infections (Idomir *et al.,* 2009).

The rise of resistance and emergence of new diseases and infections worldwide, leads to the higher health care costs especially in treating the nosocomial infections. This is because, more new and expensive drugs are required as well as longer treatment and diagnosis needed in which results in prolonged stays in the hospital. Thus, there is a crucial need for continuous research. Understanding the mechanism of reaction between the host and pathogens as well as collecting the valuable data

are required in order to develop and improve the treatments, vaccines and diagnostics.

2.2 History of antibiotic discovery

In a broad term, antibiotics are a type of antimicrobial drug which are effective in small concentration to inhibit or kills the growth of microorganism including bacteria, viruses, fungi, yeasts, protozoa, and parasites (IDSA, 2004). The development of antibiotics throughout the years is shown in Table 2.1.

Table 2.1 Discovery of the antibiotics

Year	Antibiotic discovery
1930s	Sulfonamides
1940s	Aminoglycosides
	Beta-lactams
1950s	Chloramphenicol
	Tetracyclines
	Macrolides
	Glycopeptides
1960s	Strepogramins
	Quinolones
	Lincosamides
1970s	Trimethoprim
1980s	-
1990s	-
2000s	Lipopeptides
	Oxazolidinones

Source: ECDC (2009).

The golden age of antibiotics started with the discovery of sulfonamides, penicillin and streptomycin in 1930s-1940s. Penicilin was the first beta-lactams (β-lactam) discovered by Alexander flaming in 1940s, from *Penicillium* sp.. This success was then followed by the development of chloramphenicol, tetracylines, macrolides and

glycopeptides in 1950s. The development of antibiotics was stable enough between 1930s to 1970s, as most of the antibiotics were discovered (Jagusztyn-Krynicka and Wyszynska, 2008). The antibiotic era started to enter declining phase at late 1970s to early 2000s, where only two classes of antibiotics (daptomycin and linezolid) were discovered. Daptomycin, was a cyclic lipopeptide derived from *Streptomyces roseosporus* while, linezolid was a synthetic antibiotic belongs to oxazolidinones. Both drugs were found effective against bacteria that were resistant against methicilin or vancomycin antibiotics (Pucci, 2006; Wright, 2007).

2.3 Types of antibiotics and mechanisms of action

There are three source of origin where antibiotics are derived from, which are natural, semi-synthetic and synthetic. The chemically modified natural antibiotics (semi-synthetic and synthetic) are done to enhance its efficacy (Madigan *et al.*, 2009). Most of the antibiotics produced today are derived from natural products (Newman and Cragg, 2007). These types of antibiotics can be classified into different classes according to their mode of actions, which varies between classes. Some of the major classes of antibiotics are β-lactams, tetracyclines, macrolides, and aminoglycosides.

Besides, antibiotics also can be classified according to their target group of microorganisms such as antibacterial, antifungal and antiviral agents (Sasidharan, 2006). Moreover, spectrum of activity of the antibiotics could also be used for classifications which are either broad or narrow spectrums. Broad spectrum antibiotics are able to react active against wide range of microorganisms (both

Gram-positive and Gram-negative bacteria) whereas, a narrow spectrum antibiotics only target a specific group of microorganism (either Gram-positive or Gram-negative bacteria). An effective antibiotic must have a good selective toxicity, where it only causes harm to the target microbes and not the host cells.

2.3.1 Antibacterial drugs

Antibacterial drugs only exert their effect on bacteria. The antibacterial antibiotics are classified based on their mode of action either bacteriostatic or bactericidal. Bacteriostatic antibiotics act by inhibiting the growth of bacteria wherein, bactericidal antibiotics kill bacteria. Different types of antibacterial drugs have different mechanism of actions. Generally, there are five mechanisms which are inhibition of cell wall synthesis, inhibition of protein synthesis, interference in metabolic pathway, nucleic acids synthesis and also disrupting the cell membrane. The different classes of antibiotics and summary of the mechanism action are shown in Table 2.2.

Table 2.2 Summary of antibiotics type, action and classes (Dzidic *et al*., 2008; Kohanski *et al*., 2010)

Class	Drug	Derivation	Species range	Primary target	Pathway affected
Fluoroquinolones					
DNA synthesis inhibitor	Nalidixic acid, ciprofloxacin, levofloxacin and gemifloxacin	Synthetic	Aerobic Gram-positive and Gram-negative species, some Anaerobic Gram-negative species (*Clostridium perfringes*) and *Mycobacterium tuberculosis*	Topoisomerase II (DNA gyrase), topoisomerase IV	DNA replication, SOS response, cell division, ATP generation, TCA cycle, Fe–S cluster synthesis, ROS formation, and envelope and redox-responsive two-component systems
Trimethoprim–sulfamethoxazole					
DNA synthesis inhibitor	Co-trimoxazole (a combination of trimethoprim and sulfamethoxazole in a 1:5 ratio)	Synthetic	Aerobic Gram-positive and Gram-negative species	Tetrahydrofolic acid synthesis inhibitors	Nucleotide biosynthesis and DNA replication
Rifamycins					
RNA synthesis inhibitor	Rifamycins, rifampin and rifapentine	Natural and semi-synthetic forms of ansamycins (derived from *Streptomyces mediterranei*)	Gram-positive and Gram-negative species, and *Mycobacterium tuberculosis*	DNA-dependent RNA polymerase	RNA transcription, DNA replication and SOS response

Table 2.2 Continued

Class	Drug	Derivation	Species range	Primary target	Pathway affected
β-lactams*					
Cell wall synthesis inhibitors	Penicillins (penicillin, ampicillin, oxacillin), cephalosporins (cefazolin, cefoxitin ceftriaxone, cefepime) and carbapenems (imipenem)	Natural and semi-synthetic forms of carbonyl lactam ring-containing azetidinone molecules (from *Penicillum notatum*, *Cephalosporium acremonium* and *Streptomyces cattleya*)	Aerobic and anaerobic Gram-positive and Gram-negative species	Penicillin-binding Proteins	Cell wall synthesis, cell division, autolysin activity (regulated by LytSR–VncRS-component system), SOS response, TCA cycle, Fe–S cluster synthesis, ROS formation, and envelope and redox-responsive two-component systems
Glycopeptides, glycolipopeptides					
Cell wall synthesis inhibitor	Vancomycin; teicoplanin	Natural and semi-synthetic forms of amino sugar-linked peptide chains (for glycopeptides) or of fatty acid-bearing, amino sugar-linked peptide chains (for glycolipopetides) derived from actinobacteria	Gram-positive species	Peptidoglycan units (terminal d-Ala-d-Ala dipeptide)	Cell wall synthesis, transglycosylation, transpeptidation and autolysin activation (VncRS two-component system)

* Drug efficacy can vary across species range based on drug generation. ‡When used as a combination of pristinamycin I and pristinamycin II.

Table 2.2 Continued

Class	Drug	Derivation	Species range	Primary target	Pathway affected
Lipopeptides					
Cell wall synthesis inhibitors	Daptomycin and polymixin B	Natural and semi-synthetic forms of fatty acid-linked peptide chains (from *Streptomyces roseosporus* and *Bacillus polymyxa*)	Gram-positive species (daptomycin), Gram-negative species (polymixins)	Cell membrane	Cell wall synthesis and envelope two-component systems
Aminoglycosides					
Protein synthesis inhibitors	Gentamicin, tobramycin, streptomycin and kanamycin	Natural and semi-synthetic forms of amino sugars (-mycins from *Streptomyces* spp. and-micins from *Micromonospora* sp.)	Aerobic Gram-positive and Gram-negative species, and *Mycobacterium tuberculosis*	30S ribosome	Protein translation (mistranslation by tRNA mismatching), Electron transport chain, SOS response, Tricarboxylic acid cycle, Fe–S cluster synthesis, Reactive oxygen species formation, and envelope and redox-responsive two-component systems
Tetracyclines					
Protein synthesis inhibitors	Tetracycline and doxycycline	Natural and semi-synthetic forms of four-ringed polyketides (from *Streptomyces aureofaciens* and *Streptomyces rimosus*)	Aerobic Gram-positive and Gram-negative species	30S ribosome	Protein translation (through inhibition of aminoacyl tRNA binding to ribosome)

Table 2.2 Continued

Class	Drug	Derivation	Species range	Primary target	Pathway affected
Macrolides					
Protein synthesis inhibitors	Erythromycin and azythromycin	Natural and semi-synthetic forms of 14- and 16 membered lactone rings (from *Saccharopolyspora erythraea* and *Streptomyces ambofaciens*)	Aerobic and anaerobic Gram-positive and Gram-negative species	50S ribosome	Protein translation (through inhibition of elongation and translocation steps) and free tRNA depletion
*****Sulfonamides***					
Metabolic pathway inhibitor	Sulfamethoxazole, trimethoprim	Synthetic	Aerobic Gram-positive and Gram-negative species	Folate synthesis	Biosynthesis of nucleotides (building block of DNA and RNA)

Source: **Dzidic *et al*. (2008); Kohanski *et al*. (2010)

2.4 Antioxidant activity

Antioxidant is referred to the compounds or substances that are able to prevent or minimize oxidative damages to the body tissues (Scheibmeir *et al*., 2005). Generally, they are two types of antioxidant which are natural and synthetic antioxidant. Natural antioxidants are generally refers to the compounds which originated from natural products such as phenolics, nitrogen compounds and carotenoids (Nandita and Ranjini, 2004). Synthetic antioxidants are commonly used in food processing as food preservatives (Zhang *et al*., 2010). Most common commercialized synthetic antioxidant like butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are used to prolong the storage life-span of food.

Antioxidant can be classified into two categories, based on their mechanism of action which is chain-breaking antioxidants and preventive antioxidant. The chain-breaking antioxidant acts by breaking the formation chain of the free radical by giving up an electron to the free radical and stabilize the free radicals. On the other hand, the preventive antioxidants inhibit or delay the oxidation process by scavenging the reactive oxygen species. There are three crucial enzymes needed in scavenging the reactive species namely, superoxide dismutase, catalase and glutathione (Scheibmeir *et al*., 2005).

2.4.1 Oxidative damage and diseases

Free radical denotes to any atoms with a single unpaired electron in the outermost shell that formed from the splitting of the weak bonds (Gutteridge and Mitchell,

1999). Free radicals are unstable and highly reactive as it tends to pair up with other electron to gain stability. The unpaired electron is responsible in causing cellular damage and disruptions of biological function by attacking macromolecules such as lipids, proteins, DNA and carbohydrates through lipid peroxidation (Kuhn, 2003; Wu and Cederbaum, 2003). Thus, presence of antioxidant in the body will helps in combating the free radicals.

Free radicals are produced by aerobic metabolic reactions of the body (Gutteridge, 1994), which resulted into two major types of free radicals being produced; reactive oxygen species (ROS) and reactive nitrogen species (RNS). According to Scheibmeir *et al*. (2005), reactive oxygen species (ROS) such as superoxide anion ($O_2^{-\bullet}$) and its derivatives; hydroxyl radical ($^{\bullet}OH$) and hydrogen peroxide (H_2O_2), are produced through reduction of triplet oxygen species. This reduction reaction that occurrs during electron transport chain (ETC) process in mitochondria require an enzyme called NADPH oxidase. The hydroxyl radical ($^{\bullet}OH$) produced is responsible in damaging cell membrane and lipoproteins through lipid peroxidation that leads to artherosclerosis (Kerr *et al*., 1996). The formation of the ROS types and its derivatives is illustrated in Figure 2.1.

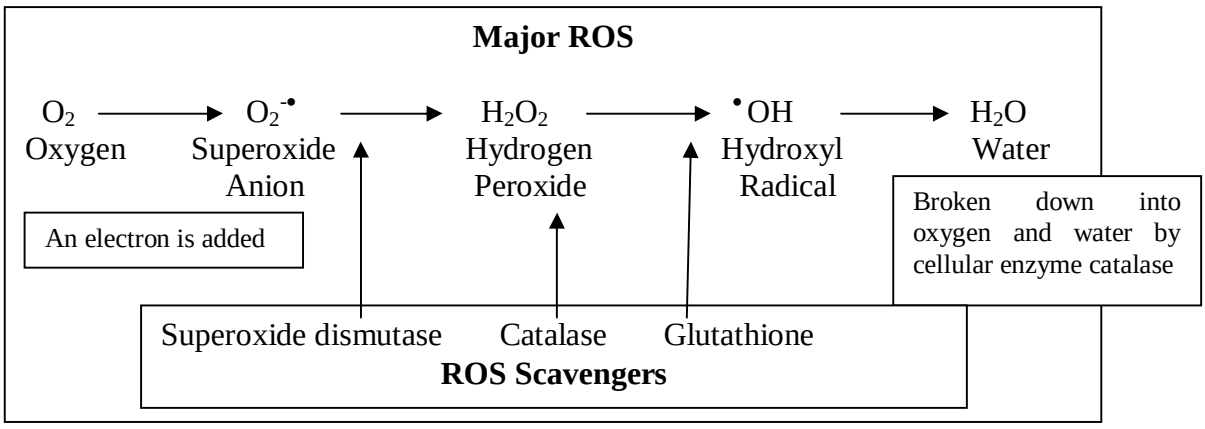


Figure 2.1 Formation of reactive oxygen species (ROS) and its derivatives. (**Source:** Scheibmeir *et al*., 2005)

Reactive nitrogen species (RNS) called nitric oxide ($NO^{\bullet}$) and its derivatives; nitrosonium cation (NO^{+}), nitroxyl anion (NO^{-}) and peroxynitrile ($ONOO^{-}$) are produced through an oxidative process of an L-arginine terminal nitrogen atom (Palmer *et al*., 1988; Stamler *et al*., 1992). On the bright side, not all free radicals are bad for body. Some of the free radicals that produced by body through metabolism reactions involved in important physiological functions as shown in Table 2.3. Liver for example, use free radical for detoxification and neutrophils produced free radicals to destroy the pathogens (Lunec *et al*., 2002).

Table 2.3 Important physiological functions involves free radicals or their derivatives

Type of radical	**Source of radical**	**Physiological process**
Superoxide anion ($O_2^{-\bullet}$) and related ROS	NADPH oxidase	Control of erythropoietin production; hypoxia-inducible functions; smooth muscle relaxation
Superoxide anion ($O_2^{-\bullet}$) and related ROS	Any source	Oxidative stress responses; maintenance of redox homeostasis
Nitric oxide ($NO^{\bullet}$)	Nitric oxide synthase	Smooth muscle relaxation (control of vascular tone); cGMP-dependent functions

*ROS; Reactive oxygen species. (**Source:** Droge, 2002).

However, unavailability of antioxidants or excessive production of free radicals in the body will lead to oxidative stress condition (Li *et al.*, 2009). This condition is responsible for development of diseases such as heart disease, congestive heart failure, Alzheimer's disease, ageing, liver damages, cancer and others (Duan *et al*., 2006).