BIOLOGICAL ACTIVITIES OF EXTRACTS FROM GREEN MARINE MACROALGAE, *HALIMEDA DISCOIDEA*

NOR AFIFAH BINTI SUPARDY

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BIOLOGICAL ACTIVITIES OF EXTRACTS FROM GREEN MARINE MACROALGAE, *HALIMEDA DISCOIDEA*

by

NOR AFIFAH BINTI SUPARDY

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CONTENT

LIST OF TABLES

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
B	butanol extract
C^+	chloramphenicol
C	chloroform extract
CFU	colony forming unit
DE	diethyl ether extract
DMSO	dimethyl sulfoxide
DPPH	diphenylpicryl-hydrazyl
EA (p)	ethyl acetate (partitioning) extract
EA (S)	ethyl acetate (Soxhlet) extract
GC-MS	gass chromatography-mass spectrometer
H	hexane extract
H_2SO_4	sulfuric acid
HMDS	hexamethyldisilazane
K	ketoconazole
LC_{50}	lethality concentration of 50%
M (p)	methanol (partitioning) extract
M (S)	methanol (Soxhlet) extract
MBC	minimum bactericidal concentration
MIC	minimum inhibitory concentration
MRSA	methicillin-resistance *Staphylococcus aureus*
NA	nutrient agar
$NAHCO_3$	sodium bicarbonate
NB	nutrient broth
PDA	potato dextrose agar
R_f	relative frequency
SD	standard deviation

SDB	Sabouraud dextrose broth
SEM	scanning electron microscope
TEM	transmission electron microscope
TLC	thin layer chromatography
TPC	total phenolic content
UV	ultra violet

AKTIVITI EKSTRAK SECARA BIOLOGI DARIPADA MAKROALGA HIJAU MARIN, *HALIMEDA DISCOIDEA*

ABSTRAK

Rumpai laut *Halimeda discoidea* telah dikaji bioaktivitinya dan pencirian sebatian bioaktif yang terlibat. Aktiviti antimikrob ekstrak-ekstrak kasar yang berlainan kutub (metanol, etil asetat, dietel eter, butanol, heksana dan kloroform) telah disaring terhadap 18 bakteria, tiga yis dan lima kulat melalui kaedah peresapan cakera. Didapati, ekstrak heksana telah muncul sebagai ekstrak paling aktif, diikuti dengan ekstrak etil asetat (Soxhlet) dan kloroform. Ekstrak heksana mempamerkan aktiviti terhadap lima bakteria Gram-positif, tiga Gram-negatif dan dua yis. Kemudian, kaedah pencairan kaldu secara makro telah dilaksanakan untuk menentukan kepekatan perencatan minimum (MIC) terhadap mikrob yang terencat. Keputusan telah menunjukkan nilai MIC dalam julat 0.25 mg/ml hingga 1.00 mg/ml. Hanya empat daripada 13 mikrob yang terencat mempamerkan nilai kepekatan maut minimum (MBC) pada nilai 1.00 mg/ml. Profil pertumbuhan bagi *Klebsiella pneumoniae* ATCC 13883, MRSA dan *Candida albicans* ditentukan untuk 48 jam pada kepekatan setengah MIC, MIC dan dua kali ganda MIC. Hasilnya, pada 48 jam dan pada kepekatan dua kali ganda MIC, *K. pneumoniae* ATCC 13883, MRSA dan *C. albicans* masing-masing telah menunjukkan pengurangan kira-kira 1.9 log, 2.3 log dan 3.0 log, berbanding dengan kawalan. Mikroskop elektron imbasan (SEM) dan mikroskop elektron transmisi (TEM) telah digunakan untuk melihat perubahan morfologi sel *K. pneumoniae* ATCC 13883 dan *C. albicans* setelah masing-masing dirawat dengan ekstrak heksana dan kloroform. Pada kepekatan MIC dan 100.00

mg/ml, perubahan morfologi seperti pengecutan dan pemecahan telah diamati pada sel-sel yang dirawat. Ekstrak heksana yang aktif disubjekkan kepada fraksinasi berpandukan biopencerakinan untuk memencilkan dan mencirikan sebatian bioaktif yang terlibat. Fraksi sf2 yang muncul sebagai fraksi aktif dengan nilai kepekatan sebanyak MIC 0.50 mg/ml bersama-sama dengan ekstrak kasar telah dianalisis melalui kaedah kromatografi gas-spektrometer jisim (GC-MS). Berdasarkan nilai peratus kesamaan ≥ 90 % berpandukan piawaian data NIST, dapat diandaikan bahawa aktiviti antimikrob telah dicirikan oleh sebatian E-15-heptadekenal. Tambahan kepada aktiviti antimikrob, ekstrak-ekstrak telah diperiksa aktiviti antioksida, jumlah kandungan fenol, dan analisa unsur-unsur utama, masing-masing melalui ujian penjerapan radikal DPPH, Folin-Ciocalteu dan 'Methods of Analysis of Association of Official Analytical Chemists (AOAC)'. Ekstrak kloroform telah menunjukkan pencapaian paling tinggi untuk kedua-dua aktiviti iaitu antioksidan dan jumlah kandungan fenol. Ujian kemautan anak udang brin telah dijalankan untuk menguji tahap akut dan kronik kesitotoksikan ekstrak heksana dan kloroform. Tahap ketoksikan yang agak tinggi telah dicatatkan oleh kedua-dua ekstrak dengan nilai kepekatan 50% maut (LC_{50}) pada 30.78 µg/ml untuk heksana dan 96.20 µg/ml untuk kloroform. Susunan keputusan analisa unsur-unsur utama daripada yang paling tinggi ke paling rendah ialah debu (66.00 ± 1.71) %, fiber (34.52 ± 2.61) %, protein (5.94 ± 0.03) %, kelembapan (3.21 ± 0.14) %, lemak (1.07 ± 0.14) %. Kesimpulannya, ekstrak tidak kutub heksana *H. discoidea* telah menunjukkan aktiviti antimikrob dan kemampuan ketoksikan yang bagus. Selain itu, ekstrak sederhana kutub kloroform telah menunjukkan sumber antikandida yang bagus, dan antioksidan dengan kandungan fenol yang tinggi.

BIOLOGICAL ACTIVITIES OF EXTRACTS FROM GREEN MACROALGAE, *HALIMEDA DISCOIDEA*

ABSTRACT

Macroalgae *Halimeda discoidea* was studied for its bioactivities and characterization of the bioactive fraction was carried out. The antimicrobial activity of 8 extracts with different polarities (methanol, ethyl acetate, diethyl ether, butanol, hexane and chloroform) was started with the screening against 18 bacteria, three yeast and five fungi, through the disc diffusion method. The result revealed that hexane extract was the most active extract, followed by the ethyl acetate (Soxhlet) and chloroform extracts. Hexane extract was active against five Gram-positive and three Gram-negative bacteria and two yeasts. Then, broth macrodilutan assay was carried out to determine the minimum inhibitory concentration (MIC) of all susceptible microbes. The results showed that the MIC values were in the range of 0.25 mg/ml to 1.00 mg/ml. Only four out of 13 susceptible microbes exhibited the minimum bactericidal concentration (MBC) of 1.00 mg/ml. The growth profile of *Klebsiella pneumoniae* ATCC 13883, MRSA and *Candida albicans* were determined for 48 hours at the concentrations of half MIC, MIC and double MIC. As the results, *K. pneumoniae* ATCC 13883, MRSA and *C. albicans* at double MIC showed reductions of approximately 1.9 log, 2.3 log and 3.0 log at 48 hours relative to the control, respectively. Scanning electron microscope (SEM) and transmission electron microscope (TEM) were used to view the morphological changes of the *K. pneumoniae* ATCC 13883 and *C. albicans* cells treated with the hexane and chloroform extracts, respectively. At the concentrations of MIC and 100.00 mg/ml,

morphological modifications such as shrinkage, lysing and breakage were observed in the treated cells. The active hexane extract was subjected to the bioassay-guided fractionation in order to isolate and characterize the bioactive compounds involved. Fraction sf2 that appeared as the active fraction with MIC value of 0.50 mg/ml, together with the crude hexane extract were analysed by the gas chromatography-mass spectrometer (GC-MS) method. Based on the basis of ≥ 90 % match with the NIST standard reference data and literature evidences, it be suggested that the antimicrobial activity of *H. discoidea* was attributed by the compound E-15-heptadecenal. In addition to the antimicrobial activity, the extracts were also investigated for their antioxidant activity, total phenolic content and proximate analysis through the DPPH-scavenging assay, Folin-Ciocalteu assay and methods of Analysis of Association of Official Analytical Chemists (AOAC), respectively. Chloroform extract showed the highest result for both antioxidant and total phenolic content. Brine shrimp lethality test was carried out to determine the acute and chronic cytotoxicity level of hexane and chloroform extracts. Relatively high of chronic toxicity level was recorded by both extracts with LC_{50} values of 30.78 µg/ml for hexane and 96.20 µg/ml for chloroform. As for the proximate analysis, the order of the component from the major to the least was ash (66.00 ± 1.71) %, fibre (34.52 ± 2.61) %, protein (5.94 ± 0.03) %, moisture (3.21 ± 0.14) % and lipid (1.07 ± 0.14) %. In conclusion, the non-polar hexane extract of *H. discoidea* showed promising antimicrobial activity and toxicity potential. In other hand, the medium polarity of chloroform extract of *H. discoidea* showed good source of anticandida and antioxidant activities with high phenolic content.

CHAPTER 1.0 INTRODUCTION

1.1 Discovery of seaweed-derived drugs

Natural products derived from both terrestrial and marine organisms play a crucial role in the treatment of ailments since time immemorial (Smit, 2004; Vishwanathan and Basavaraju 2010; Alzweiri *et al*., 2011). The biological properties of these natural products such as; antibiotic for microbial infections, antioxidant agent to combat free radicals, antiviral and anticancer agents have been widely reported. As for the antibiotic purpose, the controls of bacterial and fungal infections have been remarkably effective since the discovery of *Bacillus anthracis* to cure the anthrax disease during 1877. Research on natural antibiotic has expands and many plant-derived antimicrobial drugs have been successfully isolated (Dorman and Deans, 2000; Duraipandiyan *et al.*, 2006)

Unfortunately, together with this increment is the rise of multidrug-resistant pathogens (Wright, 2005; Theuretzbache, 2009). The phenomenon of drug resistance grows drastically and has become a serious problem than expected. The improper use of antibiotic has triggered the microbes to be genetically mutant and no longer susceptible to the drugs used. Therefore, the search for new antibiotic is targeted at new target such as the marine organisms, especially the seaweeds. The great diversity of seaweeds in the oceans represents inexhaustible sources of highly bioactive secondary metabolites, awaiting to be discovered.

Seaweeds, also known as macroalgae are being utilized as a food source and as traditional remedies by the people from many parts of the world since long time ago.

However, the revolution of macroalgae usage has increased significantly especially in the extraction of the macroalgae' compounds. The extraction of compounds from the seaweeds has started with the extraction of agar, carrageenan and alginate that are used as industrial products. Since then, scientists started to search for other compounds from the seaweed that are also biologically active. As the attention has shifted to the seaweeds, many biologically active secondary metabolites have been isolated and evaluated for their bioactivities from all over the world (Smit, 2004). Different seaweeds form different divisions; Chlorophyta (green macroalgae), Rhodophyta (red macroalgae) and Phaeophyta (brown macroalgae) have shown to possess selective or multiple bioactivities. One of them is the sulphated polysaccharides from red macroalgae *Aghardhiella tenera* that shows antiviral activities towards human immunodeficiency virus (HIV) and Herpes simplex virus (HSV) (Witvrouw *et al*., 1994). Another example is the compound Kahalalide F that that was isolated from the green macroalgae *Bryopsis* sp. that exhibited multiple bioactivities including the anticancer, antitumor, and antiviral activities (El Sayed *et al*., 2000). Almost all brown algae contain phlorotannins, phenolic compound that is very effective as the antioxidant agent. For example, the fucols in *Fucus Vesiculosus* (Koivikko *et al*., 2007) and eckols in *Eckonia stolonifera* (Kim *et al*., 2009).

The discovery of effective bioactive compounds from the macroalgae has brings hope to the pharmaceutical field. The occurrence of highly diverse macroalgae' secondary metabolite is closely related with the ability of the macroalgae to survive in the habitat.

1.2 Selection of macroalgae for drug discovery

More than 8000 species of macroalgae inhabit the oceans and coastlines of the earth (www.seaweed.ie). It is a fact that different macroalgae floras grow at different oceans based on their habitat suitability and the climate zones. Malaysia is a small country that is situated near the equator and is surrounded by South China Sea, Pacific Ocean and Andaman Sea hence experiencing the tropical climate that is hot and humid throughout the year. The variety in the type and distribution of Malaysia macroalgae is influenced by the rains and tides caused by the monsoon wind systems, the Northeast Monsoon and Southeast Monsoon that blow at different times of the year (Phang, 1998).

The study on Malaysia's macroalgae has started since 1984 (Phang, 1984), and according to Du *et al*., (2008), the distribution checklist has been updated by Phang in the following years (1994, 1998, 2006, 2007). There were also some other field works reported on the distribution of the macroalgae at particular locations, such as the Port Dickson (Mijan Uddin *et al*., 2007), Straits of Malacca (Phang *et al*., 2008) and Johor (Gan *et al*., 2011). Based on the published data, the highest taxa is conquered by the red algae group (Rhodophyta), followed by the green algae (Chlorophyta) and lastly the brown algae (Phaeophyta). Despite of the increasing records on the distribution and ecology of the macroalgae, the study on the phytochemical compounds and the bioactivities of these Malaysia macroalgae had received little attention and still very low in number. Additionally, the existed bioactivity studies of the macroalgae were only emphasized on the edible macroalgae such as *Gracilaria changi* (Sasidharan *et al*., 2009), *Acantophora spicifera* (Nurul

Aili *et al*., 2010), *Euchema denticulatum* (Al-Haj *et al*., 2009; Matanjun *et al*., 2009), *Laurencia* spp (Vairappan *et al*., 2008; Vairappan *et al*., 2010), *Caulerpa lentillifera and Sargassum polycystum* (Matanjun *et al*., 2009). Out of 373 specific and intraspecific taxa that has been identified (Phang et al., 2007), the species that have been studied represent a very small part of the Malaysian macroalgae. Despite of the large macroalgae community in Malaysia, the attention is always on the edible species. Therefore the inedible species are underutilised. One of them is from the genus *Halimeda* sp., including *Halimeda discoidea* Decaisne. This species can be found in many parts of the world oceans; Pacific Ocean (Fenical and Paul, 1984), Bahamas and the Great Barrier Reef (Verbruggen *et al*., 2007), Australian coast (http://florabase.dec.wa.gov.au), Arabian Sea (Sohrabipour and Rabiei, 2007) and Mediterranean (Braga *et al*., 1996), Caribbean Sea (Fenical and Paul, 1984)

In Malaysia, the species in the family Halimedaceae' is consists of *Halimeda discoidea, H. macroloba, H. opuntia, H. simulans* and *H. tuna* and is widely distributed at the Sabah, East and West coast of Peninsular Malaysia (Phang, 2006). There is neither nutritional value nor industrial value that has been recorded regarding to this macroalgae, except it is only being used as fertilizers for the soil amendment to make the soil less acidic (www.wildsingapore.com). However, there are reports on some bioactivities of this *Halimeda* genus, including the antimicrobial activity (del Val, 2001; Karthikaidevi *et al*., 2009), antioxidant activity (Zubia et al., 2007; Boonchum *et al*., 2011) and toxicology (Paul and Hay, 1986). The production of secondary metabolites of *Halimeda* sp. was noted to contribute to these bioactivities. Nevertheless, the isolation of the bioactive compound from *Halimeda* sp. is still limited as until now, only halimedatetraacetate and halimedatrial (Paul *et*

al., 1998a) that have been isolated and recognized as biologically active. There have been a number of reports of antibiotic activity of the *Halimeda* spp. However, for the most part these reports represented the preliminary studies of crude extracts and little follow-up work has been done to isolate the bioactive compounds. Since the bioactivities and compounds characterization of this *Halimeda* sp. is still scarce, therefore this species was selected to be studied. This species also could be the source of new compounds with highly potential bioactivities.

1.3 Problem statements

Investigation pertaining to the bioactivities of *Halimeda* spp. from various locations has been recorded, but the one that originates from Malaysia especially in the antimicrobial activity is still very limited. Therefore, the aim of this study was to evaluate the bioactivities of a species from the genus *Halimeda*, namely *Halimeda discoidea* as the antimicrobial and antioxidant agents, together with the information on its toxicity property, phenolic content, analysis of the chemical compounds of the extract and the proximate analysis. This study may provide excellent information on the biologically and chemically leads for the discovery of new drugs.

1.4 Objectives of research

The objectives of this study were:

1. To extract the bioactive compounds of *Halimeda discoidea* using various organic solvents and to screen the antimicrobial activity of its crude extracts against a series of pathogenic clinical and ATCC bacterial, yeasts and fungal cultures.

2. To study the effect of the extracts on the growth of microorganisms and their morphological changes, and also to determine the cytotoxicity against the brine shrimp, *Artemia salina*.

3. To determine the antioxidant activity, total phenolic content and proximate analysis of the *Halimeda discoidea* crude extracts.

4. To isolate and identify the bioactive compound with antimicrobial activity from the most active extract of *Halimeda discoidea.*

CHAPTER 2.0 LITERATURE REVIEW

2.1 Natural products as the healers

For thousands of years, traditional medicine practitioners have been using the natural products of plants and animals to treat the sick people. The term natural product is applicable for any biological molecule of the living organisms, but it is usually referred as the secondary metabolites that is produced for the defense mechanism, regulator molecules or as the result of nutrient limitation. Natural products exhibit many biological properties including the antibiotic for microbial infections, antioxidant agent to combat free radicals, antiviral and anticancer agents. The chemical studies of the natural products has begun in the year 1877, when Louis Pauster found the right treatment to cure the anthrax disease, by introducing the *Bacillus anthracis* strain to the infected animal. The emergence of natural product antibiotic continued with a finding by Rudolf Emmerich in 1890s that showed cholera infection can be treated with a medication called pyocyanase from microbes After that, penicillin from the mould *Penicillium notatum* was found to inhibit the growth of *Staphylococcus aureus.* The trend of searching for healer from natural product has shifted to the plant. All higher plants are capable to produce secondary metabolites as their common defence weapon to fight off herbivorous animals and microbial infections (Sirikantaramas *et al.*, 2008). Those secondary metabolites turn out to be the crucial substances for developing natural-derived drugs. For example, a plant-derived drug named curcumin from *Curcuma longa* is used to treat gastrointestinal upset and arthritic pain. Taxol and teniposide that are isolated from *Taxus brevifolia* and *Podophyllum peltatum*, respectively are used as antitumor

agent. Over the last few years, more plant-derived compounds with antimicrobial activity have appeared in the literatures (Dorman and Deans, 2000; Duraipandiyan *et al.*, 2006).

2.2 Antimicrobial agent and the need for new discoveries

The golden era of effective antibiotic therapy such as penicillin, cephalosporin, and streptomycin against pathogenic microbes has sunk slowly with the rise of new resistant strains. The need for new antimicrobial agents has become a major public health concern nowadays. The underlying factors of this phenomenon are the emergence of new diseases caused by the multidrug-resistance microbes and lack in the production of new functional antimicrobial drugs.

Within the past half century, many scientific journals and articles seem to report the discovery of wide variety of antimicrobial compounds (Rivero-Cruz, 2008, Al-Bayati, 2009; Arifuzzaman *et al.*, 2010; Engelhardt *et al.*, 2010). Academic researchers, biotechnology industries and pharmaceutical companies have been struggling to isolate, design and synthesize the new potential compounds and tested them against the top listed infectious microbes. Unfortunately during the 1990s, many pharmaceutical companies have withdrawn themselves from this antibiotic field because of some reasons (Projan, 2003; Norrby *et al.*, 2005). All these efforts were done in order to fulfil the unmet medical needs and to reduce the percentage of morbidity and mortality caused by these aggressive strains. Although there are many new compounds from various sources have been isolated and screened for their antimicrobial properties, only small part of them passed the clinical trial phase.

Drugs that enter clinical trial have to go through some complicated procedures with high qualification standard and sometimes some of them are lack of vital information (Lunde, 1990; Manheimer and Anderson, 2002). Hence, this hurdle does restrain the new promising antimicrobial drugs from being marketing out there.

Starting late 20th century up to the beginning of 21st century, the emerging of new infectious diseases has emerged regularly and caused chaotic to the mankind. The rate for this appearance is at the average of one disease per year (Rincon, 2006). The emergence of new infectious diseases can be defined as the increment of new human diseases that are caused either by the introduction of new agent to the existed unrecognized agent or due to the demographic and environmental changes (Lederberg *et al.*, 1992). New diseases that appeared not only focus on the bacteria alone, but it also involves viruses and parasitic agents. The current examples of emerging diseases are such as HIV/AIDS, Lyme diseases, food borne diseases cause by some *Escherichia coli* strains, severe acute respiratory syndrome (SARS) and Spanish flu (Woolhouse and Antia, 2008).

A series of factors are causing this life-threatening phenomenon. One of them is the ecological changes for the agricultural development and economic purposes (Patz *et al.*, 2004). People who work in environments such as the land, farm, forests and swamps are actually at risk of being infected. The interaction between the people and the environment somehow unintentionally exposed them to the infective agents (Morse, 1995). Nature disasters like flood and drought which involves water and weather are always associated with the dissemination of infections to others. For example, the Korean hemorrhagic fever among the farmers that is caused from the

contact of infected field mouse *Apodemus agrarius* with a virus called Hantaan to the farmers (Nathanson and Nichol, 1998).

It has been suggested that demographical changes and human behaviours also contribute to this new disease outbreaks. Migrated people including refugees, students, pilgrims and immigrants have the potential to unconsciously bring along the inactivated virus that present within them to the new place (Wilson, 1995). This will bring the local hosts into contact with the survived pathogens and may become epidemic. Besides, the lifestyle of people has a great influence in the emergence and re- emergence of diseases. Crowded places like day care centre, swimming pool and recreational park are the focal point of distributing contaminated water. People who go there can easily acquire the contaminated water and plagues will start to spread (Macpherson, 2005).

Sometimes new diseases arise because of the microbial adaptation itself. Evolving microbes have the ability to be resilient and adaptive to the unfavourable surrounding like scarcity in nutrient supply, climate changes and the presence of antimicrobial compounds that purposely introduced to kill the microbes (Alanis, 2005). Of them, the resistance with antibiotic problem has received a great attention worldwide (Van der Waaij and Nord, 2000; Haeno and Iwasa, 2007; Vonberg *et al.*, 2008). Inadequate or over use of antibiotics during the antibiotic therapy has exposed the microbes to the antibiotics thus giving them chance to learn and adapt to the mechanism of action of the antibiotics (French, 2005). They are not only evolved to be resistant to the antibiotics but they also could remain alive in the hospital and infect high risk group including the neonates and immune suppressed persons.

2.3 Antibiotics

Originally, antibiotic is defined as microbial metabolites with low-molecular weight substances that are capable to inhibit the growth of other microorganisms at low concentrations. However the definition has changed when the sources of antibiotics such as plant products with chemically modified natural antibiotics (semi synthetic) and synthetic products emerged. The modified version of this antibiotic definition now is any natural product or a substance that 100% or partly produced by chemical synthesis of the natural products that can inhibit the growth of other microorganisms at low concentrations (Russell, 2004). Antibiotics are classified according to the main groups of microorganisms they inhibit; for instant antibacterial, antifungal and antiviral antibiotics. The antimicrobial therapies of an antibiotic are divided into two broad groups, which is bactericidal and bacteriostatic. It is bactericidal when the microbial inhibition is irreversible, meaning that the infecting microorganism is killed permanently and it is bacteriostatic when the microbial inhibition is reversible, meaning that the growth is impaired temporarily (Lancini *et al.*, 1995a)

2.3.1 Antibacterial antibiotic and its mechanism of actions

There are various mechanisms of actions possessed by the antibiotics and this depends largely on the antimicrobial compounds of the antibiotics and their target macromolecules of the microorganisms. Once the active compound managed to reach the site of action that usually involved in cell's routine function, it will inhibit the metabolic process and eventually cause the cell to be malfunctioned and die. Antibiotics that have same basic chemical structures are grouped together and

different groups will perform different kind of actions in inhibiting the microbial growth. Mechanisms of actions of the antibiotics are such as inhibiting the cell wall synthesis, transcription, replication, translation, membrane synthesis and function and as antimetabolites (Lancini *et al.*, 1995b). Major types of antibiotics based on the chemical structures and the examples of each type are summarized in Table 2.1.

Table 2.1: Classification of main antibacterial antibiotics and the mechanism of actions (Yuri, 2010)

Antibiotics	Examples	Mechanism of actions
Penicillins	Ampicillin and amoxicillin	Inhibit cell wall formation
Cephalosporins	Cephalothin, cefazolin, ceforanide, cefuroxime, cefpodoxime cefozopran	Inhibit cell wall formation
Fluoroquinolones	Ciprofloxacin, levofloxacin, lomefloxacin, norfloxacin	Interfere with nucleic acid replication and transcription
Tetracycline	Tetracycline, doxycycline, minocycline, oxytetracycline.	Inhibit protein synthesis
Macrolides	Clarithromycin, azithromycin, roxithromycin, troleandomycin	Inhibit protein synthesis

2.3.1.1 Disruption of microbial cell wall

The first two β-Lactam antibiotics are known as penicillins (penicillin G and penicillin V) and cephalosporins (cephalosporin C), and both of them originated from the *Penicillium notatum* and *Cephalosporium* sp. respectively. This antibiotic is named based upon its basic chemical structure, which is the four-membered β-lactam ring and the structure of the second ring is the one that determine either it is

penicillin or cephalosporin. This structure is responsible in disrupting the synthesis of peptidoglycan, the vital component of the cell wall of all bacteria by interacting with the transpeptidase or d-carboxypeptidases (Weil *et al.*, 1995; Lambert, 2004; Silver, 2006). Cell wall is a network of rigid structure called peptidoglycan and other macromolecules like polysaccharides and lipoproteins and its function is to maintain the shape and bear the high internal osmotic pressure of the cell. The percentage of peptidoglycan that presents in the cell wall of Gram-positive bacteria is higher than the one in Gram-negative bacteria (Scheffers and Pinho, 2005) and this explains the varying degree of sensitivity of different bacteria towards different β-lactam antibiotics. Once the cell wall being distorted, the high cytoplasm osmotic pressure of the cell will cause the cell's shape to deform and burst to death.

2.3.1.2 Inhibition of outer membrane synthesis

An outer membrane is a part of plasma membrane structure that only presents in the Gram-negative bacteria and it is located at the outermost part of the cell. The interaction between the two major macromolecules in the cell membranes which is phospholipids and lipopolysaccharides serves as the protective barrier against harmful substances, including antibiotics. Unfortunately, antibiotics such as polymyxins and its derivatives are more susceptible to Gram-negative bacteria such as *Pseudomonas aeruginosa*, *Escherechia coli* and *Klebsiella* spp..(Behera *et al.*, 2010; Falagas *et al.,* 2010). It is because as the antibiotic is highly attracted to the outer membrane than the inner membrane. The selective activity of the antibiotics against Gram-positive and Gram-negative bacteria is because of the existence of this outer membrane.

2.3.1.3 Inhibition of protein synthesis

Ribosome plays the role as the central unit for protein synthesis of both prokaryotic and eukaryotic cells and organelles. Antibiotics act on the protein synthesis at three different stages; distorting ribosomal binding sites (Franceschi and Duffy, 2006; Murray *et al.*, 2006), inhibiting the production of aminoacyl-tRNA (Kirillov *et al.*, 1997) and interfering with the elongation factors of the ribosome (Landini *et al.*, 1993; Collignon and Turnidge, 1999). Examples of antibiotics that inhibit protein synthesis are such as mupirocin, streptomycin, cloramphenicol, erythromycin, fusidic acid and elfamycins.

2.3.1.4 Inhibition of nucleic acid replication and transcription

Deoxyribonucleic acid (DNA) is the most important macromolecule for all living things. However, the macromolecule can be interfered by antibiotics such as fluoroquinolones, sulphonamides, rifampicin and nitroimadazoles. Fluoroqinolones and rifampicin are the antibacterial agents that inhibit the synthesis of some essential enzymes for the DNA synthesis (Smith *et al.*, 2001; Tupin *et al.*, 2010). The analogues of antibiotics such as sulphanomides can act as the inhibitors of nucleic acid polymerization (Stokstad and Jukes, 1987) while reduced nitroimadazoles can cause the breakage of the DNA strand (LaRusso *et al.*, 1977).

2.3.1.5 Antimetabolites

Antimetabolite antibiotic interferes with the bacterial metabolic pathway by having similar structure as the natural metabolites, thus they compete with each other in order to bind to the natural metabolite's binding site. Once the binding site is wrongly accommodated by the antimetabolite, the metabolic pathway will be alternated and the normal process is inhibited. There are two types of antimetabolites based on their mechanism of actions. The first type is having the same chemical structure like other essential constituent thus inhibit the synthesis of other essential macromolecules, such as nucleic acid (Talwar and Srivastava, 2003). The second mechanism of action involves the fusion of antimetabolites during the polymerization of macromolecules such as DNA, RNA, and proteins (Sitaram and Nagaraj, 1999).

2.3.2 Antifungal antibiotic and its mechanism of actions

Fungi and yeast are the microorganisms of eukaryotic group and are classified under the same group with other higher level organisms. Sufficient actions of the antifungal agents against targeted macromolecules of the fungi will lead to death of the fungi. This will permanently or temporarily combat the growth of harmful fungal infections of the hosts. Despite of the successful development of many new antifungal drugs, the therapeutic failures on the fungal infections also keep on appearing dramatically. The number of life-threatening fungal infections such as the aspergillosis and candidosis caused by *Aspergillus* sp. and *Candida* sp. respectively among immunocompromised patients showed an upward trend for the past two decades

(Díaz-Fuentes *et al.*, 2001; Slavin *et al.*, 2001; Pierrotti and Baddour, 2002). Antifungal antibiotics work through several mechanism of actions, which is inhibiting the fungal cell wall or plasma membrane formation, inhibiting the synthesis genetic materials and disruption their mitotic pathway (Borgers, 1980; Odds, 2003). Table 2.2 shows the classification of main antifungal antibiotics and their mechanism of actions.

Table 2.2: Classification of main antifungal antibiotics (Gupte *et al.*, 2002)

Antibiotics	Examples	Mechanism of actions
Polyene	Amphotericin B, Nystatin	Disrupting the ergosterol of the cell membrane
Azoles	Miconazole, Ketoconazole, Fluconazole, Variconazole	Disrupting the sterols/ ergosterols of the cell membrane
Allylamine and thiocarbamate	Naftifine, Terbinafine, Tolnaftate	Disrupting the ergosterol of the cell membrane
Morpholine	Amorolfine	Inhibit sterol synthesis component
Nucleoside analogue	5-Fluorocytosine (5FC)	Inhibit DNA synthesis

2.3.2.1 Inhibitors of fungal cell wall synthesis

The fungal cell wall that serves as protective barrier and support to fungi's cellular organelles is always the target of host immune system and antibiotics during the fungal infection. In filamentous fungi such as *Aspergillus fumigatus* and yeast like *Candida albicans*, the cell wall's constituents are the chitin and the mixture of β-

glucan (Sanjuán *et al.*, 1995; Bernard and Latgé, 2001; Netea *et al.*, 2006;). This uniqueness makes the fungal cell wall to be easily targeted and destroyed by the antibiotics. Echinocandins is an example of antifungal that will disrupt the production of (1,3)-β-D-glucan synthase enzyme of the cell wall and caused disruption in the cell wall (Debono and Gordee, 1994; Deresinski and Stevens, 2003). Another promising target of antifungal drugs on the cell wall is the chitin. Polyoxins and nikkomycins inhibit the synthesis of enzyme chitin synthetase in fungal (Endo *et al.*, 1970; Li and Rinaldi, 1999).

2.3.2.2 Disruption of chromosome function and replication

The antifungal agent called 5-fluorocytosine has been shown to have inhibition activity in some fungi (Waldrof and Polak, 1983; Pfaller *et al.*, 2002; Paluszynski *et al.*, 2008). 5-fluorocytosine exerts its antifungal effect by inhibiting the thymidylate synthetase activity and synthesizing 5-fluoro-2'-deoxyuridylic acid causing an impairment of DNA synthesis (Fisher and Zaoutis, 2008)

2.3.2.3 Disruption on the membrane structure

Ergosterol (predominant sterol) and other sterol compositions are important macromolecules in the formation of fungal cell membrane (Mysyakina *et al.*, 2002; Weete *et al.*, 2010). These cell membrane components are vital to maintain the cellular functions thus allowing the fungi to survive and proliferate. The alteration of the membrane structure occurred when polyene and its members such as amphotericin B and nystatin bind to the membrane sterols of the fungal, resulting in

channels formation and loss of inner cell's molecules (Lancini *et al.*, 1995b). The presence of lipophilic site of the antibiotic and lipophilic site of the fungal sterol ring within the membrane caused them to be highly attracted to each other (Kaneshiro, 2002; Hac-Wydro and Dynarowicz-Latka, 2006). Azoles are another type of antifungal drugs that derive their action towards the membrane cells.

2.3.2.4 Inhibition of fungal cell mitosis

The diseases caused by superficial mycoses on the skin, hair and nails are usually treated with the fungicidal agent griseofulvin, orally (Hector, 2005). Griseofulvin is one of the earliest antifungal products extracted from the *Penicillium griseofulvum*. The mechanism of action for this type of drug is based on the disruption of the assembly of microtubules in the fungal cell (Rezanka and Spízek, 2005) as well as the microtubules' cytoplasmic content (Bossche *et al.*, 2003) and thus inhibiting the fungal cell mitosis pathway.

2.4 Antioxidant

Reactive oxygen species (ROS) or free radicals can be defined as derivatives of oxygen molecules with one unpaired electron (Turrens, 2003). A free radical is formed during the redox reaction of a molecule whereby one electron is removed from the paired electron and caused the molecule to become unstable and very reactive. A complete redox reaction of oxygen will produce water molecule and also some by-products, which are the ROS. Normal metabolic pathways of living organisms will generate free radicals and other ROS like hydrogen peroxide and

hypochlorite ions that tend to attack other molecules (Langseth, 1995). The most common occurrence reason of oxidation process in living things are due to the photosynthesis in plants (Dummermuth *et al.,* 2003) and aerobic respiration system (Pham-Huy *et al.,* 2008).

In aquatic plants, intense light illumination and high salt intensity will result in excessive ROS production and this can be a threat to the cells. Free radicals are very reactive and can rapidly attack lipids, carbohydrates, DNA and other protein molecules of nearby cells (Pietta, 2000). Damages to the functioning cells later on will cause chronic diseases such as cancers, coronary heart disease, Alzheimer and Parkinson (Nabatchian *et al.,* 2004; Gu *et al.,* 2005; Butterfield *et al.,* 2006; Pham-Huy *et al.,* 2008). Antioxidant is needed to counter act the oxidation process by slowing down or completely terminate the process. Antioxidants are the reductive agents that act on the oxidants thus inhibiting the oxidation of other substances (Vaya and Aviram, 2008).

Cells in human are protected by two defensive systems against these ROS, which are the endogenous and exogenous antioxidants. Endogenous antioxidants that naturally present in the body are divided into two; enzymatic antioxidants, including glutathione peroxidases, superoxide dismutases and catalase, (Langseth, 1995) and also non-enzymatic antioxidant, including α-tocopherol (vitamin E), β-carotene, ascorbate (vitamin C), and glutathione (Droge, 2002). Incomplete protection of endogenous antioxidants is covered by the antioxidants from the outside source, which is known as exogenous antioxidants. Exogenous antioxidants, such as vitamin

A, C, E, polyphenols and carotenoids are obtained through the daily food consumption (Pietta, 2000).

Knowing that antioxidant compounds are crucially needed to maintain a healthy body, the demand and production of synthetic antioxidants has gone apace across time. Buthylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), octyl gallate (OG) and dodecyl gallate (DG) and quacertin are the examples of synthetic antioxidants and they are known to have better activity than natural antioxidants. However, consumers are more preferred to consume natural antioxidants than synthetic one, concerning to the safety issues of the synthetic products (Botterweck *et al*., 2000; Pokorný, 2007). Epidemiological and clinical studies on plant-based foods such as vegetables, fruits and medicinal plants has proved that terrestrial plants are rich with antioxidants compounds (Auddy *et al*., 2003; Saha *et al.,* 2004; Montoro *et al.,* 2005).

2.5 Bioactive compounds from marine sources

Marine products have been appreciated by the mankind since many years ago. Back then, the usage of the marine products is only based on the understanding that this particular marine organism can heal the ailments and is passed down to the next descendents. No one knows the exact mechanism that underlies the treatment. The failures of existed antibiotics in killing resistance microbes have inspired the researches to search for the bioactive compounds from various sources, including the oceans. The expanding interest in marine-derived bioactive compounds has somehow reduced the criticalness of the antibiotic-resistant dilemma.

The large area covers by the oceans on this earth has contributed to the rich diversity of marine organisms and habitats, and thus encompass a wide variety of chemical classes. Eventhough a huge number of marine-derived drugs with various biological activities such as antiviral, anticancer and antibacterial have been discovered and discussed in the past few decades (Scheuer, 1973; Fusetani, 2000; Pomponi, 2001), but there is still enormous numbers of species remained unexplored. Stressful marine environment with less light, high pressure, extreme coldness and the presence of preys have induced the organisms to develop special morphological and chemical weapons to go against the stress. The toxic chemicals compounds have been utilized by the organisms to inhibit the vital metabolic process in the prey, predators and competitors. These compounds seemed to serve as highly potent candidates with promising bioactivities for the discovery of new drugs. Bacteria, fungi, macroalgae and marine invertebrates are the sources for the so-called marine natural products. Table 2.4 shows some examples of compounds isolated from marine organisms with their bioactivities (Donia and Hamann, 2003).

Table 2.3: Bioactive compounds from marine organisms and their classifications (Donia and Hamann, 2003)

Compound	Type	Source
Antibacterial activity		
Bromosphaerone	Bromoditerpenes	*Sphaerococcus coronopifolius* (red alga)
Jorumycin	Dimeric isoquinoline alkaloid	*Jorunna funebris* (sea slug)
Cibrostatin 3	Isoquinolines	*Cribrochalina* sp. (sponge)
Pestalone	Chlorinated benzophenone	*Rosenvingea* sp. (brown alga)
Antifungal activity		
Halishigamide A	Macrolide	*Halichondria* sp.(sponge)
Phorbaxazole B	Macrolide	*Phorbas* sp. (sponge)
Bengazole A	Oxazole-containing fatty acid ester	*Jaspis* sp. (sponge)
Meridine	Polycyclic alkaloid	*Corticium* sp. (sponge)
Antiviral activity		
Thyrsiferol	Triterpene	*Laurencia venusta* (red alga)
Gymnochrome D	Brominated phenanthroperylenequinone	*Gymnocrinus richeri* (pigments from fossil crinoids)
Spongiadol	Tetracyclic furanoditerpene	*Spongia* sp.
Antiprotozoal activity		
Plakortide	Cyclic peroxylactone	*Plakinastrella onkodes* (sponge)
Cyclic peroxides	Peroxide	*Plakortis aff angulospiculatus* (sponge)

2.5.1 Marine macroalgae

Macrolagae or seaweed is the marine plant, photosynthesizing multi-cellular organisms that lack specialized structures and mechanisms for reproduction. The main structure of a macrolagae consists of thallus, blades, pneumatocysts, stipe and holdfast. Macrolagae is divided into three major groups based on their different photosynthetic pigments found in their cells; green algae (Phylum Chlorophyta); brown algae (Phylum Phaeophyta); red algae (Phylum Rhodophyta). They usually attach to the surface of the rocky shores and reef, shells or any hard surface using their holdfasts. Macrolagae have the role to balance the ecosystem as the primary producer in the aquatic world. They directly use energy from sunlight for photosynthesis and produce oxygen for others and also serve as one of the food source. Besides, the existence of macroalgae in the oceans will aid in filtering the oceanic nitrate and ammonium produced by the invertebrates for the nitrogen fluxes that later will be utilized by other marine organisms (Bracken and Stachowicz, 2006). The variation in distribution of different macroalgae species have been studied in various oceans' areas and the data showed the algal growth is strongly related to the biotic and abiotic factors such as the grazer's population, seasons, elevation of the habitat, and nutrient supply (Eriksson and Bergström, 2005; Thomsen *et al.,* 2006; Kerswell, 2006).

Algae have been consumed by the people in certain countries since long ago because of the high mineral and nutrient contents. For example, "Nori" or *Poryphyra* sp. that being consumed by the Japanese since 300 years ago is proven to contain high content of soluble dietary fibre that can lower the glycemic index in human (Goñi *et*

al., 2000). Increase in demand of this species locally and internationally has contributes to the inflation of Japan economic growth, with approximately more than 2 million U.S. dollars per year (Barsanti and Gualtieri, 2005). In Malaysia, some communities who live at the coastal areas consume *Gracilaria changii*, *Gracilaria tenuispitata*, *Euchema* sp. and two *Caulerpa* spp as their salad course (Phang, 2006). Exploitation of macrolagae properties have been extended into the food industry. Rhodophyta *Euchema* spp are specially cultivated in some Asian countries to obtain the agars and carrageenans (hydrocolloids) (Phang, 2006). These two components are extracted from the species and are used in the food technology as the gelling and thickening ingredients in food products (http://www.seaplants.co.cc/2009/11/eucheuma-seaplants-in-indonesia.html).

2.5.1.1 Marine macroalgae with medicinal properties

Macroalgae is another important source of potential bioactive compounds for the marine-derived drugs. The bioactive compounds are the secondary metabolites produced by the algae, as the chemical weapons to survive in the marine ecology. The marine ecology is full of stresses like intense competitions for space, combinations of high UV radiation and oxygen level, high salinity, attack from the grazers and desiccation during high temperature (Smit, 2004). Algae from various world parts with different ecology backgrounds have been investigated and found to possess variety of therapeutic properties (Xu *et al.*, 2004; Puglisi *et al.*, 2007; Zubia *et al.*, 2007), including Malaysia which is located right on the equatorial line (Lim, 2005; Sasidharan, 2007). The therapeutic properties are such as the antiviral activity; antibiotic activity; cytotoxicity, antimitogenic, anticancer and antitumor activities;