BACTERIA - SPONGE INTERACTION: EXISTENCE OF POSSIBLE CHEMICAL CORRELATION

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BORNEO MARINE RESEARCH INSTITUTE
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DECLARATION

I hereby declare that the material in this thesis is my own except for quotations, excerpts, equations, summaries and references, which have been duly acknowledged.

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ABSTRACT

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The vast ocean has the reputation of having the greatest biodiversity. Therefore, represents an enormous resource for the discovery of chemotherapeutic agents. This particular investigation delves upon the study of bioactive compound from two marine sponges from coastal waters of Sabah, *Amphimedon sp.* and *Xestospongia sp.* and the most active sponge was then investigated for its relationship with its inherently available culturable bacteria in an effort to confirm the identity of the bioactive compound producer. All fractions except for fraction 1 and fraction 6 from the hexane crude extract from *Amphimedon sp.* showed antibacterial activity against 5 strains of marine environmental bacteria while all fractions except for fraction 1 from the 90% methanol crude extract showed antibacterial activity against 5 strains of marine environmental bacteria. *Amphimedon sp.* produced potent sterol as its bioactive compound, while Xestosponge sp produced aaptamine. Their identities were identified based on extensive H^1-NMR and MS data. Sponge-bacteria relationship was carried out by isolating culturable bacteria from sponge tissues, and a total of 40 strains of bacteria were isolated from *Amphimedon sp.* but only one strain (AA1) was found to have bioactive activity. 16S rRNA regions of AA1 were amplified using forward primer 27F (GAGTTTGATCCTGGCTCAG) while the reverse primer was JR1R (GACTACCAGGGTABCTAATC) and was compared with the top three matches provided by NCBI GenBank. Strain AA1 was found to be pro *Bacillus sp.* with 99% confidence and with e-value of zero. A mixture of fatty acids were isolated as active metabolite from AA1 and upon gas chromatography (GC) analysis and antibacterial assay with corresponding commercial fatty acids. It was confirmed that hexadecenoate was the active compound in AA1. In conclusion, culturable bacteria were not responsible for the production of active compounds in *Amphimedon* sp.

ABSTRAK

Laut yang meluas mempunyai reputasi sebagai takungan biodiversiti terbesar. Oleh itu, laut adalah sumbangan terbesar dalam penemuan agen kemoterapi. Kajian ini melihat kepada sebatian bioaktif dari span marin Amphimedon sp. dan Xestospongia sp. dari perairan Sabah dan span marin yang paling aktif dipilih untuk kajian hubungan span marin dengan bakteria boleh kultur dalam usaha untuk mengesahkan identiti penghasil sebatian bioaktif. Kesemua fraksi kecuali fraksi 1 dan 6 daripada ekstrak kasar heksan Amphimedon sp. menunjukkan aktiviti antibakteria terhadap 5 bakteria persekitaran marin sementara kesemua fraksi kecuali fraksi pertama dari ekstrak kasar metanol 90% menunjukkan aktiviti antibakteria terhadap 5 jenis bakteria persekitaran marin. Amphimedon sp. menghasilkan sterol berpengaruh sebagai sebatian bioaktifnya sementara Zestospongia sp. menghasilkan aaptamina. Identiti kedua-dua sebatian dikenalpasti berdasarkan data H^1-NMR dan MS intensif. Hubungan span-bakteria dijalankan dengan memencilkan bakteria boleh kultur dari tisu span dan 40 bakteria telah dipencil dari Amphimedon sp. dan hanya satu (AA1) menunjukkan aktiviti bioaktif. Bahagian 16S rRNA bakteria AA1 dibesarkan dengan primer kehadapan 27F (GAGTTTGATCCTGGCTCAG) sementara primer kebelakang adalah JR1R (GACTACCAGGGTABCTAATC) sebelum dijujuk dan dibandingkan oleh NCBI GenBank dengan bandingan tiga tertinggi dalam senarai. AA1 didapati pro Bacillus sp. dengan keyakinan 99% dan nilai e-sifar. Campuran asid lemak yang dipencil sebagai metabolit aktif dari AA1 diuji dengan analisis gas kromatograf (GC) dan ujian antibakteria yang berkaitan dengan asid lemak mengesahkan heksadekanoat sebagai sebatian aktif dalam AA1. Intisari kajian ini, bakteria boleh kultur tidak bertanggungjawab untuk penghasilan sebatian aktif dalam Amphimedon sp..

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LIST OF ABBREVIATIONS

BLAST	Basic Local Alignment Search Tool
$CDCl_3$	Deuterated chloroform
CFU	Colony Forming Unit
COSY	Correlation Spectroscopy
DAPI	4′,6-diamidino-2-phenylindole
dH_2O	Distilled water
DNA	Deoxyribonucleic Acid
FAME	Fatty Acid Methyl Ester
GC	Gas Chromatography
GC-MS	Gas Chromatography – Mass Spectrophotometer
HMBC	Heteronuclear Multiple-Bond Correlation
HPLC	High Performance Liquid Chromatography
HSQC	Heteronuclear Single Quantum Correlation
$MgCl_2$	Magnesium Chloride
MS	Mass Spectrophotometer
NaCl	Sodium Chloride
NCBI	National Center for Biotechnology Information
NMR	Nuclear Magnetic Resonance
NOESY	Nuclear Overhauser Effect Spectroscopy
PCR	Polymerase Chain Reaction
PTLC	Preparative Thin Layer Chromatography
R_f	Retention Factor
RNA	Ribonucleic Acid
RPM	Revolution per minute
SEM	Scanning Electron Microscopy
SiO_2	Silicon Dioxide
TBE	Tris/Borate/EDTA
TEM	Transmission Electron Microscopy
TLC	Thin Layer Chromatography
TMS	Tetramethylsilane

LIST OF SYMBOLS

%	Percentage
°C	Degrees Celsius
cm	Centimetre
cm^2	Centimetre square
DW	Dry weight
g	Grams
g/cm^2	Gram per centimetre square
IC_{50}	Half maximal inhibitory concentration
L	Litres
m	Meter
mg/cm^3	Milligram per centimetre cube
mg/g	Milligram per gram
MHz	Mega hertz
mL	Millilitres
mM	Milimolars
mm	Millimetres
mm^3	Millimetre cubed
N	Normality
ng/g	Nanogram per gram
nm	Nanometres
nM	Nanomolars
ppm	Parts per million
ppt	Parts per thousand
w/w	Weight over weight
α	Alpha
β	Beta
µg	Micrograms
µg/mL	Micrograms per millilitres
µL	Microlitres
µm	Micrometres

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CHAPTER 1

INTRODUCTION

1.1 Introduction

The world's oceans cover more than 70% of the earth's surface and are referred to as the greatest biodiversity since it represents 34 of the 36 phyla of life and contains more than 300,000 described species of plants and animals. The diversity of species are extraordinarily rich on the coral reefs where there are around 1,000 species per m^2 in selected waters (Donia and Hamann, 2003).

With its reputation as the greatest pool of biodiversity, it represents an enormous resource for the discovery of secondary metabolites and chemotherapeutic agents. Given the diversity of marine organisms and habitats, marine natural products cover a wide variety of chemical classes. Therefore, the marine environment has the potential for the discovery of valuable chemical products for the treatment of various infectious and non-infectious diseases. Unique secondary metabolites with various biological activities evolved considerably through ecological pressure such as predation, competing for space, reproduction, pollution and fouling of the surface. Over the years, researchers over looked the potential of these secondary metabolites as a remedy to control infectious and parasitic organisms.

Over the past four decades marine organisms have been in the limelight of a worldwide effort to define the novel natural products from the marine environment. A small number of marine plants, animals and microbes have already yielded more than 12,000 novel chemicals with hundreds of new compounds still being discovered the biomedical arena and pharmaceutical industry (Donia and Hamann, 2003).

To date majority of these chemicals have been identified from marine invertebrates of which sponges predominate (Lie and Zhou, 2002). In comparison with the natural products extracted from terrestrial organisms that have been investigated for many years, bioactivity information about marine natural product is scarce and limited. Therefore, when potential bioactive products are isolated from marine organisms, the bioactivity of these extracts should be tested vigorously. It is essential to develop a fast and reliable method to determine the bioactivity for the marine natural products. As one of the most interesting phyla for pharmacological active marine compounds, sponges are being investigated extensively in the last decade.

1.2 Sponge

1.2.1 Taxonomy

Sponges consist of 15,000 known species where only 1% of the known species (150 species) are fresh water species. From the 15,000 known species, only 17 species are of commercial value. Sponges represent the lowest metazoan phylum (de Caralt *et al.*, 2003) and they have shown to have metazoan structural characteristics and functional molecules (Custodio *et al.*, 1998).

Both, palaeontological and comparative cell-biological studies indicate that the phylum *Porifera* is very ancient and potentially the most simple among eumetazoans. In the recent years, the *Porifera* have been extensively studied in order to establish cellular and molecular aspects of the evolution from unicellular to multi-cellular grade of organisation in animals (Custodio *et al.*, 1998). Sponges are invertebrates where its morphological simplicity and plasticity have led to poor taxonomy where it is most distinct at the species level (Duran and Rützler, 2006). Up to date, there are only four classes of *Porifera*. The four classes are Calcarea, Hexactinellida, Demospongiae and Sclerospongiae.

Members of the group Calcarea are the only sponges that possess spicules composed of calcium carbonate (Müller *et al.*, 2006). These spicules do not have hollow axial canals. The Calcarea first appears at the base of the Lower Cambrian and has persisted until the present. Greater than 100 fossil genera are known. Like the Hexactinellida and the Demospongia, the calcarean sponges were at their most diverse during the Cretaceous. Today, their diversity is greatest in the tropics, as is the case with most marine groups. They are predominantly found in shallow waters, though at least one species is known from a depth of 4,000 meters. The fossil record of the Calcarea indicates that it has always been more abundant in near-shore shallow water settings (Oakley, 1937).

The hexactinellids or glass sponges are characterised by siliceous spicules consisting of six rays intersecting at right angles, much like a toy jack. Hexactinellids are widely viewed as an early branch within the Porifera because there are major differences between extant hexactinellids and other sponges. In particular, much of their tissues are syncitia, extensive regions of multinucleate cytoplasm. Some discrete cell types do exist, including archaeocytes. Furthermore, whereas other sponges possess the ability to contract, hexactinellids do not. Moreover, hexactinellids possess a unique system for rapidly conducting electrical impulses across their bodies, allowing them to react quickly to external stimuli. (Ley and Lauzon, 1998).

The Demospongia is by far the most diverse group of sponges. More than 90 percent of the known living sponge species are demosponges. This ratio is not maintained in the fossil record, where less than half of the known genera and families are demosponges. However, the vast majority of living demosponges do not possess skeletons that would easily fossilise, thus their fossil diversity, which peaks in the Cretaceous, is probably an enormous underestimate of their true diversity. As their great number of species would suggest, demosponges are found in many different environments of different regions, from warm nutrient dense intertidal environment to quiet cold abyssal depths. Besides that, all of the known freshwater poriferans are demosponges (Oakley, 1937).

Demosponge skeletons are composed of spongin fibers and/or siliceous spicules, though one genus (Oscarella) has neither. Demosponge spicules, if present, are siliceous, have one to four rays not at right angles, and have axial canals that are triangular in cross section. Demosponges take on a variety of growth forms from encrusting sheets living beneath stones to branching stalks upright in the water column. They tend to be large and only exhibit the leucon grade of organisation (Manuel, 2009).

Sclerospongiae are sponges that have a skeleton constructed from calcium carbonate, silica and spongin. They have a thin, living layer covering a massive underlying skeleton of aragonite-silica and spongin, which support the cells. These are the coralline sponges, which are mostly known from fossils. There are a few modern species, e.g. *Sclerospongiae* sp., which are only found on coral reefs in the West Indies and Pacific, where they contribute to the structure of the reefs (Duran and Rützler, 2006).

1.2.2 Morphology

One autopomorphic character of sponges is the presence of high levels of telomerase activity in all cells including somatic cells (Custodio *et al.*, 1998). This character is not found in higher metazoan phyla and implies that sponges do not show a clear distinction between the germ-cell and somatic-cell lineages. Most sponges are long-lived where some can reach a life span of more than 1500 years.

Sponges are generally nonselective feeders and capture food particles by pumping seawater into their internal canal system. Water entering through incurrent pores, or ostia, covering the sponge surface passes through diverging incurrent canals into flagellated choanocyte chambers, which drive the water current. Water exits the sponge through excurrent pores or oscula. Many sponges have plastic morphology where the shape is influenced by biotic and physical factors such as competition (Duckworth and Pomponi, 2005).

1.2.3 Chemistry

It is strongly believed that chemical defence plays an important role for filter feeding marine sponge. Sponges need to be free of fouling organisms in order to enable them to actively filter their food particles. As a sessile life form and a non-muscular organism, the marine sponges cannot escape predation, therefore they