CHEMICAL CONSTITUENTS AND BIOACTIVE SECONDARY METABOLITES FROM SOFT CORALS

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DECLARATION

I hereby declare that this thesis contains my original research work. Sources of findings reviewed herein have been duly acknowledged.

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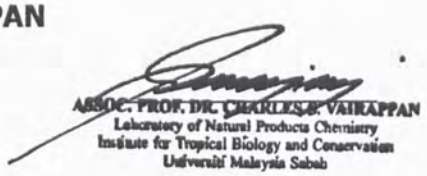

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ABSTRACT

CHEMICAL CONSTITUENTS AND BIOACTIVE SECONDARY METABOLITES FROM SOFT CORALS

Soft corals (phylum Cnidaria, class Anthozoa, subclass Octocorallia) are found worldwide, more in tropical coral reefs as compared to the temperate coral reefs. Unlike Scleractinian coral, soft coral does not have internal calcium carbonate skeleton. Therefore, soft coral contains chemical compounds in their body tissues, which are known as secondary metabolites that play an important role in their defense, and some of them also exhibited pharmacological activity. Research was carried out on four populations of *Dendronephthya* sp. from Sepanggar Island and one population of *Lobophytum* sp. from Banggi Island. Samples were extracted to obtain their crude extracts and chemical profiling of these extracts were carried out. *Dendronephthya* sp. with spiky morphology was found to contain very little secondary metabolites, with morphology similarity among the species and was not easily identified in the field. Further, microscopy studies were done using stereomicroscope and Scanning Electron Microscope (SEM). Observation showed differences in arrangement of spikes among the species. *Dendronephthya* sp^1 and sp^2 had similar morphology while spike arrangement of *Dendronephthya* sp^3 and sp^4 were different from sp^1 and sp^2. Samples were also subjected to extraction and only the primary metabolites were successfully isolated. Sterols isolated from every sample showed the same HPLC spectrum. Fatty acids, which were another isolate of these specimens, were analyzed using cluster analyses and presented in dendrogram. Dendrograms of monounsaturated fatty acids (MUFAs), polyunsaturated fatty acids (PUFAs) and selected fatty acids biomarker (C15:1, C18:1n9t, C18:1n9c, C24:1, C18:2n6t, C18:3n3, C22:6n3) showed that *Dendronephthya* sp^1 and sp^2 had the closest correlation followed by sp^3 and then sp^4. These results were similar to the findings from microscopy observation. Therefore, fatty acids can be used as chemotaxonomy biomarker for these particular four populations of *Dendronephthya* sp. Sterols isolated from these *Dendronephthya* sp could be used as a "finger-print" profile in identification of this genus. Meanwhile, the *Lobophytum* sp. showed numerous secondary metabolites from the chemical profile. Based on the chemical profiles, extracts of *Lobophytum* were subjected to isolation of their pure metabolites. Four pure metabolites were isolated and each of the metabolites was tested for their antibacterial activities. Three metabolites from *Lobophytum* sp. showed positive results against five marine environmental bacteria and two human pathogenic bacterium. Apart from that, all the metabolites tested showed insignificant antioxidant activities. Isolated metabolites were subjected to spectroscopic analysis and their structures were determined to be cembrene. Out of the four cembrenes from this *Lobophytum* sp., three were found to be novel.

ABSTRAK

Karang lembut (filum Cnidaria, kelas Anthozoa, subkelas Octocorallia) boleh dijumpai di pelosok dunia, lebih banyak di terumbu tropika berbanding dengan terumbu temperat. Karang lembut tidak mempunyai rangka dalaman kalsium karbonat seperti karang Scleractinian. Oleh itu, karang lembut mengandungi sebatian-sebatian kimia yang memainkan peranan sebagai pelindung, dan setengahnya menunjukkan aktiviti farmakologik. Kajian ini melibatkan empat populasi Dendronephthya sp. dari Pulau Sepanggar dan satu populasi Lobophytum sp. dari Pulau Banggi. Sampel-sampel diekstrak untuk mendapatkan ekstrak kasar dan dilakukan kajian profil kimia. Dendronephthya sp. yang morfologinya mempunyai duri tajam didapati mempunyai sebatian sekunder yang amat sedikit serta penampilan yang seiras antara spesies dan sukar untuk dikenalpasti di lokasi persampelan. Seterusnya, kajian morfologi yang lebih teliti dijalankan menggunakan mikroskop stereo dan mikroskop elektron penskanan (SEM). Penelitian menunjukkan terdapatnya perbezaan dalam susunan spikul di antara spesies. Dendronephthya sp^1 dan sp^2 menunjukkan morfologi yang lebih seiras sementara Dendronephthya sp^3 dan sp^4 kelihatan berbeza dengan sp^1 dan sp^2. Sampel juga diekstrak dan hanya sebatian primer diperolehi. Sterol yang dipencilkan daripada keempat-empat sampel menunjukkan spektrum HPLC yang serupa. Asid lemak yang merupakan antara metabolit yang dipencilkan telah dianalisis menggunakan analisis cluster dan dipaparkan dalam bentuk dendrogram. Dendrogram asid lemak mono tepu (MUFAs), asid lemak poli tepu (PUFAs) dan asid lemak yang terpilih (C15:1, C18:1n9t, C18:1n9c, C24:1, C18:2n6t, C18:3n3, C22:6n3) menunjukkan bahawa Dendronephthya sp^1 dan sp^2 mempunyai korelasi yang paling dekat diikuti dengan sp^3 dan sp^4. Keputusan ini adalah sama dengan keputusan yang diperolehi dari pengamatan mikroskop. Oleh itu, asid lemak boleh digunakan sebagai petunjuk taksonomi kimia bagi empat populasi Denronephthya sp. yang dikaji ini. Sterol yang dipencilkan daripada Denronephthya sp. pula dapat digunakan sebagai cap-jari dalam penentuan identiti genus ini. Sementara itu, profil kimia Lobophytum sp. menunjukkan kepelbagaian sebatian sekunder. Berdasarkan profil kimia, kajian pemencilan sebatian tulen telah dijalankan. Empat metabolit tulen telah dipencil daripada Lobophytum sp. Setiap metabolit telah diuji aktiviti antibakterianya. Tiga metabolit daripada Lobophytum sp menunjukkan keputusan aktif terhadap lima bakteria persekitaran marin dan dua bakteria patogen manusia. Selain daripada itu, semua metabolit yang diuji menunjukkan aktiviti antioksida yang tidak signifikan. Metabolit yang dipencilkan telah dikaji secara spektoskopi dan strukturnya telah dikenalpastikan sebagai "cembrene", tiga daripada empat metabolit yng dipencilkan dari Lobophytum sp ini adalah "novel".

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LIST OF ABBREVIATIONS

CC	Column chromatography
$CDCl_3$	Deuterated chloroform
HPLC	High performance liquid chromatography
HSQC	Heteronuclear single quantum coherence
HMBC	Heteronuclear multiple bond correlation
HRTOFMS	High Resolution Time of Flight Mass Spectroscopy
MeOH	Methanol
MeCN	Acetonitrile
Na_2SO_4	Anhydrous sodium sulphate
NOESY	Nuclear Overhauser effect spectroscopy
Rf	Retention factor
Si	Silica
TLC	Thin layer chromatography
PTLC	Preparative thick layer chromatography
TMS	Tetramethylsilane
UV-Vis	Ultra violet – visible
^1H-^1H COSY	Proton-proton correlation spectroscopy
^1H-NMR	Proton nuclear magnetic resonance
^{13}C-NMR	Carbon-13 nuclear magnetic resonance
2D	Two dimensions

LIST OF SYMBOLS

v/v	volume to volume
w/v	weight to volume
%	percent
°	degree
°C	degree Celsius
min	minute
kg	kilogram
g	gram
mg	miligram
L	litre
mL	millilitre
μL	microlitre
M	metre
cm	centimetre
mm	millimetre
μm	micrometre
nm	nanometre
MHz	mega hertz
Hz	hertz
δ	chemical shift
mmu	mili mass unit
ppt	parts per thousand
α	alpha
β	beta
ml/min	millilitre per minute
min^{-1}	per minute
Hz	hertz
σ	pai
mmu	mili mass unit

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CHAPTER 1

INTRODUCTION

1.1 Introduction

The Ocean, which is known as the 'mother of life' is an important source of structurally unique natural products that are mainly accumulated in living organisms (Jha and Xu, 2004). However, over 25,000 plants in the world, only 10% have been investigated for their biological activities (Harvey, 2000). The marine environment covers 80% of the world's plant and animal species (McCarthy and Pomponi, 2004).

The marine environment covers a wide thermal range (from the below freezing temperatures in Antartic waters to about 350°C in deep hydrothermal vents), pressure range (1-1000atm), nutrient range (oligotrophic to eutropic) and it has extensive photic and non-photic zones. This wide ranging environment has contributed to diversity and speciation at all phylogenetic levels, from microorganisms to mammals. However, the research into the extraction and use of marine natural products is still in its infancy due to the lack of ethno-medical history and difficulties in marine organisms sample collection (Jha and Xu, 2004). But with the development of new technologies, like new diving equipment and remote operated machines, it is possible to collect samples in any kind of environment (McCarthy and Pomponi, 2004).

In recent years, many bioactive compounds have been isolated from marine animals like soft corals, sponges, tunicates, sea hares, nudibranchs, bryozoans and marine organisms (Haefner, 2003; Donia and Hamann, 2003). In an effort of searching of new metabolites from marine organisms, 10,000 of metabolites have been isolated and there are more than 5,000 novel compounds have been identified, many of which showed potential as pharmacodynamic properties (McCarthy and Pomponi, 2004).

1.2 Soft Coral

Soft coral (phylum Cnidaria, class Anthozoa, subclass Octocorallia) are a group of marine creatures that constitute a dominant part of the biomass in the tropical reef environments. There are about 90 genera belonging to 23 families of soft corals found in shallow water of Indo-Pacific. Soft corals play an important role as dominant element of coral reef landscape in many parts of the world.

Soft corals together with hard corals are in Phylum Cnidaria having in common a very simple body plan and polyps structures. Soft coral is the common name for a group of organism in order Alcyonacea. Unlike hard coral, Scleractinian, soft coral does not have external calcium carbonate skeleton. Soft corals can also be distinguished from hard corals by the numbers of tentacles. Soft corals consist of 8 tentacles as their colony characteristics, while hard coral polyps have multiples of six tentacles.

Despite their lack of reef-builiding capability, soft coral tissue contains small hard sclerites as a body support and can strengthen the base of the soft coral. The part of the body is usually examined in the identification of the soft coral species. Most of the soft coral species inhibit by small single-celled algae known as zooxanthellae. These tiny organisms are able to photosynthesis. Therefore, the play a role as food resources that transfer food to the host coral they live inside. However, there are some azooxantallae soft coral that live as a phytoplankton feeder.

Without external skeleton as protection, most of the soft coral contains toxic chemical in their body tissues. Soft corals exude mucus with traces of chemicals to repel predators and avoid growth of other organisms such as sponges and algae too close to them. Therefore, the potential exists for soft corals to as a source of useful chemicals for pharmaceutical (Fabricius and Alderslade, 2001).

1.3 Chemical Ecology of Soft Coral

1.3.1. Chemical Taxonomy

Most of the species of soft corals produce a set of specific metabolites. This observation brings about an idea that relationship between certain species, especially those with morphology similarity, may be determined by analyzing their unique and complementary metabolites. The discipline is known as chemotaxonomy studies and may help in complement of morphological analyses of relationship within some families or even genus (Fabricius and Alderslade, 2001).

1.3.2. Protection and Defence

Soft corals are sessile marine organisms that cannot hide or run away from predators and compete for space with other living organisms. Instead they produce a variety of chemical constituents which effectively as defence mechanisms. Commonly these substances are known as secondary metabolites and they are by no mean by-products. However, in recognition of the physiological and ecological, these substances play an important role in the survival of these animals.

Most of the metabolites produced belong to a group of chemicals called terpenoids, and are produced and stored by both larvae and adult corals of a wide range of species. These metabolites also play a wide range of roles as antifeeding, antifouling and allelopathic substances. Anti-feeding substances are effective against most predators, especially damselfish, butterflyfish, nudibranches and egg cowry which often graze on the surface of the colonies. Anti-fouling substances will protect the colonies from settlement and growth of algae, fungi and bacteria while allelopathic substances enable soft corals to inhibit growth and survival of competitor. Colonies will slowly release chemical substances into water and this may cause tissues death or temporary paralysis to the intruder.

However, some species with low levels of chemical protection such as *Dendronephthya hemprichi* from Red Sea will overcome this problem by fast growth and reproduction (Fabricius and Alderslade, 2001).

1.4 *Dendronephthya* sp.

The alcyonacean *Dendronephthya* sp. of the family Neptheidae is one of the most beautiful soft corals in the world. They are soft branches corals with prominent sclerites that are brightly colored commonly in red, yellow, orange and often bicolored. The sclerites in the stalk are normally pale or white in colour, while the bright colours arise in upper branches (Fabricius and Alderslade, 2001). The *Dendronephthya* sp. can be found abundantly in Indo-Pacific basin, which includes several hundreds of reef-inhibiting species. It normally forms aggregations composed of numerous colonies with a wide array of colors (Dahan and Benayahu, 1997).

Unlike the other coral-reef octocorals, *Dendronephthya* species are azooxanthellate. They are not associated with endosymbiotic algae at any stage in their life cycle. However, this species are able to inhabit the reef sites that are dominated by zooxantellate species of stony corals, soft corals, zooanthids and sea anemones (Dahan and Benayahu, 1997).

The azooxanthellate *Dendronephthya* sp. is a phytoplankton feeder and dependent on dissolved organic matter for food. This particular species normally occur in habitats that exposed to strong water currents, but rare in wave-exposed areas (Fabricius et al., 1995). Unlike other soft corals, *Dendronephthya sp.* gives people a spiky appearance and feel. This is due to the body of the majority of polyps of this genus is supported by a group of large spindle-shaped sclerites called a 'supporting bundle'. However, due to their morphological similarity, the species of this genus is not able to be discerned in the field and must be microscopically examined. This genus has been a source of confusion to most of the taxonomist. Without close inspection, this genus may be confused with colonies of Umbellulifera and Stereonephthya (Fabricius and Alderslade, 2001).

1.5 *Lobophytum* sp.

Lobophytum is a member from family Alcyoniidae which usually found in shallow and very clear water down to 25m depth. Colonies from this family are the dominant reef of octocorals in the Indo-West Pacific. This is due to the growing pattern which is often known as 'massive' referring to the fact that their dimensions are similar in all directions, and that the polyps are joint together to form fleshy masses.

The colonies of *Lobophytum* are generally thick-encrusting, firm to touch and the polyps only present on the upper part of the surface. The size of the upper surface is usually same diameter as the colony base and often found to have lobes and ridges. *Lobophytum* mostly found in yellow-brown colour with the tips of lobes lighter than the sides.

Lobophytum is a zooxanthellate octocoral like most of the other soft corals. The zooxanthellate will contribute energy needs to the colonies through photosynthesis. However, photosynthetic efficiency from the zooxanthellate is quite low. Therefore, the colonies still need to intake food and nutrients from the environment as additional energy source (Fabricius and Alderslade, 2001).

Due to its sessile characteristic, *Lobophytum* produce a variety of chemical properties (Fabricius and Alderslade, 2001). This particular genus was found to have a number of diterpenes especially cembrane. A number of the metabolites isolated were reported to be active against various biological activities.

1.6 Objectives

This research aimed to study the chemical properties of the two genus of soft corals, *Dendronephthya* and *Lobophytum*. Detailed objectives are as follows:

(i) To perform chemical profiling of the four populations of *Dendronephthya* spp. and one population of *Lobophytum* sp. soft coral crude extract using TLC.

(ii) To carry out morphological study of four morphologically similar *Dendronephthya* sp. using Scanning Electron Microscope and Stereo Microscope.

(iii) To isolation major chemical constituents from *Dendronephthya* sp. and analysed for their potential as chemotaxonomy biomarker for *Dendronephthya* sp. due to its gross morphological similarity among the species.

(iv) To isolate and elucidate the chemical structures of secondary metabolites from *Lobophytum* sp.

(v) To identify the antimicrobial and antioxidant activities of the metabolites isolated.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Soft corals are well known as a rich source of lipids such as fatty acids and sterols and secondary metabolites with diverse chemical constituents and interesting biological activities. Soft corals produce several classes of structurally unique and complex secondary metabolites such as sesquiterpenes and diterpenes with wide variety of carbon skeletons.

However, there are also some soft corals that contain very little secondary metabolites and consist of sterols and fatty acids as their major chemical properties. This maybe due their exoskeleton which consists of hard skeleton or spikes that can play a role as defence mechanisms. This group of soft corals also shows morphological similarity among the species such as *Dendronephthya* sp. which cannot be differentiating in-situ. Therefore, the possibility of sterols and fatty acids as chemotaxonomy biomarker for this group of soft corals is worth to be look into.

2.2 Fatty Acids and Sterols as Chemotaxonomy Biomarker

Lipids which consist of fatty acids and sterols play an important role as major sources of metabolic energy and essential materials for the formation of cells and tissue membranes. They are also an important component in the physiology and reproduction processes of marine animals and reveal the special biochemical and ecological conditions of the marine environment (Berge and Barnatham, 2005). In recent year, fatty acids in marine algae have attracted increasing attention among researches and chemotaxonomists. Fatty acids compositions of red, brown and green macrophytic algae, belonging to different taxonomic classes, orders or families and genera were used as chemotaxonomy biomarker due to its ability to distinguish features of taxonomic value (Li et al., 2002). Biomarkers are basically compounds or groups of compounds that can be used as signatures of individual organisms or groups of organisms, or of certain environmental processes.

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