VOLATILE COMPOUNDS AND ANTIBACTERIAL ACTIVITIES OF THE METHANOL EXTRACTS OF SOFT CORALS (OCTOCORALLIA) FROM PULAU PAYAR AND PULAU SONGSONG

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by

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LIST OF ABBREVIATION AND SYMBOLS

ATR	Attenuated Total Reflectance
CLSI	Clinical Laboratory Standard Institute
DPPH	2,2-diphenyl-1-picrylhydrazyl
FTIR	Fourier Transform Infra-red Spectroscopy
GC/MS	Gas Chromatography and Mass Spectrometry
HL-60	Human caucasian promyelocytic leukemia
HPLC	High performance liquid chromatography
MHA	Muller Hinton Agar
MHB	Muller Hinton Broth
MBC	Minimum bacteriosidal concentration
MIC	Minimum inhibition concentration
MRSA	Methillin resistant *Staphylococcus aureus*
*msh*1	mitochondrial gene *msh*1
NMR	Nuclear magnetic resonance
NOAA	National oceanic and atmospheric administration
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
SEA	South East Asia
SEM	Scanning electron microscopy

USM	Universiti Sains Malaysia
UMP	Universiti Malaysia Pahang
UKM	Universiti Kebangsaan Malaysia
µL	microliter
mL	milliliter
L	liter
µg	microgram
mg	milligram
g	gram
m	meter
mm	millimeter
nm	nanometer
km^2	square kilometers
^oC	degree celcius
min	minutes
^oC/min	degree celcius per minute
mL/min	milliliters per minute
mg/mL	milligrams per milliliter
rpm	revolutions per minute
%	percent

CH_2Cl_2	chemical formula for dichloromethane
n-BuOH	shorthand for butanol
Tris-HCl	tris hydrochloride
TPP	tetraphenylphosphate

SEBATIAN MERUAP DAN AKTIVITI ANTIBAKTERIA BAGI EKSTRAK METANOL KARANG LEMBUT (OCTOCORALLIA) DARI PULAU PAYAR DAN PULAU SONGSONG

ABSTRAK

Karang lembut penting dalam ekosistem terumbu karang, dan diketahui menghasilkan metabolit sekunder. Sebatian ini berbeza daripada yang dihasilkan oleh organisma darat, dan boleh menghasilkan sumber baru untuk ubat-ubatan antimikrob. Tujuan kajian ini adalah untuk menjalankan pengencaman karang lembut yang diambil di Pulau Payar dan Pulau Songsong, di utara Selat Melaka mengunakan ciri morfologi dan kimia. Spesimen telah diekstrak dengan nisbah 1: 1 diklorometana: metanol dan dianalisis dengan menggunakan transformasi empatier spektroskopi infra-merah (FTIR) dan spektrometri jisim kromatografi gas (GCMS). Aktiviti antibakteria daripada ekstrak diklorometana: metanol diukur dengan menggunakan penyebaran cakera untuk diameter zon perencatan, dan menggunakan kaedah pencairan kaldu untuk mendapatkan pengukuran kepekatan minimum perencatan (MIC) dan kepekatan bakteriosidal minimum (MBC), berikutan protokol Institut Makmal Standard Klinikal (CLSI) . Tiga belas spesimen telah dikenal pasti, yang terdiri daripada 7 order, 9 famili dan 11 genus. Daripada lapan puluh lapan sebatian yang dikenal pasti, 2,4-bis (1,1-dimetiletil) - asid fenol dan n-heksadekanoik dikesan dalam semua 11 ekstrak dan 47 adalah unik kepada satu ekstrak sahaja. Contoh-contoh jenis sebatian yang diekstrak termasuk fenol, sterol, asid lemak poli tak tepu dan vitamin. Daripada dua belas ekstrak yang diuji, 50 peratus daripada ekstrak tersebut menunjukkan perencatan *Staphylococcus aureus* dan rintang metisilin *Staphylococcus aureus* (MRSA); dan kira-kira 8 peratus telah menunjukkan perencatan untuk *Enterococcus raffinosus*.

Ekstrak daripada *Sarcophyton trocheliophorum* diuji positif untuk sifat bacteriosidal terhadap MRSA, dengan ukuran MBC 10 mg /mL. Karang lembut di seluruh dunia, didapati mengandungi sebatian kimia yang bermanfaat, namun tidak banyak penyelidikan telah dilakukan di rantau ini. Kajian ini menunjukkan bahawa ekstrak karang lembut yang terdapat di Pulau Payar dan Pulau Songsong mempunyai metabolit sekunder yang berpotensi dan potensi ini dapat dimanfaatkan untuk penerokaan farmasi masa depan.

VOLATILE COMPOUNDS AND ANTIBACTERIAL ACTIVITIES OF THE METHANOL EXTRACTS OF SOFT CORALS (OCTOCORALLIA) FROM PULAU PAYAR AND PULAU SONGSONG

ABSTRACT

Soft corals are important in the coral reef ecosystem, and are known to produce secondary metabolites. These compounds are different from those produced by terrestrial organisms, and may yield new sources of antimicrobial medicines. The aim of this study is to identify using morphological and chemical characteristics of soft corals harvested off Pulau Payar and Pulau Songsong, in the northern Straits of Malacca. Specimens were extracted 1:1 ratio of dichloromethane:methanol and analysed using fourier transform infra-red spectroscopy (FTIR) and gas chromatography mass spectrometry (GCMS). Following Clinical Laboratory Standard Institute (CLSI) protocols, the antibacterial activites of the crude extracts were measured using disk diffusion for the diameter of the inhibition zone, and using broth dilution method to find the minimum inhibition concentration (MIC) and minimum bacteriocidal concentration (MBC) measurements. Thirteen specimens were identified, coming from 7 suborders, 9 families and 11 genera. Out of the eighty-eight compounds identified, 2,4-bis(1,1-dimethylethyl)- phenol and n-hexadecanoic acid were detected in all 11 extracts. Fourty-seven were unique to one extract only. Examples of the types of compounds extracted include phenols, sterols, poly unsaturated fatty acids and vitamins. Out of twelve crude extracts tested, 50 percent of them showed inhibition for *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* (MRSA); and approximately 8 percent have showed inhibition for *Enterococcus raffinosus*. The extract from *Sarcophyton trocheliophorum* tested positive for bacteriocidal properties against MRSA, with an MBC measurement of 10mg/mL. Soft corals around the world, have been found to contain beneficial chemical compounds, however not much research has been done in this region. This study shows that soft coral extracts found in the Pulau Payar and Pulau Songsong do have secondary metabolites with potential and this potential can be harnessed for future pharmaceutical exploration.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.0 General Introduction

One of the most diverse and complex ecosystems on earth are marine environments. Due to the harsh chemical and physical conditions, such as low nutrients and extreme temperatures, marine organisms produce several types of metabolites with unique features made up of a diverse array of biological activities (Jain *et al.,* 2008). These compounds have potential to be implemented in pharmaceutical or medical industries, and are of high value. Since the mid-1960s, around 20,000 marine natural products have been discovered, however, only a few has been commercially applied (Rocha *et al*., 2011). Therefore, there is a lot of potential for the discovery of new bioactive compounds in marine environments.

Numerous studies have proven that soft corals are a highly diverse group of marine organisms which are known to contain a wide variety of secondary metabolites such as diterpenoids (Blunt *et al*., 2013; Wei *et al.,* 2013) and steroids (Cheng *et al.,* 2009; Amir *et al.,*2012a). Soft corals have a high dependency on their chemical defensive system, in which secondary metabolites accumulate in their bodies or are released into their surroundings for survival (Shahbudin *et al*., 2011). This is despite the fact that they do not have an effective physical structure for protection in an environment which is very competitive and threatening.

Anti-predatory, antimicrobial, allopathic and antifouling agents are part of the inventory of their chemical defence functions (Changyun *et al*., 2008). Soft corals are a part of phylum Cnidaria (Bayer, 1956). Twenty-one percent of soft corals contains potential marine compounds for medicinal purposes (Jha & Zi-Rong, 2004). An estimated 50 percent of soft corals have been reported to produce toxins; of which 60 percent of their extracts contain bioactive compounds which have traits consistent with medicinal properties (Higa *et al*., 2001; Sheu *et al.*, 2002). There has been many novel metabolites isolated and their structures explained (Duh *et al.*, 1999; Shahbudin *et al.,* 2011). For example, *Sinularia* corals which are widely distributed, produces toxins. Approximately 60 percent of the 73 species of *Sinularia* are associated with toxins such as sesquiterpenes, diterpenes, norditerpenes and poly-amine compounds with antibacterial, anti-inflammatory and cytotoxic activities (Khalesi *et al*., 2007; Shahbudin *et al*., 2011).

1.1 Coral reefs of the world

Coral reefs are unique ecosystems and are rich in biodiversity. They can be compared to an underwater tropical forest of sorts. A coral reef is a complex ecosystem that supports many different habitats and is important in the development of many biological communities such as fish and marine invertebrates (Jones & Syms, 1998). Coral reefs are generally made of calcium carbonate, which is deposited by sclerectinian/ hard corals (reef-building or hermatypic corals).

Numerous fish and invertebrate species use coral reefs as their feeding, breeding and nursery ground. They also act as a natural barrier and are able to break waves, hence preventing coastal erosion. Coral reefs are found throughout the world, bordered by the tropical and subtropical regions between 30°N and 30°S,and usually restricted to the photic zone due to their symbiosis with microscopic algae (Stoddart, 1969; Falkowski *et al*., 1993).

Southeast Asia (SEA), is part of the Indo-West Pacific region. This region is associated with high biological significance, extensive coastlines and diverse coral reefs (Paulay, 1997). The Indo-West Pacific region alone, contains more than 50 genera with approximately 700 species of corals (Yamazato, 1991). This is in comparison to the Atlantic region which only has 26 genera with fewer than 100 species (Yamazato, 1991). In particular, the areas surrounding Indonesia, Malaysia and the Philippines form the Coral Triangle (Figure 1.1) which is known as a centre of the global coral diversity (Hoeksema & Putra, 2000; Hoeksema, 2007). Unfortunately, due to global warming, coral bleaching, overfishing, coastal pollution and coral destruction by tourists, coral coverage all over the world is in decline (McClanahan, 2002; Hughes *et al*., 2003; Gardner *et al.,* 2003; Bellwood *et al*., 2004). In a survey conducted in 2008 to determine the current status of the worlds' coral reefs, it was reported that the coral reef area in SEA was 91 700 km^2, with 40 percent destroyed, 20 percent at critical stage (50% - 90% corals lost), 25 percent at threatened stage (20- 50% corals lost) and 15 percent at low threat level (Wilkinson, 2008).

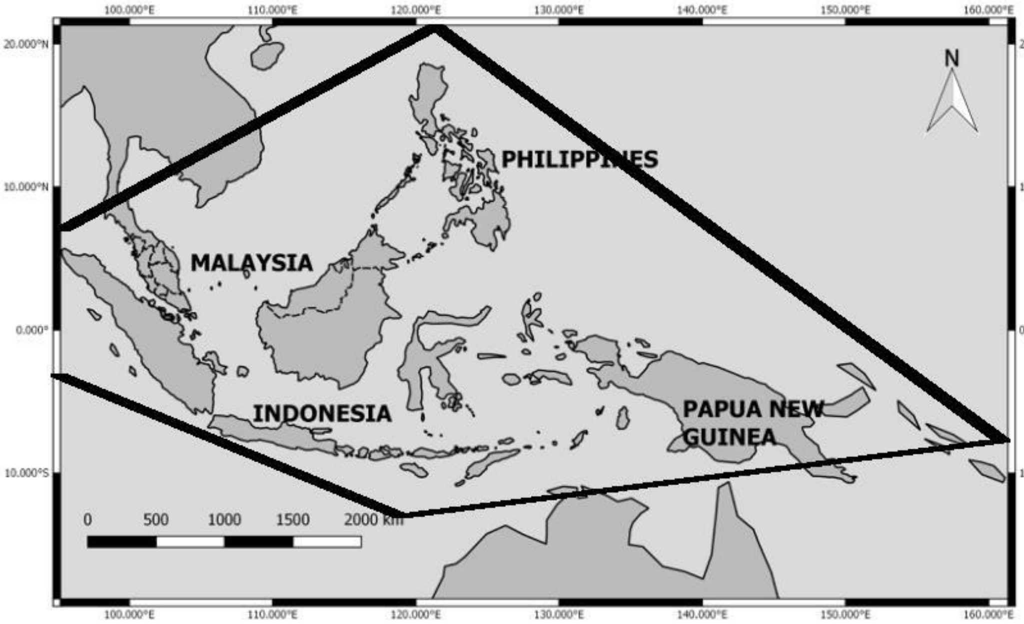

Figure 1.1 The Coral Triangle which forms a global diversity hotspot for marine tropical waters (adapted from Veron & Smith, 2000)

1.2 Biology of Octocorals

There are several features that can distinguish the different classes of Anthozoa. The characteristic feature of the octocoral, is that each polyp has eight hollow tentacles (Fabricius & Alderslade, 2001). Each of the tentacles are surrounded by either one or several rows of pinnules. Helioporacea (blue corals), Alcyonacea (soft corals and gorgonians), and Pennatulacea (sea pens) make up the three orders of Octocorallia (Alcyonaria) subclass (Fabricius & Alderslade, 2001; Zaharatul, 2014). However, the three orders of Octocorallia are separate in terms of a common ancestor, as the age and evolutionary separation time is unknown, due to the lack of fossil records (Fabricius & Alderslade, 2001). There is only one surviving species of Helioporacea; *Heliopora coerulea* which is made of crystalline aragonite skeleton (Daly *et al*., 2007).

Order Pennatulacea or more commonly known as sea pens is unfortunately understudied (Fabricius & Alderslade, 2001). Order Pennatulacea is made up of 16 families. Sea pens are mostly found in the deep ocean, but they can still be found in shallow waters, which are usually turbid. They are soft-bottom inhabitants and burrow into the substrate if there is light (Fabricius & Alderslade, 2001). Therefore, they are not usually found. Sea pens are dimorphic, meaning that they consist of both autozooids and siphonozooids polyps (Williams, 2011). Sea pens are recognised by their polyp (oozooid) which is big, axial, and central. Bearded by an internal calcareous axis, oozooids form the penduncle (stalk) and rachis. The penduncle anchors their base into the substrate for support (Fabricius & Alderslade, 2001).

The final and biggest order for subclass Octocorallia is Alcyonacea (Fabricius & Alderslade, 2001). In this order, Fabricius & Alderslade (2001) described approximately 90 genera of Alcyonacea. There are 23 families of Alcyonacea from the tropical Indo-Pacific region which is made up of the warm and shallow waters of the Red Sea, the Indian Ocean, and Central-West Pacific Ocean (Fabricius & Alderslade, 2001; Zaharatul, 2014). Alcyonacea's growth forms include stolons, axial-polyps, fleshy and gorgonians. Order Alcyonacea is made up of all soft corals and gorgonians (sea fans). Sub-order Holaxonia and Calcaxonia are two groups of complex gorgonians which differ in horizontal build (Fabricius & Alderslade, 2001). Order Alcyonacea also includes flowery soft corals (Family Nephtheidae) and leathery soft corals (Family Alcyoniidae).

Taxonomic Tree of Octocorallia data was extract from (Fabricius & Alderslade, 2001; Daly *et al*., 2007; Zaharatul, 2014).

Kingdom Animalia
- **Phylum** Cnidaria
  - **Class** Anthozoa
    - **Sub-class** Octocorallia
      - **Order** Helioporacea
        - **Family** Helioporidae
        - **Family** Litothelostidae
      - **Order** Pennatulacea
        - **Sub-order** Sessiliforae
          - **Family** Anthopthilidae
          - **Family** Chunellidae
          - **Family** Echinoptilidae
          - **Family** Funiculinidae
          - **Family** Kophobelemnidae
          - **Family** Protopthilidae
          - **Family** Renillidae
          - **Family** Scleroptilidae
          - **Family** Stachyptilidae
          - **Family** Umbellulidae
          - **Family** Veretillidae
        - **Sub-order** Subselliflorae
          - **Family** Pennatulidae
          - **Family** Pteroeididae
          - **Family** Virgularidae
      - **Order** Alcyonacea
        - **Sub-order** Alcyoniina
          - **Family** Alcyoniidae
          - **Family** Nephtheidae
          - **Family** Nidaliidae
          - **Family** Paralcyoniidae
          - **Family** Xeniidae

Sub-order Calcaxonia

- **Family** Chrysogorgiidae
- **Family** Dendrobrachiidae
- **Family** Elliselidae
- **Family** Ifalukellidae
- **Family** Isididae
- **Family** Primnoidae

Sub-order Holaxonia

- **Family** Acanthogorgiidae
- **Family** Gorgoniidae
- **Family** Keroeididae
- **Family** Plexauridae

Sub-order Protoalcyonaria

- **Family** Taiaroidae

Suborder Scleraxonia

- **Family** Anthothelidae
- **Family** Briareidae
- **Family** Corallidae
- **Family** Melithaeidae
- **Family** Paragorgiidae
- **Family** Parisididae
- **Family** Subergorgiidae

Sub-order Stolonifera

- **Family** Acrossotidae
- **Family** Clavulariidae
- **Family** Coelogorgiidae
- **Family** Cornulariidae
- **Family** Pseudogorgiidae
- **Family** Tubiporidae

Sub-order (Unassigned)

- **Family** Acanthoaxiidae
- **Family** Haimeidae
- **Family** Viguieriotidae

1.2.1 Anatomy of soft corals

Autozooids are a type of polyp with eight branched tentacles, which are present in most octocorals (Fabricius & Alderslade, 2001; Devictor & Morton,2010). All octocoral are made up of colonial polyps, except for *Taiarao tauhou* (Daly *et al.,* 2007). The autozooid is a dual-function polyp as it takes part in both feeding and reproduction. Octocorals are either monomorphic or dimorphic. Monomorphic corals only have autozooids, while dimorphic corals have two different polyps; autozooid and siphonozooid. Dimorphic octocorals are generally larger in size. The primary function of the siphonozooid is to flood the colonies with seawater (Fabricius & Alderslade, 2001).

An autozooid is tubular or cylindrical in shape, and has a mouth with tentacles at the outer end (Fabricius & Alderslade, 2001). The gastrovascular cavity has three thick layers of wall, and is part of the extended section of the polyp (Fabricius & Alderslade, 2001). The finger-like tentacles surrounding the mouth are known as pinnules. Feather-like pinnules extend towards the water body, maximising the surface area of the polyp. These pinnules have sensory cells, stinging capsules and are usually integrated with zooxanthellae (symbiotic algae). The pinnules have sensing and grabbing skills to help with food capture. The number and configuration of pinnules vary between octocoral species (Fabricius & Alderslade, 2001).

Octocorals polyps are divided into the anthocodia, which is the visible portion that can extend and retract, and the anthostele, which is an extension of the gastrodermal canal (Figure 1.2) into the coenenchymal mass (Devictor & Morton, 2010). In some stoloniferous soft coral species, the anthocodia is situated in a very long calyx in which there is a small depression in the stolon at the end of it. The anthosteles are available in more sophisticated octocorals, while the ordinary soft corals usually just have fleshy coenenchymal colony mass (Fabricius & Alderslade, 2001).

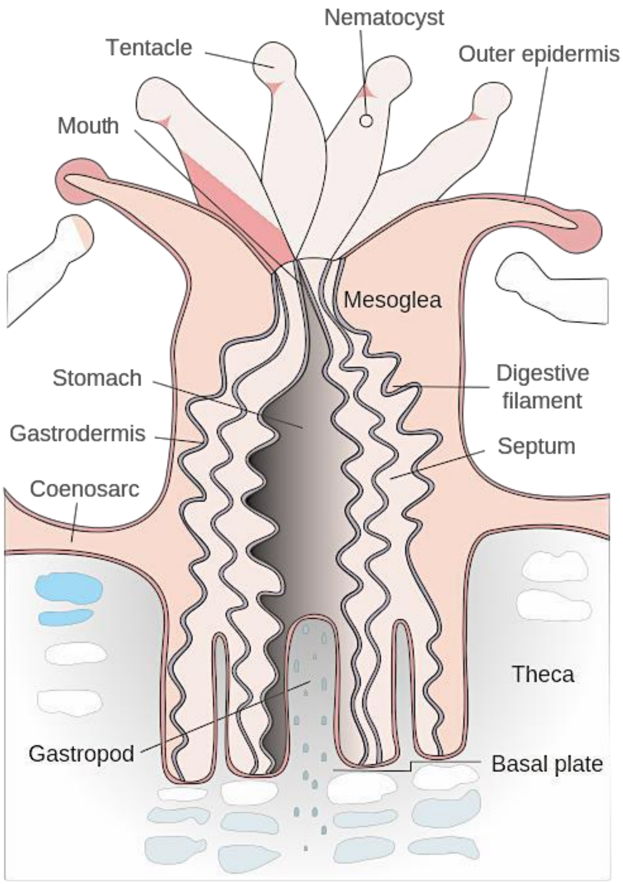

Figure 1.2 Cross-section of octocoral anatomy (adapted from Gini Kennedy, NOAA website)

Octocoral colonies take on many forms, such as branching, encrusting, whip-like, feather-like, fleshy or even completely calcereous types (Devictor & Morton, 2010). Branching types include dichotomous, monopodial, whip-like branches, rod-like branches, open irregular, innate, planar, and lobed, with clusters of polyps (Fabricius & Alderslade, 2001; Devictor & Morton, 2010). Unbranched colony forms include flagelliform (whip-like), capitate, foliose, club-like, pen-like and stoloniferous (Fabricius & Alderslade, 2001; Devictor & Morton, 2010). Growth form of ocotocorals can also be affected by the environment (Bayer, 1961; Devictor & Morton, 2010), and this varies between regions. Soft corals use the hydroskeleton as an internal hydrostatic pressure, which supports the colonies (Daly *et al*., 2007; Fabricius & Alderslade, 2001).

Sclerites are calcified microscopic elements embedded in the coenenchyme of soft corals (Bayer *et al*., 1983). The hard skeleton sclerites can be found on the outside surface of most octocorals to protect them. The sclerites are found embedded in different parts of the coral surface to give some support and it also acts as a defense mechanism for the octocorals.

The arrangement of sclerites is important for the classification and identification of octocorals. Both shape and size of the sclerites must be taken into account, as well as the arrangement of the sclerites on the polyps (Bayer *et al*., 1983; Fabricius & Alderslade, 2001). Sclerite shapes include capstan, spicule, spindle, double head, rod, club, thornstar, amongst others. Bayer *et al*. (1983) provided details images representing the different morphologies of more than 150 types of octocoral sclerites (Devictor & Morton, 2010).

1.2.2 Identification of soft corals by molecular methods

The utilization of molecular methods continues to revolutionize our understanding of evolutionary relationships within and among the cnidarians and sponges (Fukami *et al.,* 2004; Boury-Esnault, 2006; McFadden *et al*., 2006a; McFadden *et al*., 2009). In the 1950s, taxonomic classification and phylogenetic inference in these groups was difficult (Bayer, 1956). This was because of the apparent simplistic morphological approach and a lack of characteristics suitable for analysis (McFadden *et al*., 2009). McFadden *et al*. (2009) stated that many of the morphological characters usually used for higher level classification in these taxa have now been shown to disagree with molecular phylogenetic evidence, and is likely to represent homoplasy which is a shared character of species which is not present in the ancestor.

Molecular studies on soft corals have shown that the traditional morphology-based taxonomy for order and family levels, does not reflect phylogenetic relationships (McFadden *et al*., 2006a). However, reevaluation of certain families has allowed for the identification of new characteristics that are consistent with the molecular data. For example, Sanchez *et al*. (2003) found that characteristics such as axial structure and the size and shape of surface sclerites (traditionally considered most important for classifying octocorals belonging to the holaxonian group of sea fans) were not congruent with the phylogeny. Instead, those of minor taxonomic importance such as the presence of polyp sclerites and sclerite ornamentation were diagnostic for the major molecular clades. He did this by mapping morphological characters onto a molecular phylogeny. Following a similar trend, McFadden *et al*. (2006b) reported that polyp sclerites and subtle aspects of colony growth form allowed for the identification of a previously cryptic genus of octocoral.

In addition, colony growth morphology is usually very variable (Benayahu *et al*., 1998), even within the same colony (Alderslade & Shirwaiker, 1991; Benayahu, 1998), and there is the need for the extraction and subsequent examination of sclerites in the tissues. Therefore, many species remain undescribed and the validity of other identifications is uncertain (van Ofwegen, 2002).

1.3 Distribution of Octocorals in Malaysia

The most recent survey of coral reefs in Malaysia was conducted by ReefCheck Malaysia in 2016. According to the survey, the percentage of substrate covered (n=206) by hard corals were 39.63%, while that covered by soft corals was 4.07%. This was a decrease of 0.59% in substrate covered by soft corals in 2013, also surveyed by ReefCheck Malaysia.

The distribution of soft corals in Malaysia is more prevalent on the east coast of the Malaysian Peninsula; in islands such as Pulau Redang (n= 12), Pulau Perhentian (n=10) and Pulau Tioman (n=18) , with soft coral coverage of 7.55%, 0.81% and 2.57% respectively (ReefCheck Report, 2016). Nevertheless, there are soft corals on other reefs in Malaysia as well.

The distribution of soft corals in the Straits of Malacca differs from that of the east coast of Peninsula Malaysia and also in East Malaysia (Sabah and Sarawak). Three islands were surveyed, however the number of survey sites differed between the islands. The soft coral coverage for Pulau Sembilan (n=9), Pulau Pangkor Laut (n=1) and Pulau Payar (n=3) was 5.07%, 0% and 0% respectively.

Mohammad *et al*. (2016) identified 23 specimens of soft corals collected from reefs off the Northern Straits of Malacca. The specimens belonged to 8 families, in which 15 different genera were identified. Hong & Sasekumar (1981) found soft coral communities off Cape Rachado, Port Dickson. The area was dominated by soft corals from the family Alcyoniidae. It was reported that this was due to their ability to tolerate the high turbidity, and exceptionally low tides in the waters of Cape Rachado (Hong & Sasekumar,1981).

Genus *Dendronephthya* from Family Nephtheidae was found to be most common in the reefs of Pulau Pemanggil, off the east coast of Johor, Malaysia. Mohammad *et al*. (2004) identified six species of *Dendronephthya*, in which *D. binongkoensis*, a relatively rare species, was most abundant. Meanwhile, the blue coral

(*Heliopora coerulea*) and those from family Stolonifera, were most common off the reefs of Pulau Aur, Johor (Dunn, 1970).

1.3.1 Physical factors affecting distribution of corals

The growth of corals on a reef is not random. The zonation of coral assemblages on reefs is one of the reef ecosystem's most striking features, and most intertidal reefs exhibit a zonation pattern (Baird *et al*., 2003). This zonation can be caused by different factors, such as light, water movement, and competition (Baird *et al.,* 2003).

The distribution of soft corals is affected by physical factors such as waves and currents, light, temperature, sedimentation and nutrients (Fabricius & Alderslade, 2001). Octocorals in particular are vulnerable to dislodgment, and other damage by storm waves. Due to this, not many soft corals are found in outer-reef shelf areas.

A study by Chanmethakul *et al*. (2010), indicated that different genera of soft corals show habitat preferences. It was found that those from family Nephtheidae were more widely distributed on rocky shores and submerged rocks than on true reefs, while the Alcyoniidae were widely distributed on true reefs.

The angle of substrate also has an influence on the distribution and abundance of soft corals, due to the availability of light. For example, corals from the family Alcyoniidae, were found on substrates ranging from 0 to 90 degrees, owing to the fact that it has zooxanthellae in its system that require light to photosynthesize.. On the other hand, corals from several genera such as *Neptheidae* and *Nidaliidae* solely survive on filter feeding and can be found on substrates of all angles. This makes them better space colonizers (Chanmethakul *et al.,* 2010).

1.4 Marine natural products

Natural products have been one of the most productive source for drug and vaccine development. More than 100 new products are in clinical development, with emphasis on anti-cancer agents and anti-infection drugs. In the past 50 years, there has been a shift in the focus of natural products from the land to the sea. This is mostly due to the fact that the ocean has higher biodiversity compared to land, and the advances in technology, allowing researchers to explore the deeper realms of the oceans (Harvey, 2000). Living in a harsh and unforgiving environment, marine organisms have developed different mechanisms to face the tough competitive situations. Marine invertebrates can survive in the highly competitive and hostile environments, mostly relying on their chemical defensive system in which they accumulate a series of secondary metabolites in their bodies or release the compounds to the surroundings (Wang *et al.*, 2005; Xu &Yan, 2006; Wang *et al.*, 2008).

Marine natural products (MNP) have huge potential in the development of pharmaceuticals, cosmetics, nutraceuticals, agrochemicals, and others. The application of MNP to drugs has been researched on since the early 1970s, but only a few have been successfully developed into pharmaceuticals (Newman & Cragg, 2004). Other than applications in pharmacy, the chemical substances or secondary metabolites produced have been used in research and has been a big contribution to the progress of life sciences (Fusetani, 2010).

The bioactive secondary metabolites with potential have mainly been isolated from marine sponges, algae, jellyfish, sea anemones, corals, bryozoans, molluscs, echinoderms, tunicates and crustaceans. These metabolites can be divided into several categories; steroids, terpenoids, isoprenoids, nonisoprenoids, quinones, brominated compounds, nitrogen heterocyclics, and nitrogen sulphur heterocyclics (Bhakuni & Rawat, 2006).

In a review by Coll (1992) where he collated previous research on secondary metabolites isolated from soft corals and their corresponding activities, it was summarised that these compounds had roles in antifeeding, reproduction, prevention of fouling, cytotoxicity and allelopathy.

A review conducted on marine bioprospecting trends (Leal *et al.,* 2012) stated that approximately 9800 new marine natural products were discovered over a span of 20 years from 1990 to 2009. It indicated that the natural products came from a total of 11 phyla, 6 subphyla, 20 classes, 20 subclasses, 74 orders, 253 families, 569 genera and 1354 species. Phylum Porifera comprised the bulk of it at 48.8%, with phylum Cnidaria coming in second at 28.6%. The class Anthozoa comprised 99.0% of the new natural marine products recorded from Cnidaria. From this, the sub-class Octocorallia accounted for 95.5%, and order Alcyonacea accounted for 98.1% of that (Leal *et al.*, 2012).

1.4.1 Marine natural products of Malaysia

Research on marine natural products in Malaysia is still relatively new. There have been reports of research on bioactive compounds in sea cucumbers and red algae in the nineties. Since then, research has also been done on specimens from pylum Porifera, Cnidaria and Echinodermata.

1.4.1(a) Sponges (Phylum Porifera)

Sponges are the most primitive invertebrates in the marine environment but are considered a rich phylum with novel bioactive compounds (Blunt *et al.,* 2007). It was reported that more than 90 compounds were isolated from sponges in the period of

2003 to 2005 (Mayer *et al.,* 2007), which represented 36% of the total isolated compounds reported in the studies where they worked on isolating natural products. Sponges are known to have an efficient immune system. They produce secondary metabolites known to repel and deter predators (Qaralleh *et al.,* 2010). The compounds extracted exhibited a wide range of biological activites such as antibacterial (Fostel & Lartey, 2000), antifungal (Fostel & Lartey, 2000), antiviral (Rinehart *et al.,* 1984), and antitumour (Monks *et al*., 2002). The secondary metabolites isolated consisted of terpenes, peptides, alkaloids and fatty acids (Mayer *et al.,* 2007; Qaralleh *et al*., 2010).

In a study by Qaralleh *et al.* (2010) done on four marine sponges collected from the coastline of in Langkawi and Snake Island, Malaysia; it was found that extracts from all the sponge species showed antibacterial activity against at least one of the test microbes. *Neopetrosia exigua* was found to be the most promising species tested.

Study on marine sponge *Aaptos* sp. along the east coast of Peninsula Malaysia in 2009 by Mohammad *et al.* (2009) indicated the preliminary results of the study were promising as it showed that there was significant antibacterial activity against several of the test species. Specimens were collected from the Terengganu islands of Bidong, Kapas, Redang and Perhentian.. Result of the study indicated that extracts from specimens collected from the island of, Bidong, Kapas and Perhentian yielded better antibacterial properties compared to specimens collected from Redang Island.

In another study by Mohamad *et al.* (2009) on *Xestospongia* sp. also collected off Bidong Island yielded a novel discovery. In the hexane extract, a long-chain saturated fatty acid compound, i.e. octacosanoic acid was identified. The compound was found to be inactive against antioxidant activity (DPPH), of moderate activity against *Klebsiella pneumonia* in antibacterial activity and had strong cytotoxicity against Human Caucasian Promyelocytic Leukemia (HL-60) cell line.

Neopetrosia exigua collected from Langkawi were studied by Majali *et al.* (2015). Specimens were subjected to extraction and fractionating using different solvents and later tested for their antimicrobial activities. Results obtained showed that the active metabolites were present in n-hexane, dichloromethane, n-butanol and water fractions; and all showed antibacterial activity to at least one bacteria species.

These studies showed that the Malaysian waters are a potential source for marine sponges that are worthy as a main compound for the development of therapeutic drugs for various diseases.

1.4.1(b) Soft corals (Phylum Cnidaria)

Soft corals are a very diverse group of marine invertebrates which have been known to produce a wide variety of secondary metabolites. These compounds protect the soft corals in the highly competitive and hostile marine environment (Shahbudin *et al.*, 2011). The chemical defensive functions of these secondary metabolites include anti-predatory, antimicrobial, allopathic and antifouling agents (Changyun *et al.*, 2008). Within the phylum Cnidaria, 21% of the species contain potential marine medicinal compounds (Jha & Zi-Rong, 2004). About 50% of octocorals have been documented to produce toxins. Out of this, 60% of the extracts contain bioactive secondary compounds with potential (Higa *et al.*, 2001; Sheu *et al.*, 2002). Organo-solvent (such as dichloromethane-methanol mix) extracts of octocorals are made up of 90% lipid and sterols while 10% of the organic extracts are biologically active diterpenes or sesquiterpenes (Edrada *et al.*, 2000). There has been a lot of new and novel metabolites isolated from soft corals (Duh *et al.*, 1999; El-Gamal *et al.*, 2005; Yin *et al.*, 2005).

There have been several studies conducted on octocorals in Malaysia. A few of these studies have been focused on the genus *Nephthea* where most of the works were performed on soft corals obtained from islands off the coast of Sabah, Malaysia. Ishii *et al.* (2010) extracted three colourless oils and a white solid from *Nephthea* sp. The four compounds had reacted differently against the tested human pathogenic bacteria which included *Pseudomonas aurelis*, *Salmonella typii* and *Vibrio cholera*. Compounds were identified by High Performance Liquid Chromatography (HPLC) and Nuclear Magnetic Resonance (NMR) spectroscopy. Subsequently in 2009 and 2010, three novel products were discovered (Ishii *et al.*, 2009; Ishii *et al.*, 2010). In the 2009 study, Ishii *et al.* extracted a new germacrane-type norsesquiterpenoid and also a new 4α-methylated sterol from the *Nephthea* specimens collected from the Sibuan Island, Sabah. In 2010, a new cembrane diterpene was found in the methanol extract of the *Nephthea* sp. specimen collected from Layangan Island, Sabah.

Bahl *et al*. (2014) found a novel brianene diterpenoid compound extracted from *Pennatula aculeate*, more commonly known as a sea pen (WorMs) which exhibited moderate COX-1 and COX-2 inhibitory activity. This study was also conducted in Sabah, Malaysia.

In a study on *Dendronepthya* sp. from Pulau Payar, Langkawi, Shahbudin *et al.* (2011) found that the crude extracts contained an antioxidant compound based on a rapid screening using dot-blot and DPPH staining (Huang *et al.*, 2005; Chang *et al.*, 2006).

1.4.1(c) Seagrass

Thirteen out of approximately 57 major seagrass species have been reported in Malaysia. However, *Enhalus acroides* and *Halophalia ovalis* are the most common (Bujang *et al.,* 2006). Seagrasses can be wave breakers (Rönnbäck *et al.,* 2007), habitats and nurseries for marine life such as crustaceans, bivalves and sea cucumbers (Hovel & Lipcius, 2002; Mercier *et al.,* 2000); and a food source for grazing animals such as green turtles and dugongs (Hines *et al.,* 2005). In Tamil Nadu, India*, Enhalus acoroides* has been used in traditional medicine to treat cuts, wounds and scabs (Parthasarathy *et al.,* 1991). Qi *et al*. (2008) reported that *E. acoroides* has antioxidant properties and antimicrobial properties against gram positive bacteria.

In a study by Ismail *et al*. (2012) the leaf extract of *Enhalus acroides* was subjected to antimicrobial susceptibility tests and cell cytotoxicity tests. Results showed that the ethyl acetate extract was more effetive than the ethanol extract. The ethyl acetate extract at concentration $\geq$ 6.25 mg/ml was able to inhibit the growth of all the tested bacteria and also killed 50% of the HeLa cells. This preliminary study showed that *E. acroides* found in the south coast of Malaysia has bioprospecting potential.

1.4.1(d) Seaweed and algae

Seaweeds are multicellular macroalgae that have potential to be a renewable resource in the pharmaceutical and commercial field (Cardozo *et al.,* 2007). Pharmacologically important compounds such as flavonoids, carotenoids, dietary fiber, protein, essential fatty acids, vitamins and minerals, can be found in seaweeds (Ganesan *et al.,* 2008). In addition to pharmaceutical purposes, seaweed are also utilised as daily dietary food supplements and is beneficial to human health (Ganesan *et al.,* 2008). Naturally, seaweeds contain novel antioxidant compounds which control the free radical formation from metabolic reactions (Balakrishnan & Pillai, 2017). Edible seaweeds include *Sargassam* sp., *Padina* sp. and *Eisenia bicyclis* which contain phlorotannins, fuxoxanthin, polyphenols and phylopheophylin (Foon *et al*., 2013). The bioactive secondary metabolites most likely are involved in metabolic regulation and standard mechanisms (Jo *et al.*, 2005; Foon *et al*., 2013).

Vairappan (2003) investigated the antibacterial activity of halogenated metabolites from Malaysian red algae *Laurencia majuscule*. The compounds extracted were in the form of elatol and iso-obtusol, which was the same as that reported by Vairappan *et al.* (2001). Elatol exhibited antibacterial activity against six of the tested bacteria while iso-obtusol exhibited antibacterial activity against four bacterial species.

Vairappan *et al.* (2008) studied the *Laurencia* species in Malaysia. *Laurencia* sp. specimens were collected from Pulau Tioman, Pahang; Pulau Korah and Pulau Nyireh, Terengganu. This study was the continuation of a series of studies on the *Laurencia* sp. in Malaysian waters since 1997. The compounds extracted resembled those previously found in specimens collected in different countries such as the Philippines and Australia. From the specimens collected at Pulau Tioman, novel brominated metabolites, tiomanene, acetylmajapo-lene B, and acetylmajapolene A as well as known majapolene B and majapolene A were isolated. In addition, from the specimens collected at Pulau Kerah and Pulau Nyireh, three known halogenated sesquiterpenes and two known halogenated C15 acetogenins were found respectively. Several of these halogenated metabolites showed moderate antibacterial activity against the tested marine microbes.

In a study by Foon *et al.* (2013), seaweed specimens *Eucheuma cottonii* and *Padina* sp. were collected from Pulau Besar, Mersing, Malaysia and investigated. The antioxidant activity of the seaweed discovered was most likely due to the presence of phenolic compounds in the methanolic extract. FTIR spectroscopy analysis showed the presence of alcohols, phenols, carboxylic acids, aromatics and alkene functional groups. The extracts were tested for their antioxidant activity using a DPPH free radical scavenging assay by Zakaria *et al*. (2011). It was reported that the seaweeds tested have potent bioactive compounds that are important for the food and medical industries.

Also in 2013, Mansor *et al.* isolated marine algae *Oscillatoria* sp. from the Kuantan coastal area. The secondary metabolites were extracted in ethanol and the compounds identified through GC-MS. The ethanolic extract was also subject to antibacterial testing. It was reported that there were flavonoids, esters, phenolics and fatty acids in the extract. The antibacterial results of this study showed that the extract had different effectiveness in antibacterial activities with the highest inhibition against *Pseudomonas aeruginosa*, and the least against *Staphylococcus aureus*.

1.4.1(e) Sea cucumber (phylum Echinodermata)

Sea cucumbers are marine invertebrates that fall under the phylum Echinodermata and the class *Holothuroidea*. They can be found worldwide. There are approximately 45 identified sea cucumber species in Malaysian waters, with some of these species already being used in traditional medicine to treat wounds, eczema, arthritis and hypertension (Ridzwan, 2007). Several studies have shown the multiple biological activities of sea cucumbers. These include wound healing promoters and exhibiting antibacterial, antifungal and antioxidant properties (Ridzwan *et al.,* 1995; Fredalina *et al.,* 1999; Hawa *et al*., 1999; Ridzwan *et al*., 2001; Hing *et al*., 2007). *In-*

vitro studies showed that compounds such as saponin glycosides inhibit several types of cancer cell growth including CEM-SS T-lymphoblastic cell (Kawasandi *et al.,* 2004) and four human cancer cell lines; human non-small lunch carcinoma (A549), human cervical cancer (C33A) and human esophageal carcinoma (TE1) (Althunibat *et al.,* 2013).

One of the early studies done in 1995 by Ridzwan *et al.*, showed the presence of antibacterial activity in the phosphate-buffered saline (PBS) extract from two sea cucumber specimens; *Holothuria atra* and *Bohadschia argus*. However, the lipid extract and methanol extract from *H. atra*, *Holothuria scabra* and *B. argus* did not show any activity.

In a study done in 2009 by Althunibat *et al.*, samples of three sea cucumber species*; Holothuria scabra*, *Holothuria leucospilota* and *Stichopus chloronotus* were collected from coastal areas in Terengganu, Malaysia. The results showed that the aqueous extract of *H. leucospilota* contained the highest level of total phenols, while the lowest level was obtained from organic extract of *H. scabra*. The aqueous extracts of *H. scabra*, *H. leucospilota* and *S. chloronotus* exhibited high antioxidant activities (77.46%, 64.03% and 80.58%, respectively) against linoleic acid free radicals. Only the aqueous extract of *S. chloronotus* inhibited growth of C33A (human cervical cancer) and A549 (human non-small lung carcinoma) cancer cells. Onthe other hand, when tested against C33A and A549 cancer cells, the proliferative effects of all organic extracts depended on the dosage.

1.4.1(f) Fungi

Over the past two decades, marine resources such as plants, bacteria, cyanobacteria, algae and fungi have become a significant source of new and pharmacologically active chemical compounds for the development of new drugs, antibiotics, pesticides and antifouling substances. This is possible because of the chemical diversity of their secondary metabolites (Kjer *et al.,* 2010; Debbab *et al.*, 2012; Jones & Pang, 2012; Debbab *et al.*, 2013; Paliany *et al.,* 2014). Fungi from marine sources are now globally accepted to play an important role as a promising source for the discovery of new chemical compounds, together with the growing need of new natural products (Lu *et al.*, 2010; Rateb & Ebel, 2011; Debbab *et al.*, 2013; Paliany *et al.*, 2014). Symbiotic fungi have been reported to produce unique compounds with interesting bioactivities. In the past few years, there has been an increasing interest in the research of marine derived endophytic(symbiotic) fungi for novel compounds which are medically important (Jones *et al.*, 2008; Kjer *et al.,* 2010; Debbab *et al.*, 2013).

Paliany *et al.* (2014) investigated the microbial activities of endotyphic and manglicolous fungi (marine fungi found in mangroves) from the coastlines of Malaysia. Endophytic fungi were isolated from the leaves of healthy *Vitex rotundifolia* and *Ipomoea pes-caprae* collected at two different locations; Kijal Beach on the east coast and at Port Dickson Beach on the west coast, respectively. Manglicolous fungi were obtained from the Institute of Biological Sciences, University of Malaya culture collection. Out of the 31 fungi isolated, five and ten of the endophytic fungi strains from *V. rotundifolia* and *I. pes-caprae* respectively; and nine of the manglicolous isolates displayed positive antibacterial activity.

1.5 Objectives

There are two main objectives to this research. The first objective is to identify soft corals collected in the northern Straits of Malacca based on morphological charactheristics that circumscribe each taxon. The second objective is to identify volatile compounds extracted from the collected octocoral specimens and to test their potential antibacterial properties.

1.6 Thesis outline

This thesis is divided into five chapters. Chapter 1 is essentially the introduction and literature review, and is aimed to outline the objectives of the current study. Chapter 2 is a detailed writeup of all the methodology used in this study. Classical taxonomy was used to identify the octocoral specimens, and same volatile compounds were identified. Compounds were identified by running gas chromatography mass spectrometry (GCMS), and disk diffusion tests were run to check the antibacterial capabilities of the extracts. Chapter 3 summarises the results obtained from the study. It provides information about the identity of the soft corals, as well as its corresponding secondary metabolites. Chapter 4 allows for a discussion of the results obtained, linking them with previous studies, and the likes. Finally, Chapter 5 brings the whole thesis together with conclusions, and future direction for the study.

CHAPTER 2

METHODOLOGY

2.1 Study sites

The Straits of Malacca is a channel connecting the Andaman Sea of the Indian Ocean and South China Sea of the Pacific Ocean. The Straits is located at latitude 6.0333, longitude 99.9167, and is approximately 800 km long with a 65 km width. However it expands northward to about 250 km wide. The northern region is approximately 200 metres deep and is deeper than the south region (Encyclopaedia Britannica ("Strait of Malacca," 2005)).

This study was carried out at two different islands in the northern Straits of Malacca; specifically islands off the coast of Kedah (Figure 2.1). The two research areas are Pulau Payar Marine Park, which is a marine protected area, and Pulau Songsong, which is not a protected area. The islands were chosen based on the accessibility, as well as biodiversity of octocorals off the reefs.