CHEMICAL PROFILING AND ANTIBACTERIAL SCREENING OF CRUDE EXTRACTS OF SOFT CORAL *Lobophytum* sp. FROM STRAITS OF MALACCA

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2021

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Name of Candidate: **TAN YEE YEIN**

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Field of study:

MARINE BIOTECHNOLOGY

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CHEMICAL PROFILING AND ANTIBACTERIAL SCREENING OF CRUDE EXTRACTS OF SOFT CORAL *Lobophytum* sp. FROM STRAITS OF MALACCA

ABSTRACT

Marine natural products have gained popularity in the pharmaceutical field over the years as the ocean is vast and is extraordinarily rich in species diversity, making it the ideal target for exploration. Marine naturals products, isolated from marine sponges, algae and soft corals have been reported to exhibits biological activities such as anti-inflammatory, antitumor, and antimicrobial. In recent years, the emergence of antibiotic resistant bacteria has posed a large threat to healthcare worldwide and new antibacterial compounds are in demand. This study focuses on *Lobophytum* sp. harvested from The Straits of Malacca, to isolate secondary metabolites and test for antibacterial properties against gram-positive (*Staphylococcus aureus* and *Bacillus subtilis*) and gram-negative bacteria (*Escherichia coli* and *Salmonella typhi*) using crude extracts. Cholesterol has been isolated and FAME analysis showed the major fatty acid constituents of the sample is palmitic and arachidonic acid, 29.905% and 18.891% respectively. Crude extracts and fractions obtained from column chromatography shows weak inhibition against *B. subtilis.*

Keywords: Marine natural products, soft corals, antibacterial

PROFIL KIMIA DAN PENYARINGAN ANTIBAKTERIA EKSTRAK KASAR KARANG LEMBUT *Lobophytum* sp. DARI SELAT MELAKA

ABSTRAK

Sejak kebelakangan ini, produk semula jadi marin menjadi semakin popular dalam bidang farmaseutikal. Ini adalah kerana saiz lautan yang luas dan cukup kaya dengan kepelbagaian spesies menjadikannya sasaran ideal untuk penerokaan. Produk semula jadi marin, yang dipencilkan dari span laut, alga dan karang lembut dilaporkan menunjukkan aktiviti biologi seperti terhadap -keradangan, tumor, dan mikrob. Beberapa tahun lepas ini, dilaporkan kemunculan bakteria tahan antibiotik telah menyebabkan ancaman besar bagi penjagaan kesihatan di seluruh dunia dan sebatian antibakteria baru semakin menjadi permintaan. Kajian ini memberikan tumpuan kepada *Lobophytum* sp. dituai dari Selat Melaka, bertujuan untuk memencilkan metabolit sekunder dan menyaring sifat antibakteria terhadap bakteria gram-positif (*Staphylococcus aureus* dan *Bacillus subtilis*) dan bakteria gram-negatif (*Escherichia coli* dan *Salmonella typhi*) dengan menggunakan ekstrak kasar. Kolesterol yang telah diasingkan dan analisis FAME menunjukkan sampel unsur asid lemak utama adalah asid palmitik dan arakidonik, masing-masing 29,905% dan 18,891%. Ekstrak dan pecahan kasar yang diperoleh dari kromatografi lajur menunjukkan perencatan yang lemah terhadap *B. subtilis*.

Kata kunci: Produk semula jadi laut, karang lembut, antibakteria

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ABBREVIATIONS

amu	Atomic mass unit
AGE	Agarose gel electrophoresis
CHCl3	Chloroform
COI	Cytochrome c oxidase I
EI-MS	Direct insertion electron ionization mass spectrometry
EtAOc	Ethyl acetate
etc	Et cetera
FAME	Fatty acid methyl esters
FFNSC	Flavours and Fragrance of Natural and Synthetic Compounds
GCMS	Gas Chromatography/Mass Spectrometry
gDNA	Genomic DNA
H2O	Water
HCl	Hydrochloric acid
Hex	Hexane
HIV	Human immunodeficiency virus
HPLC	High Performance Liquid Chromatography
MDR	Multi drug resistance
MeOH	Methanol
MHA	Mueller Hinton
MRSA	Methicillin-resistant *Staphylococcus aureus*
MUFA	Monounsaturated fatty acid
NCBI	National Center for Biotechnology Information
NIST	National Institute for Standard and Technology

NMR	Nuclear Magnetic Resonance
PCR	Polymerase chain reaction
ppm	Parts per million
PTLC	Preparative thin layer chromatography
PUFA	Polyunsaturated fatty acid
Rf	Retention factor
ROVs	Remotely operated vehicles
SCUBA	Self-Contained Underwater Breathing Apparatus
SFA	Saturated fatty acid
sp.	Species
SYBR	Synergy Brands, Inc.
TAE	Tris-acetate-EDTA
TLC	This layer chromatography
Tol	Toluene
UV	Ultraviolet

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CHAPTER 1 : INTRODUCTION

1.1. Natural products

Natural products are secondary metabolites extracted from various types of organisms including terrestrial plants, terrestrial microorganisms, marine organisms, and terrestrial vertebrates and invertebrates (Chin et al., 2006). Secondary metabolites are small molecules, often having molecular weights less than 2000 amu, synthesised by organisms as a result of the organism adapting to its surrounding environment or are produced to act as a possible defense mechanism against predators to assist in the survival of the organism (Dias et al., 2012; Sarker, 2006). Secondary metabolites can be classified groups such as alkaloids, flavonoids, coumarins, glycosides, lignins, steroids, terpenoids and quinone. These metabolites can be obtained by extraction, fractionation and purification of the crude extracts of the organisms.

Natural products have been incorporated into healthcare for thousands of years in treating and preventing human diseases (Chin et al., 2006; Dias et al., 2012) especially drugs derived from terrestrial plants. Commercially important drugs like acetylsalicyclic acid (aspirin) and morphine is isolated from the bark of the willow tree *Salix alba* and *Papaver somniferum* respectively and were developed into single chemical drugs and are still clinically used (Chin et al., 2006; Dias et al., 2012). Natural products and their derivatives make up 90% of therapeutic classes and are used by roughly two-thirds to threequarters of the world's population and are used to treat 87% of all categorized human diseases including as antibacterial, anticancer, anticoagulant, antiparasitic, and immunosuppressant agents, among others (Chin et al., 2006; McChesney et al., 2007). Towards the end of 2014, 49% of small

molecules approved as anticancer either natural products or directly derived therefrom (Newman & Cragg, 2016).

1.2 Marine natural products

Marine natural products entered the pharmaceuticals much later compared to terrestrial plants, even though the ocean which covers over 70% of the world and are extraordinarily rich in species diversity, especially in tropical environments (Gerwick & Moore, 2012; Jiménez, 2018). High level of uncertainty towards the ocean and its inhabitants might be the reason why researches are not exploring them, however, the introduction of SCUBA in the 1970s, to the use of manned submersibles in the 1980s and more recently the use of remotely operated vehicles (ROVs) since 1990s changed the dynamic of new natural products emergence (Dias et al., 2012). The exploration of marine organisms and their structurally diverse natural products begins with the need to detect and understand the chemical basis of various toxins with which people came into contact, such as saxitoxin, tetrodotoxin, and the brevetoxins (Gerwick & Moore, 2012). This opens the door for researches to dive down deeper into studying the diverse secondary metabolites secretes by marine organisms.

Some of the earliest discovered biological active compounds were reported back in early 1950s, spongouridine and spongothymidine shown in Figure 1.1, categorised as nucleosides, from the Caribbean sponge *Cryptotheca crypta* and 15 years later, approved as antiviral and anticancer drugs respectively (Chin et al., 2006; Cragg & Newman, 2005; Dias et al., 2012). Marine sponges do not have working systems and survive by filtering water current, obtained food and removed waste, and vulnerable towards predators. This enhances the diversity of secondary metabolites secreted by them, leading to further studies on its metabolites structure and bioactivities (Dias et al., 2012). Over time, other groups of organisms, such as red algae, hard and soft corals, sea cucumber, marine cyanobacteria, marine fungi, and diverse other

groups of marine eubacteria, were studied, and the repertoire of unique molecular species grew (Gerwick & Moore, 2012).

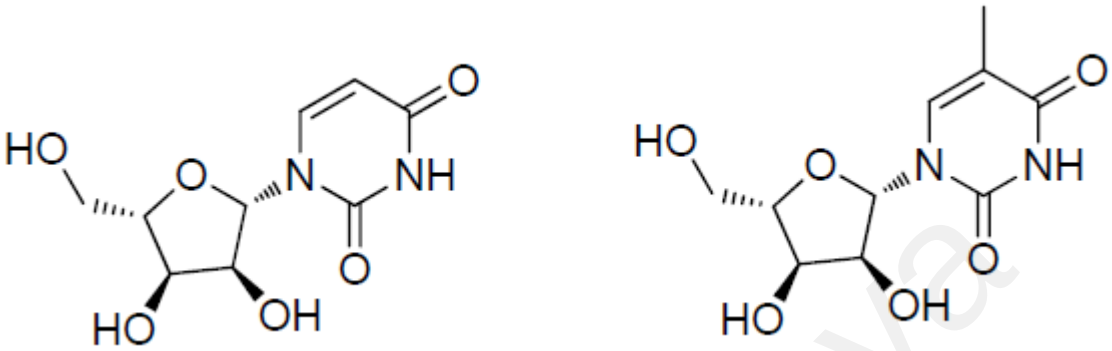


Figure 1.1 Molecular structure of Spongouridine (a) and Spongothymidine (b)

1.3 Soft corals and its bioactive properties

The ocean is believed by many to have the highest biodiversity of any ecosystem on the planet and in it the coral reefs house more than 25% of the marine life. Hard corals are the building blocks of coral reefs, made of hard exoskeleton of calcium carbonate and living symbiotically with tiny algae called zooxanthellae (Coral Reef Alliance, 2020) and have hollow feather-like tentacles of six or multiples of six. Soft corals, also known as Alcyonacea, do not possess the calcium carbonate skeleton found in hard corals are the most colourful and diverse groups of invertebrates found on coral reefs with eight hollow feather-like tentacles and similarly and have symbiotic relationships with photosynthesizing zooxanthellae (Wang et al., 2016; Bruckner, 2014). In many parts of the Indo-Pacific, a wide variety of alcyonacean soft corals can dominate the benthic community, sometimes surpassing the scleractinians (hard corals) in percent cover (Sammarco & Coll, 1988). As they lack a hard exoskeleton, soft corals obtain their support and protection from calcified spine-like spicules called sclerites. Alcyonacea corals are an extraordinarily rich and diverse source of novel secondary metabolites (Wang et al., 2016; Sammarco & Coll, 1988) that possess biological activities

such as antimicrobial, antiviral, anticoagulant, antidepressant, antihypertensive, antileukemic, and anthelmintic.

Soft corals are recognized as a rich source of secondary metabolites, exhibit potential bioactivities such as cytotoxic, anti-inflammation and antibacterial. However, the emergence of antibiotic resistant bacteria worldwide has been labelled as the awakening of a giant which is a threat to everyone. Pharmaceutical companies and researchers are now in race against time to develop new antibiotics to overcome this.

Antibiotics are drugs used to prevent and treat bacterial infections. Antibiotic resistance occurs when bacteria change in response to the use of these medicines (Antimicrobial resistance, 2020). Antibiotic resistance in bacteria emerges when bacteria mutate and become resistant towards antibiotics. This occurs due to overuse, inappropriate prescribing, and extensive agriculture use; which accounts for at least 50,000 deaths each year in Europe and the United States and it is anticipated that drug resistant infections will be responsible for the deaths of 10 million people worldwide by 2050 (Choudhary et al., 2017; Mobarki et al., 2019).

The first antibiotic penicillin was discovered in 1928 Sir Alexander Fleming, shortly thereafter, penicillin resistance became a substantial clinical problem and new antibiotics were discovered, developed, and deployed, restoring confidence (Mobarki et al., 2019; Antimicrobial Resistance, 2020). Methicillin-resistant *Staphylococcus aureus* (MRSA) was first reported back in 1962 (UK) and 1968 (US), ever since then, the continuously evolving antibiotic-resistance of microbial pathogens has raised demands for the development of new and effective antimicrobial compounds (Choudhary et al., 2017). Pharmaceutical companies turn their focus on marine invertebrates such as soft corals. They are the main components of coral reef known to be prolific producers of secondary metabolites and have been the target

of bioactive natural product sourcing (Murti and Radjasa, 2012; Piddock, 2012), in the hope to discover new antibiotics and re-stimulate drug development.

1.4 Bioactive properties of soft corals

Lobophytum sp., NCBI: txid205095, belong to the order Alcyonacea and family Alcyoniidae (Cheng et al., 2008, 2009; Yin et al., 2006). Widely known as Devil's Finger Coral, there are 46 valid species of *Lobophytum* where it's polypbearing area are narrower than the stalk with prominent lobes or ridges on its upper surface; small and oval sclerites with the tubercles arranged in regular, transverse girdles (McFadden et al., 2006), Figure 1.2 shows examples of *Lobophytum* sp. and appearance of sclerites. *Lobophytum* sp. are generally brown are found in the Indo-Pacific and Red Sea, inhabits depths of 20 to 60 feet and are usually found near the shore in shallow waters on reef flats, where they are most prolific, and can also be found in turbid lagoons attached to rubble, and other diverse locations (Animal-World, McBirney, C., & Brough, C., 2021). *Lobophytum* sp. is a rich source of macrocyclic cembrane-type diterpenoids and their cyclized derivatives. Previous studies reported that some cembranoid analogues have been shown to exhibit diverse biological properties such as cytotoxic, anti-inflammatory, anti-HIV, and antibacterial activities (Cheng et al., 2009; Lin et al., 2009; Quang et al., 2011; Quang et al.., 2011; Yin et al., 2006). In the later sections, research and compounds isolated from *L. crassum*, *L. pauciflorum* and *L. durum* will be further discussed highlighting their bioactivities.

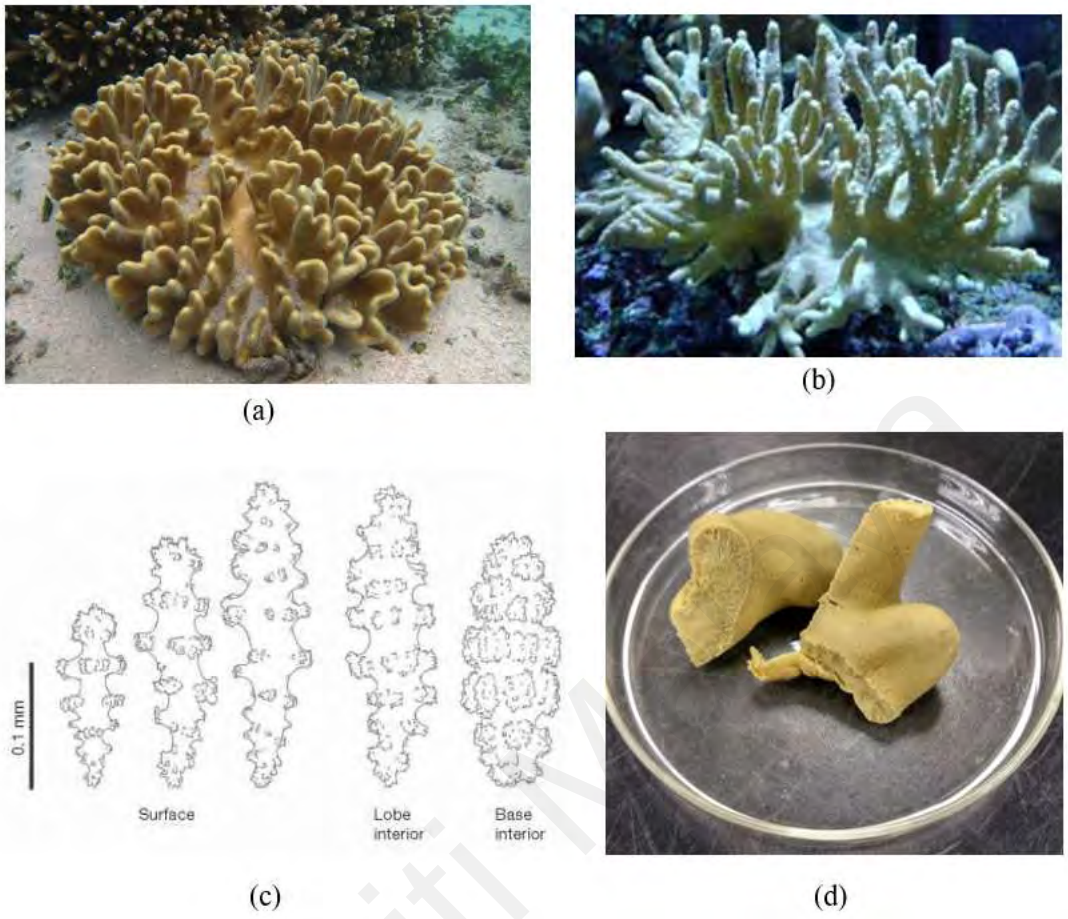


Figure 1.2 Examples of *Lobophytum* sp. and structure of sclerites. (a) *Lobophytum crassum*, (b) *Lobophytum pauciflorum*, (c) Sclerites of *Lobophytum* sp., (d) Samples of *Lobophytum* sp.

1.5 Research question

Does *Lobophytum* sp. found in The Straits of Malacca secretes secondary metabolites which possess antibacterial properties against Gram-positive and Gram-negative bacteria and how effective are they against these bacteria?

1.6 Research objectives

a. To quantify the yield of *Lobophytum* sp. crude extract

b. To perform chemical profiling of *Lobophytum* sp. crude extract and fractions

c. To screen the antibacterial activity of the *Lobophytum* sp. crude extract and fractions

CHAPTER 2 : LITERATURE REVIEW

2.1 Marine natural products

The ocean, home to 34 animal phyla identified taxonomically where most of them are native to the marine environment leads to the production of a great variety of molecules bearing unique structures in terms of diversity, structural, and functional features and also in higher incidence of significant bioactivity in relation to natural products from terrestrial life forms (Jiménez, 2018). Covering 70% of the Earth, only 5% of the ocean is explored and pharmaceutical companies began to realize that the ocean would possess unique biodiversity and may be a possible source for potential drug candidates (Dias et al., 2012). Corals, marine sponges, jellyfish, sea anemones, bryozoans, molluscs, echinoderms, tunicates and crustaceans are the main focus where bioactive secondary metabolites are isolated from; this includes alkaloids, steroids, terpenoids, isoprenoids, nonisoprenoids, quinones, halogenated compounds, nitrogen heterocyclics, nitrogen sulphur heterocyclics and lipids (Chin et al., 2006; Elshamy, 2016; Kanase & Singh, 2018; Sammarco & Coll, 1988), mostly been sesquiterpenes or diterpenes, many of which are bioactive.

To date, there are a total of seven approved drugs from marine origin and five of them are anticancer agent, example Cytarabine (Ara-C), while the other two are pain management (Ziconotide) and antiviral drugs (Vidarabine (Ara-A)) as shown in Table 2.1. 13 marine natural product or derivatives are in clinical trials as summarised in Table 2.2, mostly as anticancer. The source of these metabolites are mainly sponges, molluscs, tunicates and marine microorganisms (Chin et al., 2006; Cragg & Newman, 2005; Gerwick & Moore, 2012; Kanase & Singh, 2018; Li et al., 2019; Pereira, 2019; Rangel & Falkenberg, 2015).

Table 2.1 U.S. Food and Drug Administration approved drugs of marine origin stating their source and indication(s).

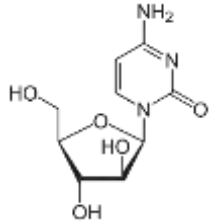
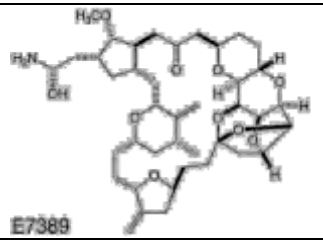
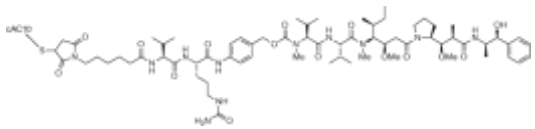
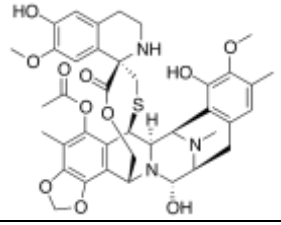
Drug	Natural Product or Derivative	Source	Biosynthetic Class	Indication(s)	Structure
Cytarabine (Ara-C)	Derivative	Caribbean sponge *(Cryptotheca crypta)*	Nucleoside	Leukemia	
Eribulin mesylate (E7389)	Derivative	Sponge *(Halichondria okadai)*	Complex polyketide	Metastatic breast cancer	
Brentuximab vedotin (SGN-35)	Derivative	Sea hare *(Dolabella auricularia)*	Antibody drug conjugate (MM auristatin E)	Anaplastic large T-cell systemic malignant lymphoma, Hodgkin's disease	
Trabectedin (ET-743)	Natural product	Tunicate *(Ecteinascidia turbinate)*	NRPS-derived alkaloid	Soft tissue sarcoma and ovarian cancer	

Table 2.1, continued.

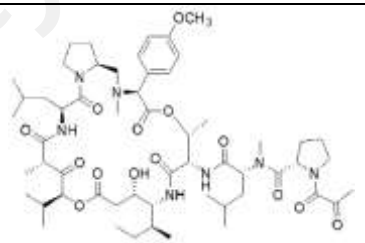
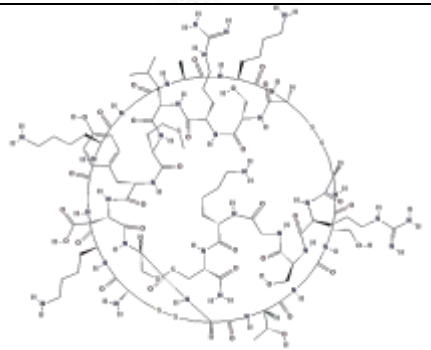
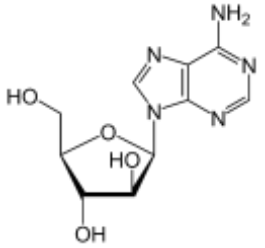
Drug	Natural Product or Derivative	Source	Biosynthetic Class	Indication(s)	Structure
Plitidepsin (Aplidin)	Natural product	Mediterranean tunicate *(Aplidium albicans)*	Cyclic depsipeptide	Multiple myeloma	
Ziconotide	Natural product	Cone snail *(Conus magus)*	Cysteine Knot Peptide	Severe and chronic pain	
Vidarabine (Ara-V)	Derivative	Caribbean sponge *(Cryptotheca crypta)*	Nucleoside	Herpes simplex virus infection	

Table 2.2 Promising marine natural products in clinical trials.

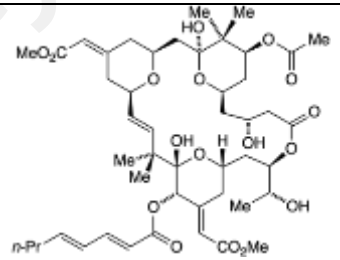
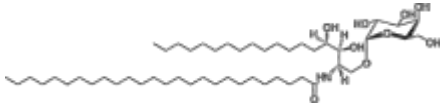
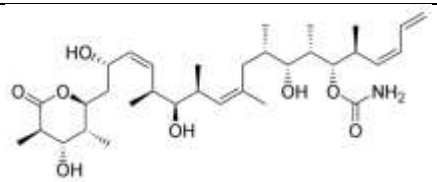
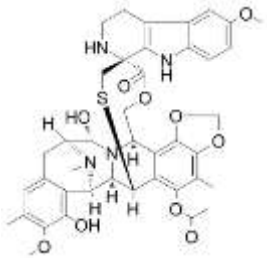
Drug	Natural Product or Derivative	Source	Biosynthetic Class	Disease area	Structure
Byrostatin 1	Natural product	Bryazoan *(Bugula neritina)*	Polyketide	Cancer, Alzheimer's	
KRN7000	Derivative	Okinawan sponge *(Agelas mauritianus)*	Glycosphingolipids	Antitumor	
Discodermolide	Natural product	Sponge *(Discodermia dissoluta)*	Polyketide	Microtubule-stabilizing drug, antitumor	
Trabectedin analog (PM01183)	Derivative	Tunicate *(Ecteinascidia turbinate)*	NRPS-derived alkaloid	Anticancer	

Table 2.2, continued.

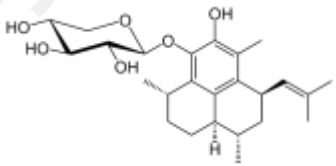
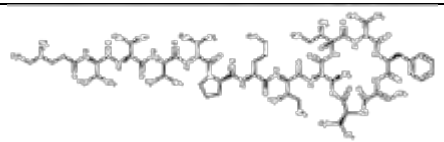
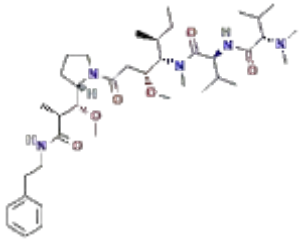
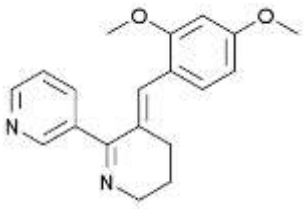
Drug	Natural Product or Derivative	Source	Biosynthetic Class	Disease area	Structure
Pseudopterosin A	Natural product	Gorgonian sea whip *(Pseudopterogorgia elisabethae)*	Diterpene glycoside	Wound healing	
Elisidepsin	Derivative	Mollusk *(Elysia rufescens)*	Cyclic Depsipeptide	Anticancer	
Soblidotin (TZT-1027)	Derivative	Sea hare *(Dolabella auricularia)*	Dolastatins	Antitumor	
DMXBA (GTS-21)	Derivative	Pacific nemertine *(Paranemertes peregrina)*	Alkaloid	Cognition, Schizophrenia	

Table 2.2, continued.

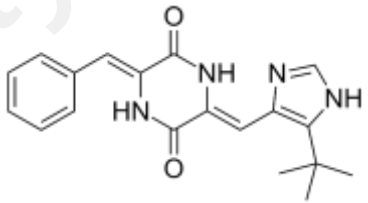
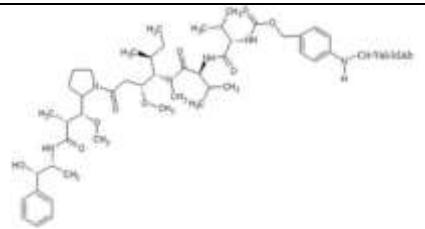
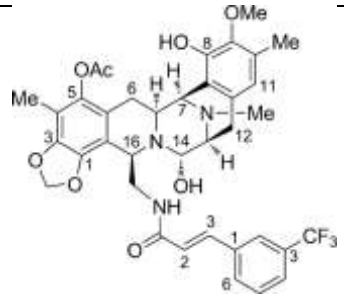
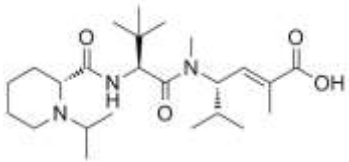
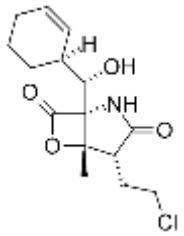
Drug	Natural Product or Derivative	Source	Biosynthetic Class	Disease area	Structure
Plinabulin (NPI 2358)	Derivative	Fungus	Diketopiperazine	Anticancer	
Glembatumumab vedotin (CDX-011)	Derivative	Mollusk	Antibody drug conjugate (MM auristatin E)	Anticancer	
Zalypsis (PM00104)	Derivative	Nudibranch	Tetrahydroisoquinolone alkaloid	Anticancer	
Hemiasterlin derivative (E7974)	Derivative	Sponge	Modified linear tripeptide (NRPS-PKS)	Anticancer	

Table 2.2, continued.

Drug	Natural Product or Derivative	Source	Biosynthetic Class	Disease area	Structure
Marizomib (Salinosporamide A; NPI-0052)	Natural Product	Marine actinomycete	NRPS with b-lactone & g-lactam	Anticancer	

2.2 Research on Genus *Lobophytum*

Alcyonarian corals are an extraordinarily rich and diverse source of novel secondary metabolites, in which allelopathy has been experimentally demonstrated to occur in the marine environment (Sammarco & Coll, 1988). Soft corals of the genus *Lobophytum* (Alcyoniidae) have proven to be a rich source of macrocyclic diterpenoids, and have been reported in the past decades to possess HIV-inhibitory, cytotoxic and anti-inflammatory activities (Tseng et al., 2011). The first compound, lobophytolide (Figure 2.1), a cembrane-type diterpenoid, was identified from *Lobophytum cristagalli* leads to further investigations for decades and more than 250 compounds were isolated and identified from the genus *Lobophytum* (Lai et al., 2017; Lin et al., 2009).

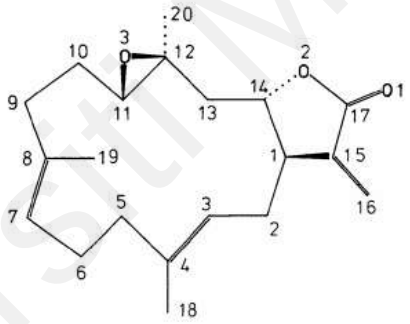


Figure 2.1 Lobophytolide, first cembrane-type diterpenoid isolated from *Lobophytum cristagalli*

The compounds reported are categorized into cembrane-type diterpenoids, other types of diterpenoids, lipids, sterols, tocopherols, triterpenoids, and type alkaloids (Lai et al., 2017; Lin et al., 2009; Peng et al., 2018; Yin et al., 2006), commonly described as defensive substances against predators such as other corals and fishes. Figure 2.2 shows the examples of different classes of terpenoids, structural characteristics of cembrane-type diterpenoids and example of sterols. To date, not much researches that have been done on the soft coral *Lobophytum* sp., and in Malaysia only one study has been reported by Chan et al.

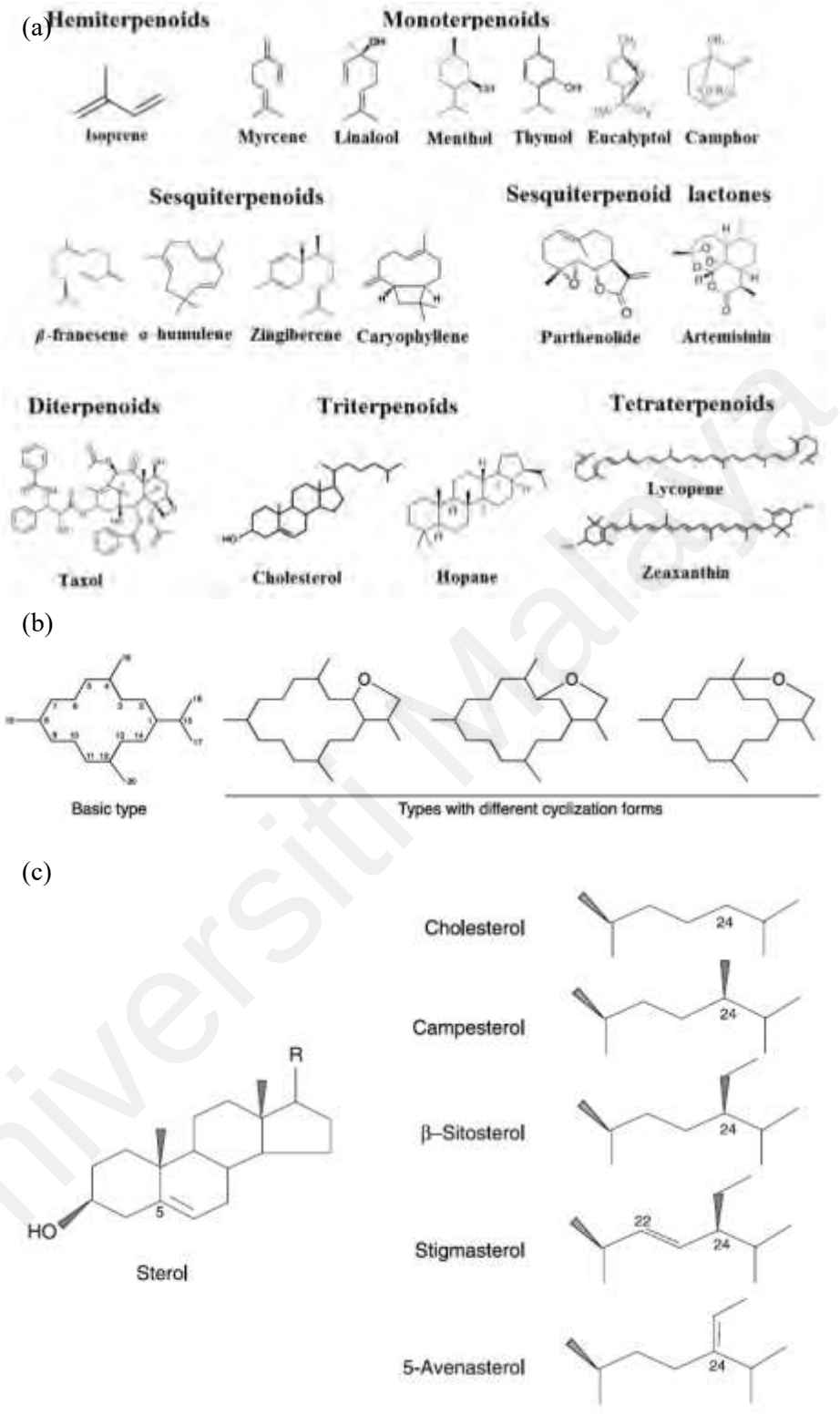


Figure 2.2 Examples of different classes of terpenoids (a), structural characteristics of cembrane-type diterpenoids (b), and examples of sterols (c)

2.2.1 Research on *Lobophytum crassum*

Lobophytum crassum are commonly found Indo-West Pacific: Australia, New Caledonia, Taiwan and Ryukyu Island. Species *L. crassum* is well known to produce oxygenated cembranoids due to geographic variation and environmental conditions (Rodrigues et al., 2019). A total of 22 cembrane-type diterpenoids were isolated and identified from *L. crassum* of Taiwan between 2008 – 2012, and *L. crassum* was the most studied soft coral species during 2016 – 2018 especially those harvested from South China Sea, Indian Ocean, the Red Sea and from aquaculture (Rodrigues et al., 2019; Wei et al., 2013). Table 2.3 shows the compounds isolated from *L. crassum* with their biological activities and location harvested from 2008 – 2018.

Table 2.3 Compounds isolated from *L. crassum* with their biological activities and location harvested from 2008 – 2018.

Compound	Biological activities	Location	Reference
Crassumolide A – F	Anti-inflammatory	Coast of Kenting	(Chao et al., 2008; Wei et al., 2013)
Lobohedleolide			
17-Dimethylaminolobohedleolide			
Sinulariol A			
Dentivulatolide			
Culobophylin A – C	Anti-inflammatory and cytotoxic	Aquaculture	(Lee N. L. & Su J. H., 2011)
Lobophylin A and B	Anti-inflammatory		
Culobophylin D and E	Anti-proliferation		(Peng et al., 2018)
Lobocrassin A – E	Lobocrassin B – anti-inflammatory	Coast of northeast Taiwan	(Kao et al., 2011)

Table 2.3, continued.

Compound	Biological activities	Location	Reference
Lobocrassin F	Anti-inflammatory	Coast of northeast Taiwan	(Lee et al., 2012; Tseng et al., 2011)
Lobophyolide A and B	Anti-inflammatory and cytotoxic	Coast of Pingtung, Taiwan	(Rodrigues et al., 2019)
Crassumolides G – I	Anti-inflammatory	Dongsha Atoll, northeastern South China Sea	(Tseng et al., 2011)
Durumolide B and C			
Sinularolide B			
20-acetylsinularolide B	N/A	Coast of Sanya, Hainan Province, China	(Zhang et al., 2008)
Presinularolide B			
3-dehydroxylpresinularolide B			
3-dehydroxyl-20-acetylpresinularolide B			
Sinularolide B – E			
Lobocrasol		Dongsha Island, Taiwan	(Lin et al., 2009)

2.2.2 Research on *Lobophytum pauciflorum*

Lobophytum pauciflorum is studied extensively by researchers and compounds such as cembrane-type terpenoids, diterpenes some acids have been successfully isolated and purified. Some shows promising biological activities like anti-inflammatory, cytotoxic, anti-fungal and antibacterial. Table 2.4 shows compounds isolated from *L. pauciflorum* with their biological activities and location harvested from 1979 – 2016.

Table 2.4 Compounds isolated from *L. pauciflorum* with their biological activities and location harvested from 1979 – 2016.

Compounds	Biological activities	Location	Reference
Polyhydroxylated sterols (II), (IV) and (VI)	N/A	Japan	(Yamada et al., 1979)
Cembranolide diterpenes (I) and (II)			(Yamada et al., 1980)
Epoxy cembranolide diterpenes (I) and (II)			(Yamada et al., 1980)
Diterpene metabolites (I) – (V)		Cairns on the Great Barrier Reef.	(Dunlop & Wells, 1979)
14,17-Epoxyloba-8,10,13(15)-trien-18-ol 18-acetate	Anti-fungus	Mindoro Island, Philippines	(Edrada et al., 1998)
Loba-8,10,13(15)-triene-17,18-diol 18-acetate			
18-Methoxyloba-8,10,13(15),16(17)-tetraene			
14,18-Epoxyloba-8,10,13(15)-trien-17-ol			
lobophytones A – G	Anti-inflammatory	Sanya Bay, Hainan Island of China	(Yan et al., 2010)
Lobophytones U – Z	Anti-inflammatory and antibacterial		(Yan et al., 2011)
Methyl sartortuoate and nyalolide	N/A		
Cyclolobatriene, lobatriene, eunicol and fuscol	Cytotoxic	Okinawan coast of Japan	(Govindam et al., 2012)
Nephthenol and Gorgost-5-ene-3β-ol	Anti-inflammatory	Hurghada at the Egyptian Red Sea coast	(Hassan et al., 2016)
Heptadecan-1-ol and batilol (6).	Anti-bacterial		
Palmitic acid and stearic acid			

2.2.3 Research on *Lobophytum durum*

Lobophytum durum have been a focus among researchers as there are a handful of novel cembranolides isolated and purified which exhibits bioactivities like anti-inflammatory, cytotoxicity and antibacterial (Chan et al., 2018; Cheng et al., 2011; Cheng et al., 2008, 2009). The compounds isolated from L. durum is summarised in Table 2.5 showing the biological activities and location harvested in Taiwan from 2008 – 2012.

Table 2.5 Compounds isolated from *L. durum* with their biological activities and location harvested from 2008 – 2012.

Compound	Biological activities	Location	Reference
Durumhemiketalolides A-C	N/A	Dongsha Islands, Taiwan	(Cheng et al., 2009)
Durumolides A – E	Anti-infammatory		(Cheng et al., 2008)
Durumolides F – L	Antibacterial		(Cheng et al., 2009)
Sinularolide D	Antineoplastic		
13S-Hydroxylobolide	Anti-inflammatory		(Wei et al., 2013)
13R-Hydroxylobolide			
Deacetyl-13-hydroxylobolide			
(7E,11E)-13,18-Dihydroxy-3,4-epoxy-7,11,15(17)-cembratrien-16,14-olide			
(3E,7E,11E)-18-Acetoxy-3,7,11,15(17)-cembratetraen-16,14-olide			
Durumolides M – Q	Cytotoxic and antiviral		(Cheng et al., 2011)

2.3 Antibacterial properties of Genus *Lobophytum*

Antibiotics played a huge role in improving the quality of our healthcare and life. However, the emergence of antibiotic resistant bacteria has posed a large threat to healthcare worldwide. As mentioned earlier, the antibiotic resistance crisis has been attributed to the

overuse and misuse of these medications, as well as a lack of new drug development by the pharmaceutical industry due to reduced economic incentives and challenging regulatory requirements (Choudhary et al., 2017; Lee Ventola, 2015; Piddock, 2012). Bacteria evolve quickly to adapt to their everchanging environment and the surge of new multi-drug resistanes (MDR) especially in Gram-negative bacteria which includes *Escherichia coli* and *Neisseria gonorrhoeae* causes serious global health crisis (Piddock, 2012). The first methicillin-resistant *Staphylococcus aureus* (MRSA) was recorded in the UK and US in 1960s, resistance has eventually been seen to nearly all antibiotics that have been developed as shown in Figure 2.3 (Piddock, 2012).

Lobophytum sp. has been reported to produce secondary metabolites with promising antibacterial properties, this includes the bacterial symbionts in them. Bacterial isolates, LBTGA2, from *Lobophytum* sp. harvested from North Java Sea was found to inhibit the growth of two MDR strains bacteria, *E. coli* and *S. aureus* (Murti and Radjasa, 2012). Zhao et al., reported two new α-methylene-γ-lactone-containing cembranoids, (1R*,7S*,14S*,3E, 11E)-7-hydroperoxycembra-3,8(19),11,15(17)-tetraen-16,14-olide, and (1R*,7S*,14S*, 3E,11E)-18-acetoxy-7-hydroperoxycembra-3,8(19),11,15(17)-tetraen-16,14-olide, isolated from *Lobophytum* sp. harvested from Sanya Bay, Hainan Island of China, were found to be moderate inhibitors against the bacteria *S. aureus* and *Staphylococcus pneumoniae* (Zhao et al., 2013).

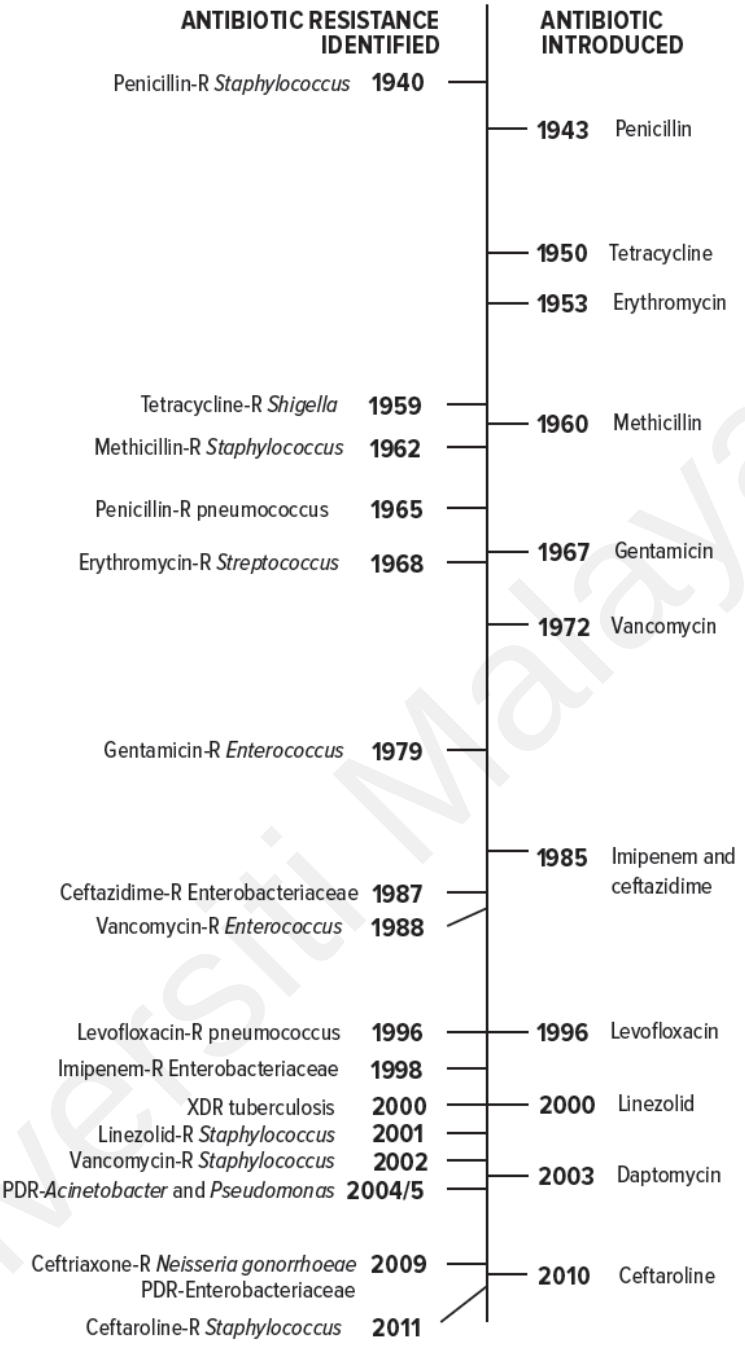


Figure 2.3 Developing Antibiotic Resistance: A Timeline of Key Events. Dates are based upon early reports of resistance in the literature. In the case of pan-drug-resistant *Acinetobacter* and *Pseudomonas*, the date is based upon reports of health care transmission or outbreaks. Note: penicillin was in limited use prior to widespread population usage in 1943 (Lee Ventola, 2015). PDR = pan-drug-resistant; R = resistant; XDR = extensively drug-resistant.

Crude extracts of *Lobophytum* sp. from the Selayar Islands (South Sulawesi), from n-Hexane, n-Butanol, ethyl acetate and aqueous extractions, all showed antibacterial activity towards bacteria from Gram-positive (*Bacillus subtilis* and *S. aureus*) and Gram-negative (*E. coli* and *Vibrio eltor*) (Putra et al., 2016). Another study from Saudi Arabian Red Sea Coast at Jeddah by Al-Footy et al., reported strong antibacterial activity of sesquiterpene alismol and cembranoid-type diterpene, cembrene A against gram-positive bacteria (*S. aureus*, *S. epidermis* and *S. pneumonia*) and gram-negative bacteria (*P. aeruginosa*) (Al-Footy et al., 2016). To date, *Lobophytum* sp. harvested from Malaysia's coast line have not been studied extensively. Recently, Chan et al., reported that the hexane-extract of *Lobophytum microlobulatum*, harvested from coastal area of Port Dickson, Negeri Sembilan, exhibits strong antibacterial activity against *S. aureus* and *B. cereus* (Chan et al., 2018). This discovery highlights the potential of marine natural products in the coastal areas of Malaysia especially their antibacterial properties.

Table 2.6 Examples of compounds with antibacterial properties isolated from Lobophytum sp.

Compound	Location	Reference
Durumolide F – L	Cembranoids	(Cheng et al., 2009)
(1*R**,7*S**,14*S**,3*E*,11*E*)-7-hydroperoxycembra-3,8(19),11,15(17)-tetraen-16,14-olide	South China Sea	(Zhao et al., 2013)
Alismol	The Red Sea	(Al-Footy et al., 2016)
Heptadecan-1-ol and batilol (6), Stearic acid		(Hassan et al., 2016)
RA: Aqueous; NH: *n*-Hexane; EA: Ethyl acetate; NB: *n*-Butanol extracts	South Sulawesi	(Putra et al., 2016)

2.4 Research gap

As the demand for new antibacterial compounds increases, researchers and large pharmaceutical companies turn towards the marine ecosystem. Numerous studies on soft corals had been reported. Some of these studies showed the potential of the secondary metabolites secreted by soft corals as antibacterial compounds. The studied organisms are mainly from the *Sinularia* and *Lobophytum* genus. Marican et al. (2016) reported antibacterial activities of soft coral extracts from *Sarcophyton* sp., *Carijoa* sp., *Coelogorgia* sp., *Junceella* sp. and *Virgularia* sp. harvested from the north of the Straits of Malacca. However, there is only one study reported which is done in Malaysia by Chan et al. (2018) on *Lobophytum* sp. This is an opportunity for local researchers to study on the soft corals found in Malaysia's waters in hope to discover interesting compounds. As the environment and diet of the corals might affect the types of secondary metabolites secreted, there is a large potential in the less discovered areas such as the Straits of Malacca.

CHAPTER 3 : MATERIALS AND METHODS

3.1 Sample collection and extraction

Approximately 1.2 kg of a colony of soft coral, morphologically resembling *Lobophytum* sp. was collected via SCUBA at the depth of 2 – 3 meters in Tanjung Tuan (2.4089° N, 101.8494° E), Port Dickson in the Straits of Malacca on 24th January 2019. The collected sample was cleaned, macerated and extracted over methanol (MeOH) in room temperature. Two replicates of sample tissue, each approximately 1 cm in size was separately extracted for molecular and microscopic identification.

Figure 3.1 Underwater photograph of collected sample (left) and macerated sample (right)

The sample was macerated and extracted in industrial grade methanol (MeOH) in room temperature. The extracts were filtered and evaporated to obtain the soft coral crude extract. Evaporation was done using Buchi Rotavapor. The crude extract obtained was subjected to liquid-liquid partitioning using ethyl acetate (EtAOc) and water (H_2O) at the ratio of 1:3. The non-polar fraction was dried by rotavapor and subjected to further profiling. The H_2O layer was discarded as it was used as a medium to remove access salt component introduced through salt water. The crude extract obtained after liquid-liquid partition were weighed and the percentage yield is calculated using the formula:

$$\%yield = \frac{\text{weight after extraction process}}{\text{material weight taken for extraction}} \times 100\%$$

3.2 Identification

3.2.1 Microscopic identification

Approximately 1 cm of soft coral tissue was digested in 10% hydrochloric acid (HCl) over a duration of 14 days. Once the tissue was digested, the whitish remains which is called spicules were observed under a light microscope to visualize the shape of spicules to aid in identification at least to genus level. The spicules observed was compared to the hand drawn illustration by Versevedelt (1983).

3.2.2 Molecular identification

3.2.2.1 Genomic DNA extraction

Genomic DNA (gDNA) was extracted using the NucleoSpin® Tissue (Macherey-Nagel), following the manufacturer's instructions with modifications. 50 mg of tissue was cut with a clean, sterile razor. The cut tissue was placed in a 1.5 mL microcentrifuge tube before proceeding with the rest of the kit's protocol. The concentration and purity of extracted gDNA was quantified using the NanoDrop™ spectrophotometer (Thermo Fisher Scientific Inc, (USA), and its integrity was assessed by running in a 0.8% agarose gel electrophoresis (AGE) with SYBR Safe staining (Thermo Fisher Scientific Inc, USA).

3.2.2.2 Quality Assessment of Extracted gDNA

0.8% agarose gel was prepared by dissolving molecular-grade agarose powder (NextGene, MYS) in 1x Tris-acetate-EDTA (TAE) buffer (1st Base, SGP) stained with10 1:10,000 (v/v) SBYR Safe staining (Thermo Fisher Scientific Inc, USA). AGE was performed by loading gDNA mixed with 6X loading dye (Thermo Fisher Scientific Inc, USA), and loading 5 uL of 1kb DNA ladder (Thermo Fisher Scientific Inc, USA) as the size marker. AGE was conducted at 100 V for 25 min using electrophoresis power supply EV243 (Consort, BEL). Upon completion, the gel was visualized using the gel documentation system Platinum HD2 (UVItec, GBR) and UVItec software (UVItec, GBR).

3.2.2.3 DNA Marker Amplification

The cytochrome c oxidase I (*COI*) gene was selected for the molecular identification and phylogenetic analysis using selected primer sequences listed in Table 3.1. DNA was amplified by polymerase chain reaction (PCR) using the MultiGeneTM gradient PCR Thermo Cycler (Labnet, USA) following the master mix in Table 3.2 and thermocycling conditions in Table 3.3. Amplified products were then stored at -20°C until further analysis. AGE was then conducted to assess the quality of PCR products and to confirm single sequence amplification by loading 5 µL PCR products (Figure 2).

Table 3.1 Selected primer sequences for DNA marker *COI*

Orientation	Primer	Primer Sequence	References
Forward	jgLCO1490	$^{5'}$TITCIACIAAYCAYAARGAYATTGG$^{3'}$	(Geller et al, 2013)
Reverse	jgHCO2198	$^{5'}$TAIACYTCIGGRTGICCRAARAAYCA$^{3'}$	

Table 3.2 Components of PCR master mix

Reagents	Volume per reaction (µL)
PCR water	16.3
10x PCR buffer	2.5
10mM dNTPs	0.5
Forward primer (10 µM)	1.25
Reverse primer (10 µM)	1.25
ExPrime Taq Polymerase (5 U µL-1)	0.2
DNA template	3.0
Total volume	**25.0**

Table 3.3 Cycling parameters for PCR

PCR stage	Temperature (°C)	Time (minute (s))
Initial Denaturing	94	2.00
Denaturing	94	1.00
Annealing	48	1.00
Extension	72	1.00
Final Extension	72	5.00
PCR Cycles	35	

3.3 Chemical Profiling

3.3.1 Column chromatography

A FAVORIT® glass column with the socket size of 29/32 and a diameter of 30 mm with the length of 300 mm was used. Silica gel, with mesh size 0.060-0.200 mm, 60A was used as packing material with Hexane (Hex) as the packing solvent. Upon packing, the column was left overnight. (Column Chromatography Procedures). Separately, a range of Hex:EtOAc solvents with 5 different ratio followed by Chloroform ($CHCl_3$):MeOH:H_2O (65:25:4) were prepared to be used as mobile phase. The eluent volume was 300ml. Each solvent ratio is shown in Table 3.4. A total 2.5703 g of crude EtOAc extract was loaded into the column and fractionated using the pre-prepared eluent based on an increasing polarity sequence. Upon completion, each fraction was concentrated to obtain crude fraction. The fractions would be used for subsequent analysis.

Table 3.4 Mobile phase solvent ratio for column chromatography of crude extract.

Fraction	Solvent ratio (Hex:EtOAc)
1	9:1
2	8:2
3	7:3
4	6:4
5	1:1
6	65:25:4

3.3.2 Thin layer chromatography (TLC)

TLC was performed to identify the best separation and visually observe presence of chemical constituents in respective the crude extract and fraction. Quantified amounts of crude extract and fraction is loaded onto MERCK TLC Silica gel 60 F_{254} aluminium sheets and developed using varying solvent systems as mobile phase shown in Table 3.5. Upon development, the TLC plates are viewed under Ultraviolet light (254 nm) and stained with 5% molybdophosphoric acid, targeted to derivatize reduced metabolites such as terpenoid and sterols. Figure 3.1 shows a schematic flow diagram of TLC. Data from TLC were used as reference to isolation procedure via preparative thin layer chromatography. (PTLC)

Table 3.5 Solvent systems for TLC of crude fractions from column chromatography

TLC	Solvent system	Solvent ratio
1	$CHCl_3$	1
2	Toluene (Tol)	1
3	Hex:EtOAc	3:1
4	$CHCl_3$:EtOAc	9:1
5	$CHCl_3$:EtOAc	8:2

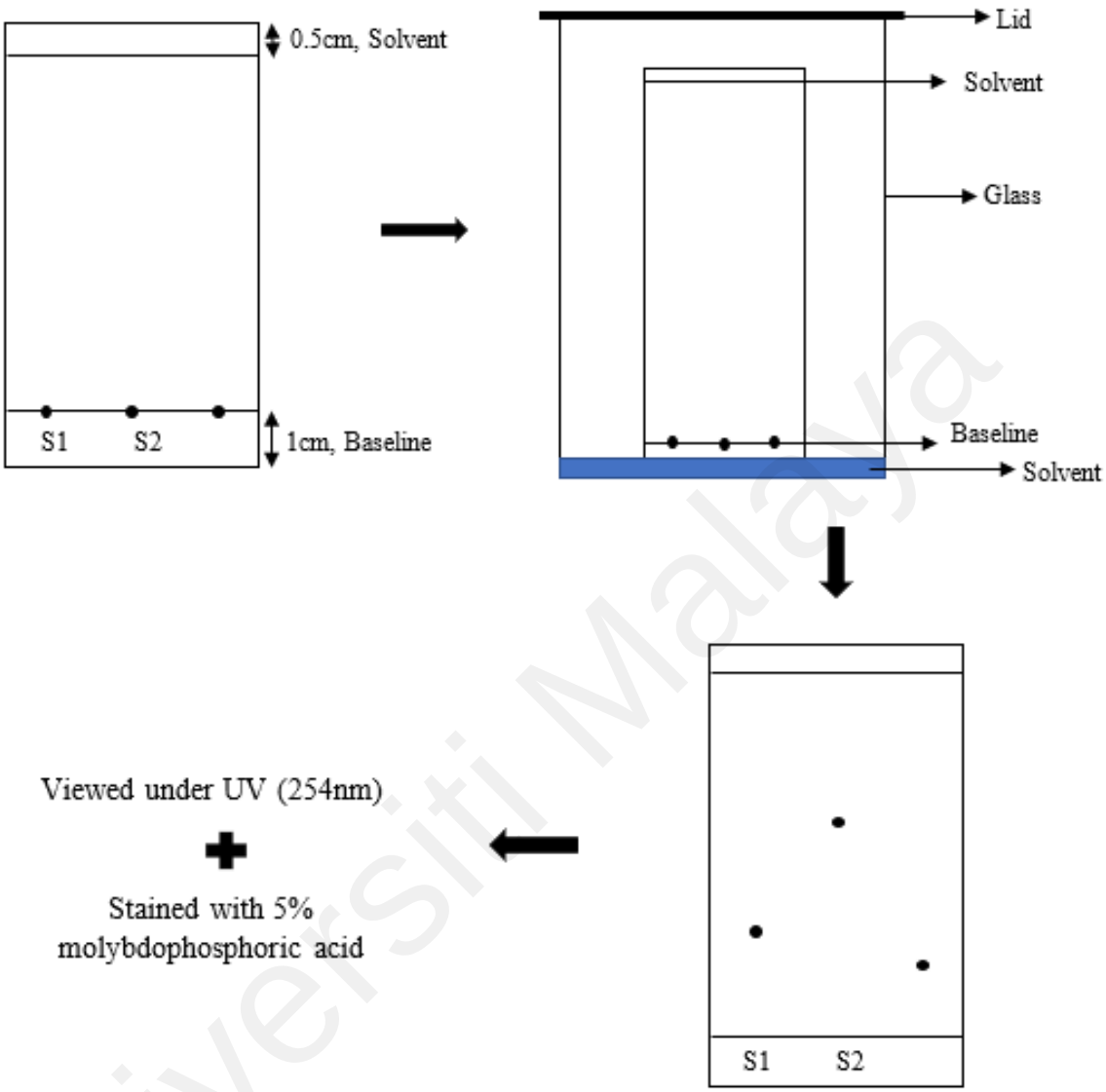


Figure 3.2 Schematic flow diagram of TLC

3.3.3 Preparative this layer chromatography (PTLC)

PTLC was performed using glass plates based on suitable solvent systems determined by TLC. Approximately 60 mg of extract was loaded onto the Merck Milipore TLC silica gel 60 F_{254} glass plate and developed using the best selected choice of solvent system. Ultraviolet (254 nm) visible and 5% molydophosphoric acid derivatized bands were selectively scraped off the plates, dissolved and eluted with EtOAc. These isolates were dried using Rotavapor and analysed using H^1 Nuclear Magnetic Resonance spectrometry (H^1 NMR). Figure 3.2 shows the schematic flow diagram of PTLC.

3.3.4 H^1 Nuclear Magnetic Resonance (H^1 NMR)

Quantified isolates were separately dissolved completely in 600 µl of chloroform-D, producing a clear solution and transferred to 3.0 mm NMR tubes for 1H-NMR experiment. All experiments were done using 600 MHz Bruker NMR spectrometer.

3.3.5 Fatty Acid Methylation

100 mg of fatty acid containing fraction 1 extract was converted to FAME through methylation by adding 2.7 ml of Hex and 0.3 ml of 2 M sodium methylate solution in a methylation vial. The vial is then subjected to constant mixing using an orbital shaker at room temperature for 3 hours. $CHCl_3$:MeOH (1:1) mixture were added into the FAMEs obtained to stop the reaction. The mixture is dried using a rotary evaporator and a yellowish oil was obtained. The methyl ester was then subjected to gas chromatography.

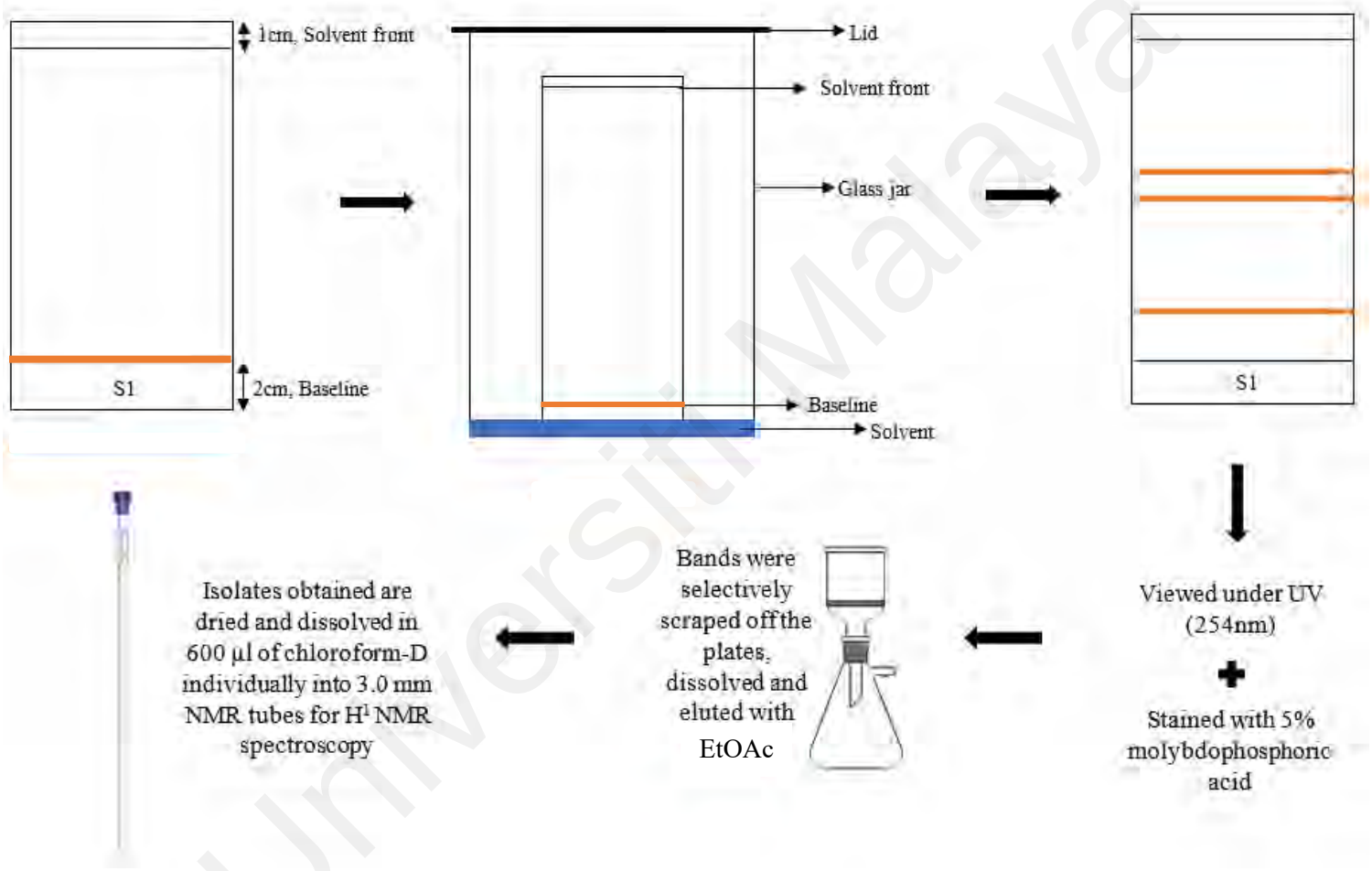


Figure 3.3 Schematic flow diagram of PTLC

3.3.6 Gas Chromatography analysis (GC)

The yellowish oil (FAMEs) obtained is dissolved in 3 ml of GC grade Hex and filtered using Minisart RC 4, 0.45µm single use syringe filter. Analysis of the FAME was performed using Shimadzu QP-2010 gas chromatograph attached to a Shimadzu GCMSQP-2010 plus detector (Shimadzu Corp., Japan) with an SGE BPX-5 (30.0 m X 0.25 µm i.d., film thickness 0.25 µm) fused silica capillary column. High purity helium was used as the carrier gas at a constant flow rate of 0.8 mL min^{-1}. 1uL of sample was injected (split ratio 1: 30) into the GCMS using an AOC5000 auto-injector. The initial temperature was set 50 °C, then heated at a rate of 3 °C min-1 to 280 °C and held isothermally for 5 min. The ion source temperature was set at 200 °C and the interface temperature at 280 °C. The mass spectrometer was set to operate in EI mode with an ionizing energy of 70 eV and an acquisition mass range from 40 a.m.u to 450 a.m.u. at 0.25 scan s-1. Identification of volatile fragments was confirmed using EI-MS and National Institute for Standard and Technology (NIST) 1998 and Flavours and Fragrance of Natural and Synthetic Compounds (FFNSC) version 1.2 computerized mass spectral libraries. Summary of the GC profile is displayed in Table 3.6.

Table 3.6 GS settings and protocol

Column Oven Temperature		
Ramp Rate, °C /min	**Final temperature, °C**	**Hold time, min**
-	50.0	5.00
4.00	280.0	5.00
0.00	0.0	0.00
0.00	0.0	0.00
MS Condition		
Ion Source Temperature, °C		200.0
Interface Temperature, °C		280.0
Solvent cut time, min		2.00

3.4 Antibacterial activity

Antibacterial test was performed using disc diffusion assay as described by Putra *et al*. (2016). 20µL of crude extract and fractions of sample of different concentration was loaded onto filter paper disc and then place on Mueller Hinton (MHA) agar spread with bacteria in a disposable petri dish. The antibacterial test was carried out using test bacteria such as *E. coli*, *S. aureus, B. subtilis* and *P. aeruginosa*. The petri dish is placed in an incubator at 30°C for 24 hours and the diameter of inhibition zone is observed and measured using Vernier caliper.

3.4.1 Bacterial strains

Four strains of bacteria were used *Escherichia coli* (ATCC25922), *Salmonella typhi*, which are Gram-negative bacteria, *Staphylococcus aureus*, and *Bacillus subtilis* are Gram-positive bacteria. The bacteria strains were obtained from Dr. Bong Chui Wei from the Institute of Biological Sciences, University Malaya. These four strains of bacteria were chosen due to their ability in causing infectious diseases in human.

3.4.2 Preparation of Crude extracts

The crude extracts with all the fractions (F1 – F6) were diluted into five different concentrations. 20µl of fractions of different concentration were loaded onto blank cartridges (Oxoid, UK) and dried. Table 3.7 showed the calculation for crude extracts. The antibiotics used for Gram-positive bacteria is Vancomycin and the antibiotics used for Gram-negative bacteria is Imipenm.

Table 3.7 Preparation of crude extracts for MHA test plates.

Formula used: $M1V1 = M2V2$			
Fraction	Starting concentration, µg/ml	Final concentration, µg/ml	Calculation
Crude	5500	500	$5500\chi = 500(3000)$ $\chi = 272.7\text{ml}$
	500	250	$500\chi = 250(3000)$ $\chi = 1500\text{ml}$
	250	125	$250\chi = 125(3000)$ $\chi = 1500\text{ml}$
	125	62.5	$125\chi = 62.5(3000)$ $\chi = 1500\text{ml}$
	62.5	31.25	$62.5\chi = 31.25(3000)$ $\chi = 1500\text{ml}$

3.4.3 Preparation of broth culture and MHA agar plates

The four bacteria were grown in a petri dish for 24 hours in a 35°C incubator. 3 well-isolated colonies that showed the same morphology of the bacteria is removed from the dish using a loop and is transferred into a tube containing 5ml of Muller Hinton broth. The broth culture is kept in 35°C incubator for 24 hours or until it achieves or exceeds the turbidity of the 0.5 McFarland standard. Sterile distilled water was used to adjust the turbidity of the broth culture to McFarland standard.

MHA plates are prepared according to the manufacturer's instructions. 1500ml of MA agar is prepared by weighing 12g of agar powder and dissolved in 1.5L of distilled water. The agar solution is autoclaved and allowed to cool to 45°C - 50°C. Using a 20ml pipette,

the warm agar solution was pipetted into 100 mm diameter plastic petri dish and left to cool or solidify in a laminar flow hood.

3.4.4 Preparation of test plates

60 plates of MHA plates were prepared and labelled as shown in the Figure 3.3. Sterile cotton swab was dipped into the bacterial culture and swab onto MHA plate.

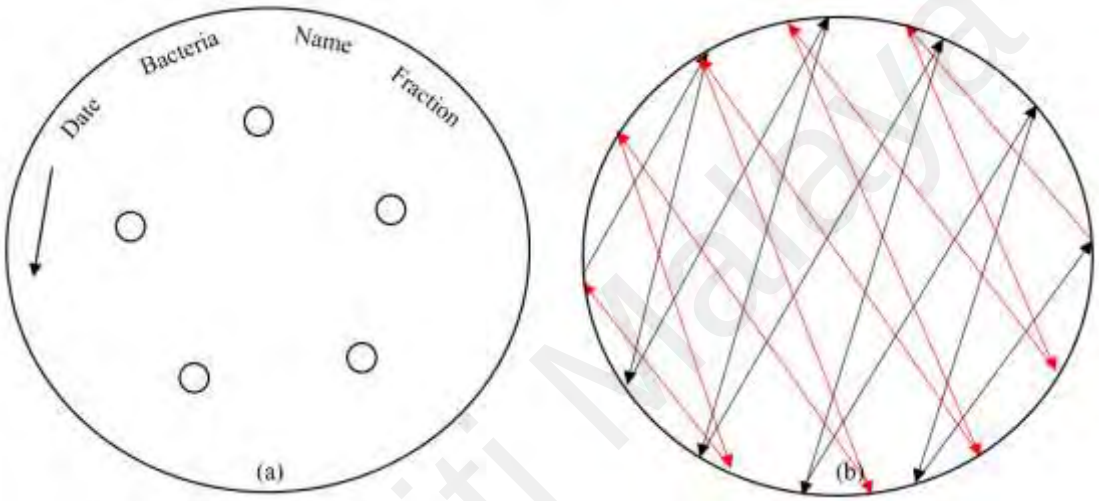


Figure 3.4 (a) MHA test plates and (b) lawn streaking diagram.

3.4.5 Identification of inhibition zone

Inhibition zone of crude extract and fractions of *Lobophytum* sp. were observed and measured using Vernier caliper at two points which are perpendicular to one another. The average diameter obtained is recorded. Figure 3.4 shows an example of inhibition zone and the measurements of its diameter.

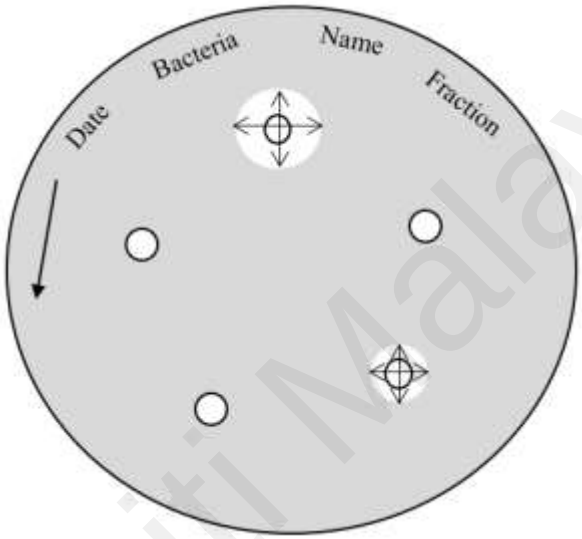


Figure 3.5 Inhibition zone appearance and the measurement of its diameter.

CHAPTER 4 : RESULTS AND DISCUSSION

4.1 Sample collection and extraction

The soft coral population collected off Tanjung Tuan, Port Dickson waters via SCUBA at the depth of 2 – 3 meters was morphologically identified as *Lobophytum* sp. The crude extract obtained after liquid-liquid partition weighed 4.7513g which is equivalent to 0.396% yield. The % yield of crude extract obtained from sample is low as isolates extracted from *Sarcophyton glaucum* yield up to 3% of the dry weight (Afifi et al., 2016). Wang et al. (2016) reported the extraction yield of soft coral *Dendronephthya* sp. from Jeju Island, Korea to be at least 8.68 ± 2.06%. However, a direct comparison with the date from other studies is not feasible as there are no studies that reported percentage yield of wet weight of sample.

4.2 Microscopic and molecular sample identification

4.2.1 Microscopic identification (Sclerite)

The spicules for soft coral fingers and base were viewed under light microscope. The spicule images are shown in Figure 4.1. The spicules observed were compared to the hand drawn illustrations in a revision of the genus *Lobophytum* Von Marenzeller (Octocorallia, Alcyonacea) by Verseveldt J., 1983. Figure 4.2 shows the example of hand-drawn spicules for *Lobophytum* sp., *Sarcophyton* sp. and *Sinularia* sp. for comparison purpose. Further identification to species level was attempted using the DNA barcoding gene *COI*.

The sclerites from the base appeared to be larger compared to those found in the stalk and the lobes with two girdles of big, spiny warts and clusters at the ends. The spicules from lobes and stalk appeared spindle-like with spiny warts. The observed spicules showed striking resemblance to the hand-drawn spicules of the genus *Lobophytum* (Verseveldt, 1983). Based

on this comparison, the sample collected, was confirmed to be from the genus *Lobophythum*. More comparison is not possible as there are no other studies which has reported the appearance of sclerites harvested from Malaysia's waters.

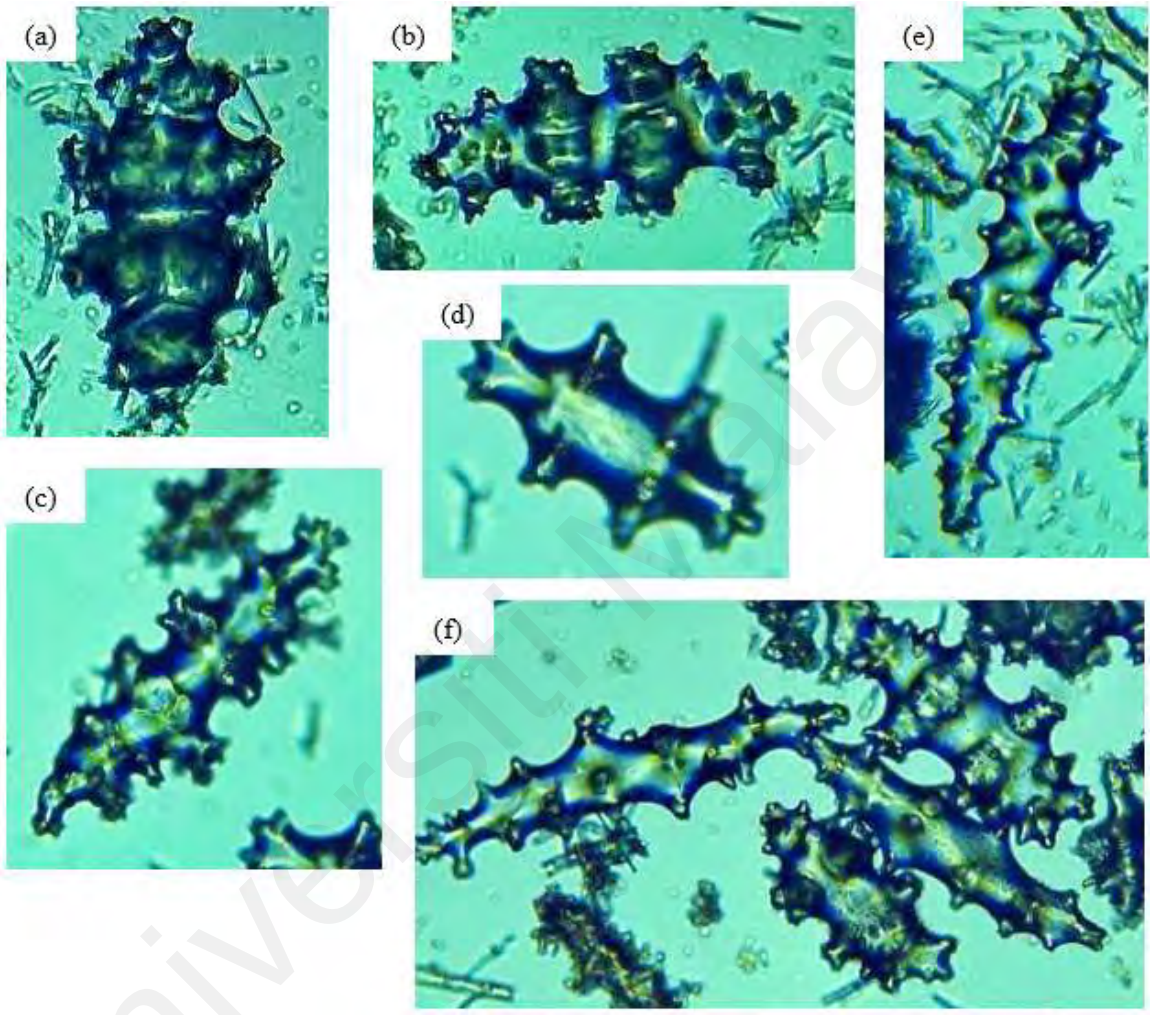


Figure 4.1 Spicules observed under light microscope at the magnification of ×100. (a) and (b) shows spicules from the interior of the base, (c) and (d) shows spicules from the interior of the lobes, (e) and (f) shows spicules from the interior of the stalk.

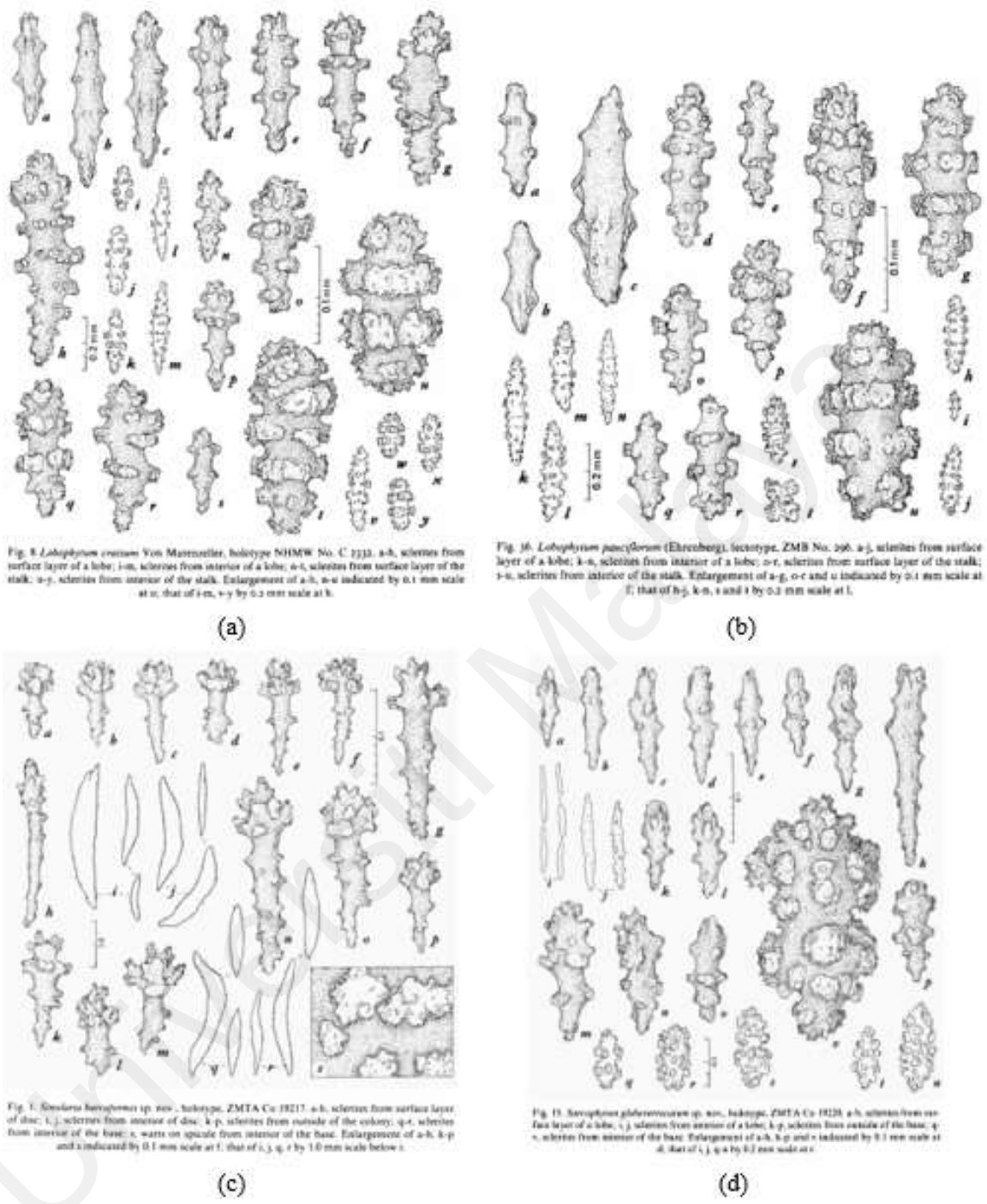


Figure 4.2 Hand-drawn spicules of soft corals (Alcyonacea) (Verseveldt, 1983); (a) *L. crissum,* (b) *L. pauciflorum*, (c) *Sinularia barcaform* and (d) *Sarcophyton globoverrucat.*

4.2.2 Molecular identification using the DNA barcoding gene *COI*

Table 4.1 showed the concentration and purity of the extracted gDNA. The 260/280 ratio represents the purity of the DNA extracted and the acceptable value is approximate 1.8 to be considered pure (Thermo Fisher Scientific, & Matlock, B. 2015). The ratio obtained is 1.41, which is lower than the acceptable value. The same goes to 260/230 ratio, the value obtained is very low compared to 2.0 – 2.2 which is the acceptable ratio for pure nucleic acid (Thermo Fisher Scientific, & Matlock, B. 2015). This this indicates the presence of contaminants from the reagents used in extraction as well as some other organic contaminants.

Table 4.1 The concentration and purity of extracted gDNA

Sample	DNA concentration (ng/µL)	260/280	260/230
SC1	83.3	1.41	0.45

AGE was performed by loading gDNA mixed with 6X loading dye (Thermo Fisher Scientific Inc, USA), and loading 5 uL of 1kb DNA ladder (Thermo Fisher Scientific Inc, USA) as the size marker. Upon completion, the gel was visualized using the gel documentation system Platinum HD2 (UVItec, GBR) and UVItec software (UVItec, GBR). The fragment size and intensity of gDNA products were compared to 1kb DNA ladder (Figure 4.3). Genomic DNA extracted from SC1 showed smearing, indicating low DNA integrity. Nevertheless, presence of DNA warranted further

The cytochrome c oxidase I (*COI*) gene amplified by polymerase chain reaction (PCR) and AGE was then conducted to assess the quality of PCR products and to confirm single sequence amplification by loading 5 µL PCR products (Figure 4.4). Based on the AGE, no band was observed indicating failed amplification of the *COI* gene which is possibly due to the low gDNA quality due to fragmentation. Another possibility could be the method used might not be the best suited one for soft corals as the positive control used in this is sea anemone sample from Padang, Indonesia (PDG-01). Therefore, we were not able to further identify the soft coral sample to species level due to limited resources and time. From this point onward, the sample will be referred to as *Lobophytum* sp.

Figure 4.3: Assessment of SC1 gDNA quality

Figure 4.4: PCR products of amplified *COI* using Geller primers (SC1 had no band)

4.3 Chemical profiling of *Lobophytum* sp. crude extract and fractions

4.3.1 Column chromatography

A total 2.5703 g of the crude extract was subjected to column chromatography in an increasing polarity solvent system as highlighted in the methodology. A total of 6 fractions were collected, concentrated and quantified. The yield of each fraction is exhibited in Table 4.2.

Table 4.2 Yield of fractions obtained from column chromatography of crude extract

Fraction	Solvent ration (Hex:EtOAc)	Weight, g
1	9:1	0.1395
2	8:2	0.2178
3	7:3	0.2469
4	6:4	0.3098
5	1:1	0.4193
6	Washing solvent	1.2214

4.3.2 Isolation via chromatography and spectroscopy techniques

Crude extract and column fractions were each subjected to TLC developed over varying solvent system as highlighted in methods section, 3.5. Figure 4.5 shows the output from each solvent system. Based on this output, we modified the solvent system to obtain a well resolved separation of spots. Well separated spots will enable ease of metabolite isolation. Eventually, the mobile phase $CHCl_3$:EtOAc with the ratio 9:1 provided the most presentable profile. From the TLC, we were able to observe the presence of multiple spots, after derivatization using 5 % molybdophosphoric acid. As shown in Figure 4.5 (e), one dark spot possibly containing 2 constituents were visible at Rf 0.85 of F1 (red circle), for F2, there are 1 dark spot and 2 spots observed at Rf value 0.61 and 0.73 respectively (yellow circles). One

dark spot can be seen clearly for F3 at Rf value 0.45 (purple circle) and for F4, the are one spot at Rf value 0.25 and 0.42 (green circles).

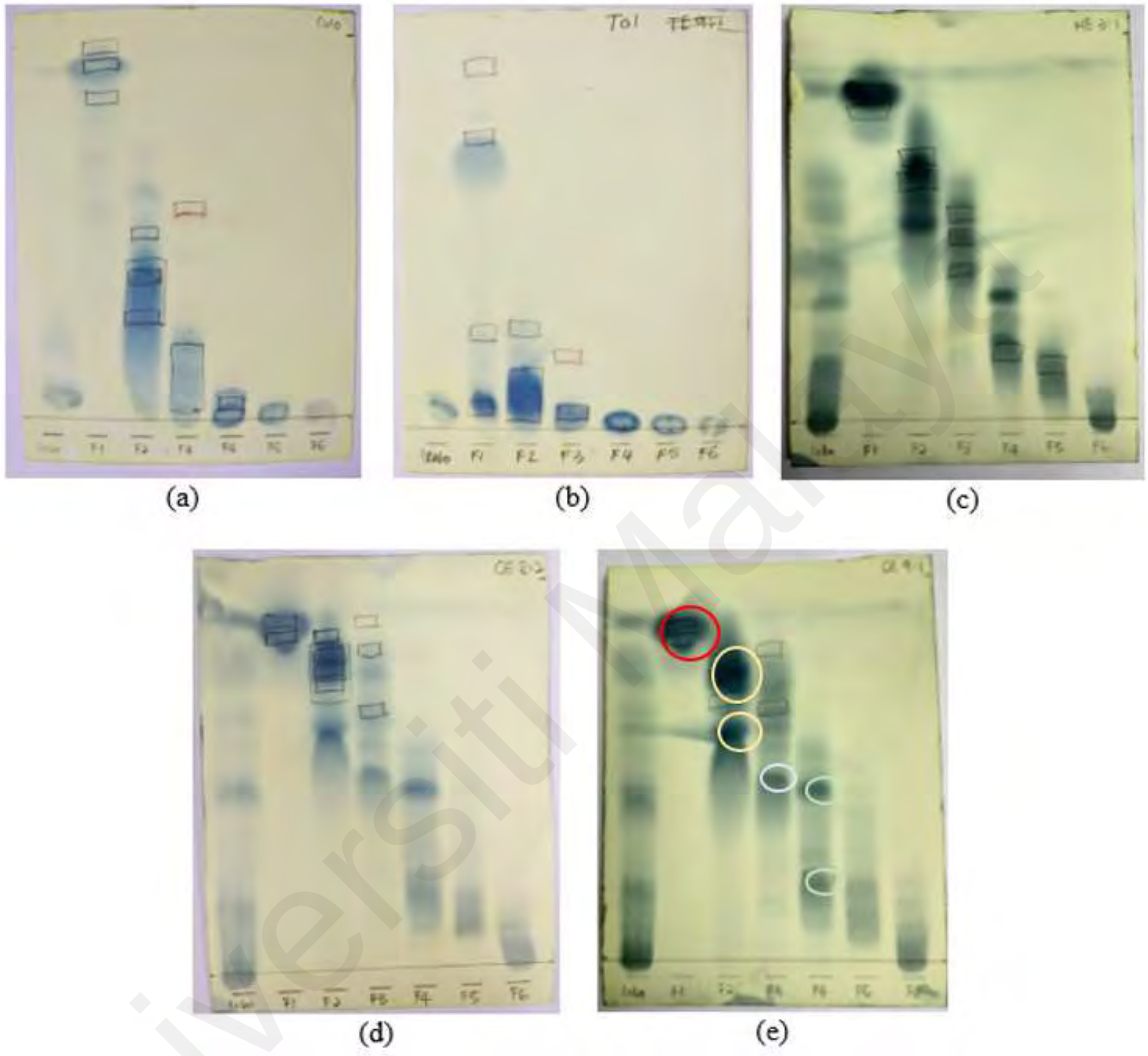


Figure 4.5 TLC plates of fractions obtained from crude extracts through column chromatography using different solvent systems; (a) 100% $CHCl_3$, (b) 100% Tol, (c) Hex:EtOAc, 3:1, (d) $CHCl_3$:EtOAc, 8:2, (e) $CHCl_3$:EtOAc, 9:1.
Spots: From left; Crude extract, Fraction 1 (F1), Fraction 2 (F2), Fraction 3 (F3), Fraction 4 (F4), Fraction 5 (F5) and Fraction 6 (F6)

Fraction 2 is subjected to further purification using PTLC with the solvent system $CHCl_3$:Tol:EtOAc of the ratio 8:1:1 and a total of seven isolates are obtained (Figure 4.6). The isolates are subjected to TLC to investigate the purity of each fractions using the solvent system Hex:EtOAc of the ratio 9:1. Fraction 2.6 showed satisfactory separation (Figure 4.7) and was further subjected to PTLC with solvent system Hex:EtOAc with ratio 8.5:1.5, a total of seven isolates obtained.

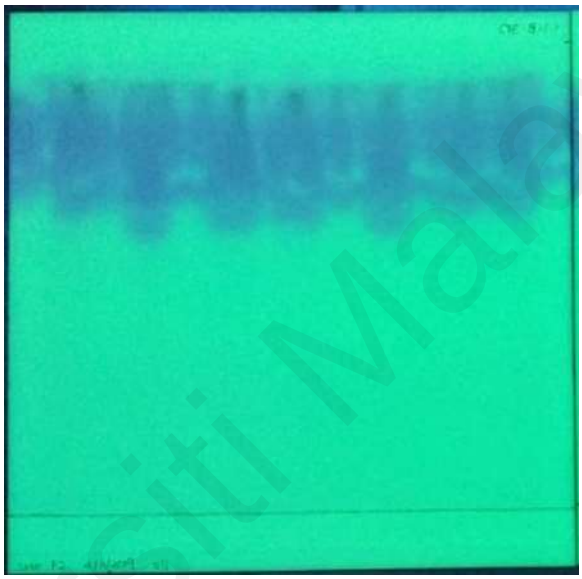


Figure 4.6 PTLC plate of crude Fraction 2 (F2) viewed under UV, 254 nm.

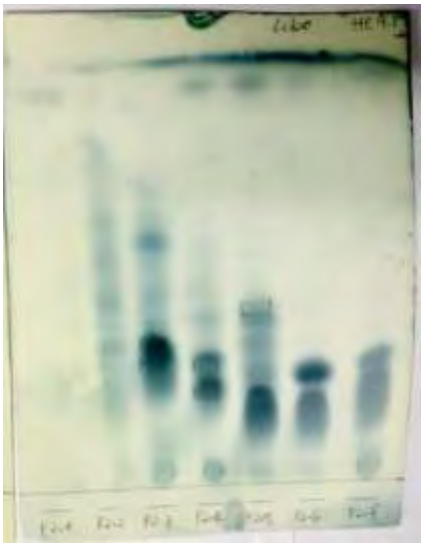


Figure 4.7 TLC plate of isolates from F2, solvent system Hex:EtOAc 9:1.
From Left; isolates F2.1, F2.2, F2.3, F2.4, F2.5, F2.6 and F2.7

As shown in Figure 4.7, F2.6 showed a single black spot at Rf value 0.25 and a tail at Rf value 0.2. F2.6 is subjected to further separation using TLC with solvent system Hex:EtOAc with the ratio 8.5:1.5. F2.6.6 and F2.6.7 displayed similar separation, hence they were combined (Fraction Combine 1) and subjected to further purification using solvent system $CHCl_3$:EtOAc with the ratio 9.5:0.5. A total of three fractions (Figure 4.8) were obtained from the combined substances and subjected to NMR. Table 4.3 summarizes the PTLC performed on F2 with the weight obtained for each isolate.

Figure 4.8 PTLC plate of Fraction Combine1 with the solvent system $CHCl_3$:EtOAc of ratio 9.5:0.5

Table 4.3 Isolates obtained from crude Fraction 2 using PTLC

Fraction	Solvent system	Weight, g
Fraction 2		
F2.1	$CHCl_3$:Tol:EtOAc 8:1:1	0.0180
F2.2		0.0042
F2.3		0.0138
F2.4		0.0190
F2.5		0.0245
F2.6		0.0172
F2.7		0.0074
Fraction 2.6		
F2.6.1	Hex:EtOAc 8.5:1.5	0.0041
F2.6.2		0.0064
F2.6.3		0.0016
F2.6.4		0.0069
F2.6.5		0.0034
F2.6.6		0.0053
F2.6.7		0.0021
Fraction 2.6.6 + Fraction 2.6.7		
Combine 1.1	$CHCl_3$:EtOAc 9.5:0.5	0.0007
Combine 1.2		0.0046
Combine 1.3		0.0014
Fraction C1.2		
Combine 1.2.1	$CHCl_3$:EtOAc 9.5:0.5	0.0010
Combine 1.2.2		0.0021
Combine 1.2.3		0.0017
Fraction C1.3		
Combine 1.3.1	$CHCl_3$:EtOAc 9.5:0.5	0.0006
Combine 1.3.2		0.0010

TLC was performed with the solvent system $CHCl_3$:EtOAc for Fraction 3 (F3), with the ratio 8:2. A satisfactory separation is observed and the same solvent system was used to perform PTLC with 60 mg of the fraction with the same solvent system, resulting in six fractions as shown in Figure 4.9 (a). Fractions obtained is further examine and purified with the solvent system $CHCl_3$:EtOAc with the ratio 9.5:0.5 as shown in Figure 4.9 (b) for TLC and Figure 4.10 (c) for PTLC. In Figure 4.9 (b), two dark spots clearly separated at Rf value of 0.48 and 0.55 hence, was subjected to PTLC with the same solvent system. Table 4.4 summarizes the PTLC performed on F3 with the weight obtained for each isolate.

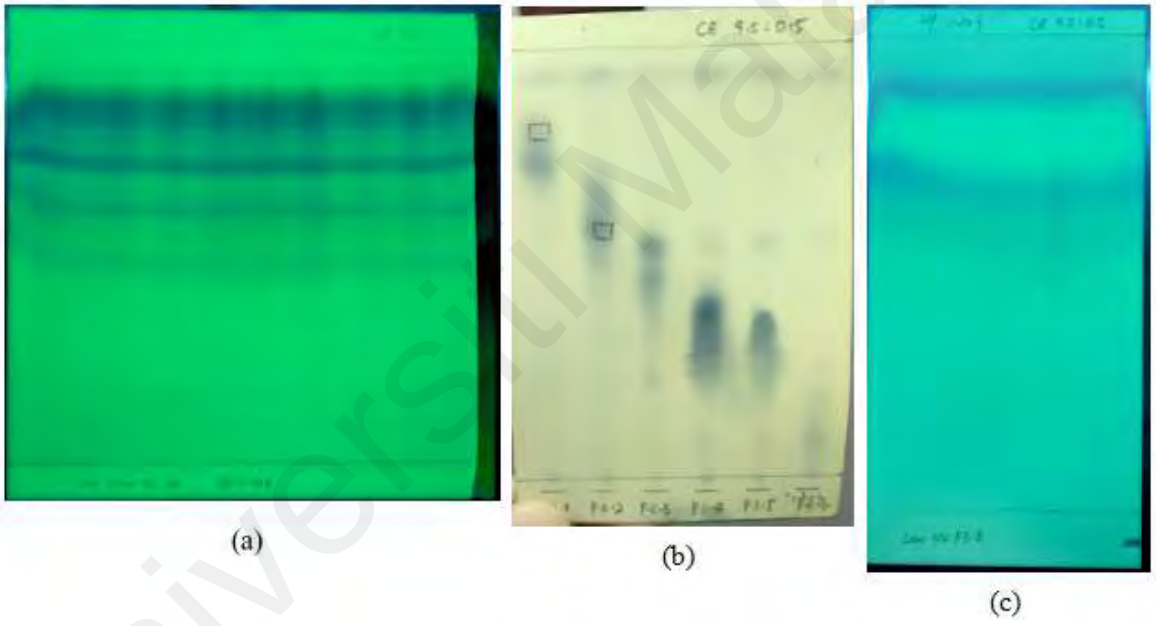


Figure 4.9 PTLC plate of crude Fraction 3 (F3) with solvent system $CHCl_3$:EtOAc of ratio 8:2 (a); TLC plate of crude F3 with solvent system $CHCl_3$:EtOAc of ratio 9.5:0.5 (b); PTLC plate of Fraction 3.3 (F3.3) with solvent system $CHCl_3$:EtOAc of ratio 9.5:0.5 (c).

Table 4.4 Isolates obtained from crude Fraction 3 using PTLC

Fraction	Solvent system	Weight, g
Fraction 3		
F3.1	$CHCl_3$:EtOAc 8:2	0.0051
F3.2		0.0044
F3.3		0.0094
F3.4		0.0031
F3.5		0.0028
F3.6		0.0017
Fraction 3.3		
F3.3.1	$CHCl_3$:EtOAc 9.5:0.5	0.0014
F3.3.2		0.0026
F3.3.3		0.0016

TLC was performed with the solvent system $CHCl_3$:EtOAc for Fraction 4 (F4) with the ratio 7:3. A satisfactory separation was observed and the same solvent system was used to performed PTLC with 60 mg of the fraction. Table 4.5 shows isolates obtained from F4 with the weight obtained for each isolate. The isolates were stored in –20°C for future studies.

Table 4.5 Isolates obtained from crude Fraction 4 using PTLC

Fraction	Solvent system	Weight, g
Fraction 4		
F4.1	$CHCl_3$:EtOAc 7:3	0.0018
F4.2		0.0023
F4.3		0.0103
F4.4		0.0302
F4.5		0.0284
F4.6		0.0124

TLC was performed with the solvent system $CHCl_3$:Hex:EtOAc for Fraction 5 (F5) with the ratio 5:1:4. A satisfactory separation is observed and the same solvent system is used to performed PTLC with 60 mg of the fraction. Table 4.6 shows isolates obtained from F5 with the weight obtained for each isolate. The isolates were stored in –20°C for future studies.

Table 4.6 Isolates obtained from crude Fraction 5 using PTLC

Fraction	Solvent system	Weight, g
Fraction 5		
F5.1	$CHCl_3$:Hex:EtOAc 5:1:4	0.0105
F5.2		0.0078
F5.3		0.0112
F5.4		0.0084
F5.5		0.0091
F5.6		0.0134

4.3.3 ^{1}H Nuclear Magnetic Resonance (NMR)

After evaluating all the fractions and isolates using chromatography, fraction 2.6.6 (F2.6.6) and Fraction 2.6.7 (F2.6.7) were selected for NMR spectroscopy based on their similar separation in TLC. Figure 4.10 shows the NMR spectrum for F2.6.6 and Figure 4.11 shows NMR spectrum for F2.6.7. Both spectra showed similar peaks appearing in identical chemical shift regions which are highlighted in boxes labelled 1, 2 and 3. Proton NMR studied the magnetic behavior of hydrogen. The peaks shown at specific chemical shift are based on tetramethylsilane (TMS, $(CH_3)_4Si$) as the internal standard with the unit parts per million, ppm, and its largely affected by the position of H-nuclei and of other electronegative atoms. Chemical shift gives an insight on the functional group present and the number of peaks showed the environments the H-nuclei are in the combination and further purification resulted in fraction Combine 1.2.2 which yielded the NMR spectrum as shown in Figure 4.12.

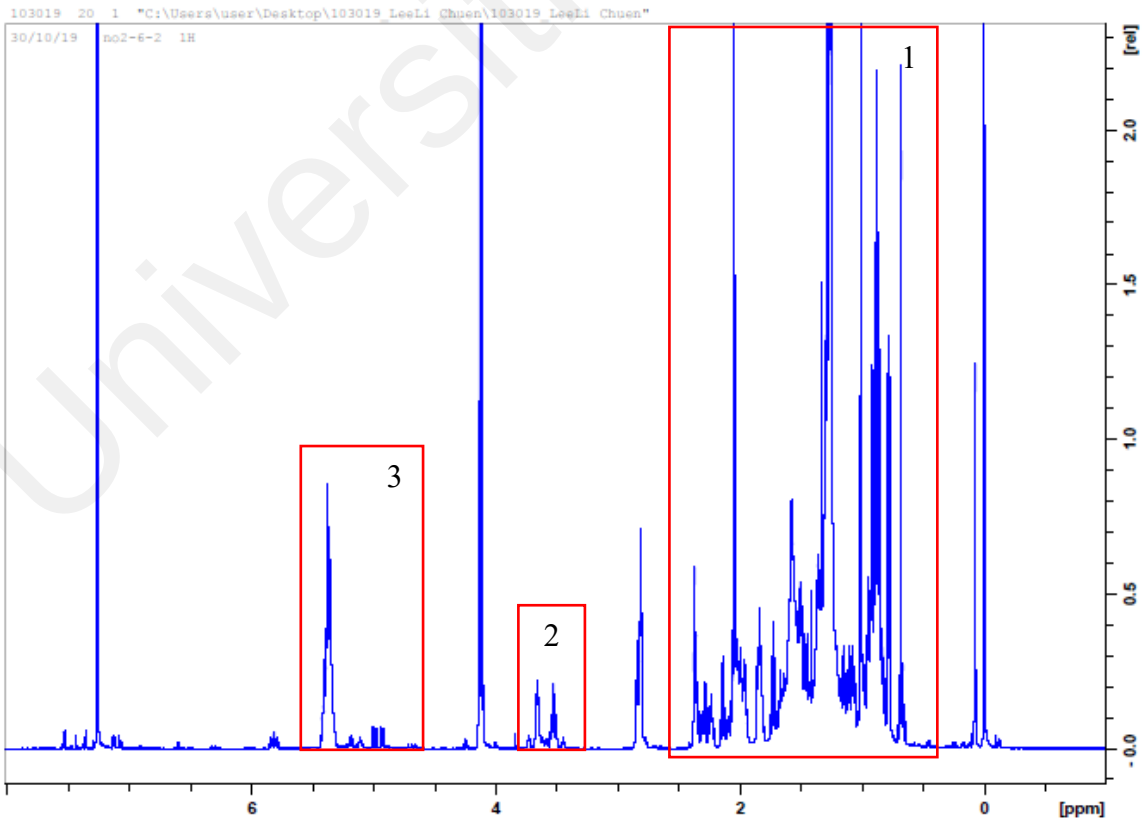


Figure 4.10 NMR spectrum of Fraction 2.6.6

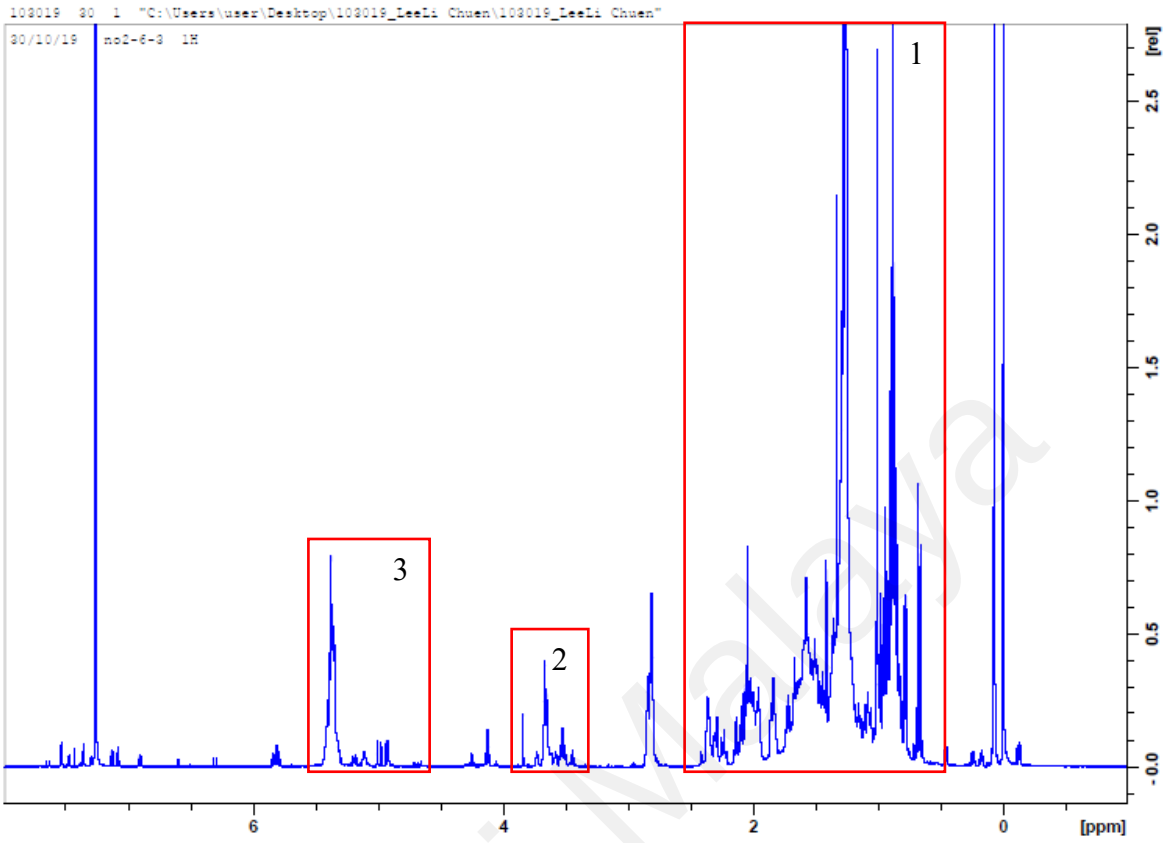


Figure 4.11 NMR spectrum of Fraction 2.6.7

Table 4.7 shows the functional group present at few chemical shifts based on the NMR spectra obtained for F2.6.6 and F2.6.7. Both the fractions showed prominent peaks at the same chemical shifts; hence, they are combined as one to be analysed together. The combined fraction was purified further and sent for NMR to obtain a cleaner spectrum as shown in Figure 4.13.

Table 4.7 Functional group represented by chemical shift.

Chemical shift	Functional group
0.5 – 2.0	-CH>-CH_2>-CH_3
2.0 – 3.0	H-C-N
3.0 – 4.0	H-C-O
4.5 – 5.5	H-C=C H-C(-O)(-O)

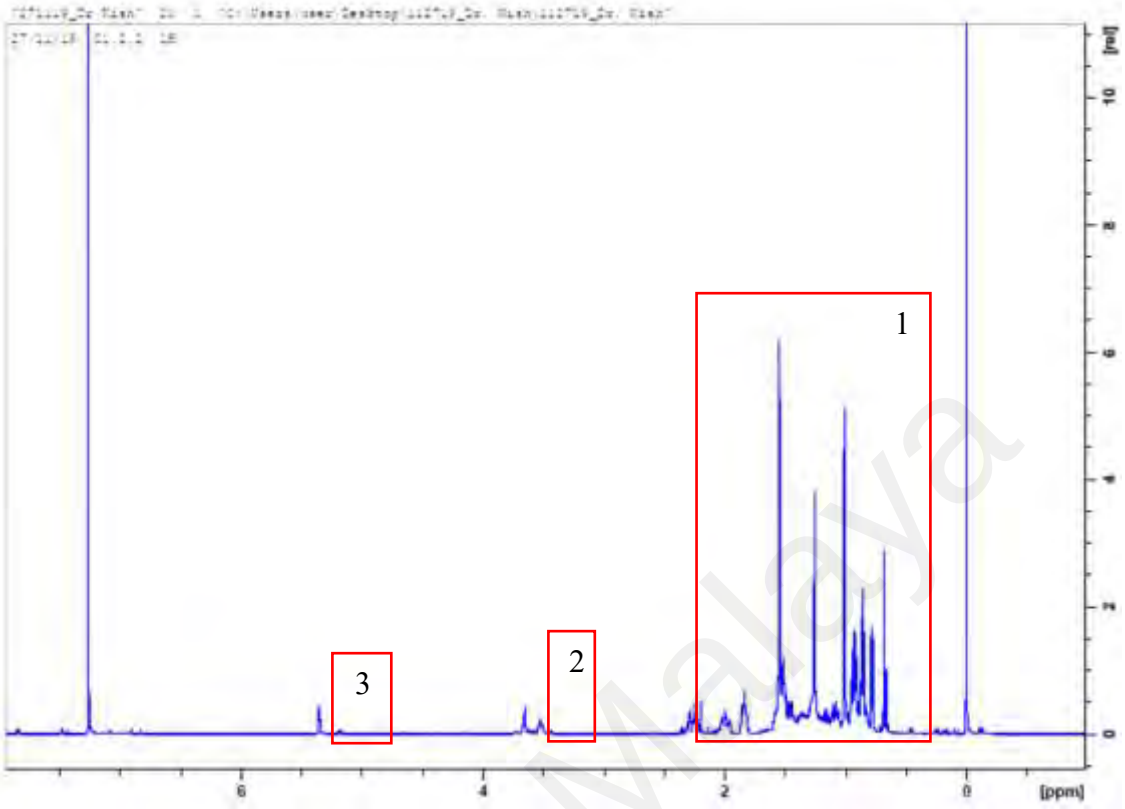


Figure 4.12 NMR spectrum of Fraction Combine 1.2.2

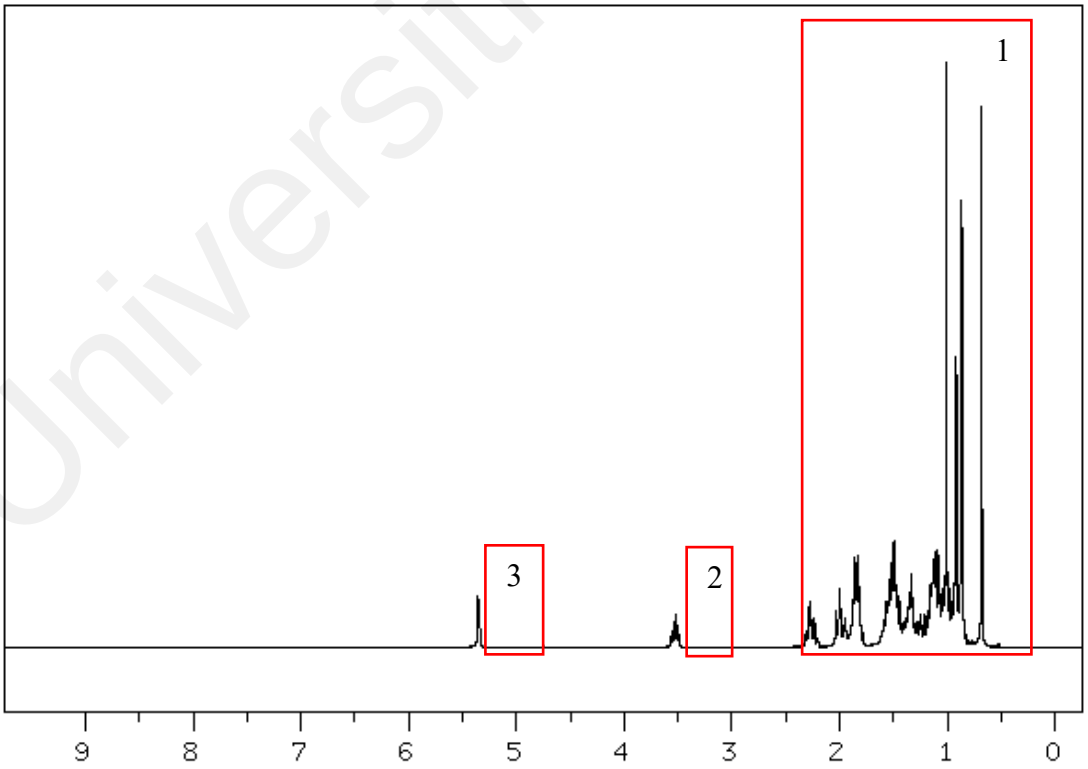


Figure 4.13 NMR spectrum of cholesterol (Chemical Book)

NMR spectrum of fraction Combine 1.2.2 showed high resemblance to the NMR spectrum of cholesterol (Figure 4.13). Prominent peaks in the boxes labelled 1, 2 and 3 shows the similarity between Fraction combine 1.2.2 and cholesterol (Figure 4.14).

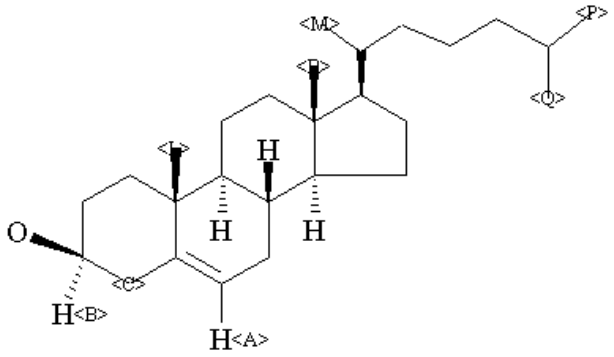


Figure 4.14 Cholesterol

Table 4.8 NMR shift of cholesterol

Box	Shift, ppm	Assign.
1	0.677	R
	0.862	Q
	0.867	P
	0.915	M
	1.006	L
	2.28	C
2	3.524	B
3	5.349	A

Apart from cholesterol (C.1.1.2), the NMR spectrum of F2.6.6 and F2.6.7 in Figures 4.10, and 4.11 resembles patterns of fatty acids signals in between 0.5 – 2.5 ppm. Compared to Figure 4.15, the ^{1}H NMR spectrum of some fatty acids with similar peaks detected in between 0.5 – 2.5 ppm as well. Therefore, we decided to document the diversity of fatty acid species in the investigated sample via GCMS profiling of methylated fatty acids.

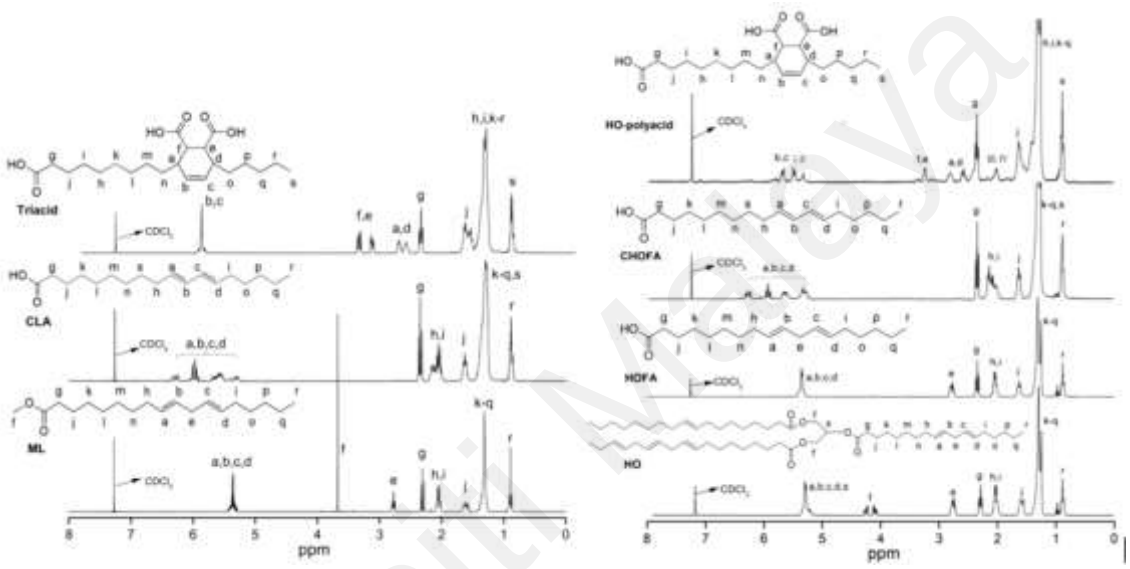


Figure 4.15 ^{1}H NMR spectra of fatty acids, (Li et al., 2018)

4.3.4 Fatty Acid Methyl Ester analysis (FAME)

As discussed above, fatty acids signals are shown on NMR spectrums of Fraction 2, hence FAME analysis were performed. 100mg of fatty acid containing fraction 1 were used and 2.7 ml of Hex and 0.3 ml of 2 M sodium methylate solution was added into the fraction to convert the fatty acids into fatty acid methyl ester (FAME) for analysis using GCMS. The composition of fatty acid methyl esters obtained from sample shows similarity to the composition of fatty acid methyl esters reported in *Lobophytum pauciflorum* (Hassan et al., 2016), as shown in Figure 4.16. Lipid composition can be an approach to distinguish organisms at the family level. The genus *Lobophytum* can be distinguished from other genera such as *Sinularia* by studying the polyunsaturated methyl esters (Imbs and Dautova, 2008).

Lipids and fatty acids make up 40% of the dry mass of soft corals (Imbs et al., 2007, 2010, 2016), the lipid composition of soft corals changes and reflect changes in the ecology, nutrition, and health of these animals including their diet (Imbs et al., 2007). The major constituents of methyl esters found in Fraction 1 are saturated palmitic acid, methyl ester, with the retention time (R.Time) of 40.446 and a concentration of 29.905% and the unsaturated arachidonic acid with the R.Time 48.210 and a concentration of 18.891%. Similarly, Hassan et al. (2016) and Imbs (2013) reported palmitic acid, methyl ester as the major constituent in soft coral *Lobophytum pauciflorum* harvested from the Red Sea along with 5,8,11,14-eicosatetraenoic acid, methyl ester, (all-Z)-, and in lower concentration, palmitic acid and stearate acid. Based on the GSMC spectrum, the fatty acids and fatty acids methyl ester can be categorized into saturated fatty acid (SFA), monounsaturated fatty acid (MUFA) and polyunsaturated fatty acid (PUFA). Hassan et al (2016), reported that stearic acid isolated from *Lobophytum pauciflorum* showed antibacterial properties. This suggested the possibilities where the fatty acid composition obtained from sample might have bioactivities such as antibacterial.

The lipid composition of the sample comprises of 47.7% of SFA, 8% of MUFA and 43.9% of PUFA. Currently, there are no available data on the fatty acid composition of soft corals from Malaysian waters as a reference for identification. The data obtained in this study can be used as a reference for future investigation of fatty acid as tool to complement identification of soft corals. Table 4.9 summarizes the GCMS analysis of fatty acid methyl esters for crude fraction 1, categorized into their respective types of fatty acids and their structure.

Table 1: Results of GC/MS analysis of the fatty acid methyl esters of *Lobophytum pauciflorum*.

Peak No.	Identified compound	*RT	**RRT	% Area
1	Methyl tetradecanoate	18.58	0.78	3.74
2	Tetradecanoic acid, ethyl ester	19.69	0.83	1.23
3	1,1,3-Trimethylurea	20.87	0.88	9.26
4	1,5,9-Cyclotetradecatriene, 1,5,9-trimethyl-12-(1-methylethenyl)	22.69	0.96	3.72
5	Hexadecanoic acid, methyl ester (Methyl palmitate, compound 4)	23.71	1	25.01
6	Methyl hexadec-9-enoate	24.52	1.03	2.41
7	Hexadecanoic acid, ethyl ester (Ethyl palmitate, compound 4)	24.68	1.04	9.39
8	7,10-Hexadecadienoic acid, methyl ester	25.51	1.08	5.38
9	Hexadecanoic acid, 15-methyl-, methyl ester	26.08	1.09	1.87
10	Z,Z-10,12-Hexadecadien-1-ol acetat	26.37	1.11	3.66
11	Octadecanoic acid, methyl ester (Methyl stearate, compound 5)	28.41	1.19	6.84
12	Urea	29.03	1.22	8.57
13	Octadecanoic acid, ethyl ester (Methyl stearate, compound 5)	29.28	1.23	3.02
14	8,11-Octadecadienoic acid, methyl ester	30.13	1.27	1.02
15	5,8,11,14-Eicosatetraenoic acid, methyl ester	35.81	1.51	3.35
16	Cyclohexane, 1-ethenyl-1-methyl-2, 4-bis (1-methylethenyl)-, [1*S*-(1α, 2β,4β)]	36.22	1.53	11.53
	Total identified compounds			100

*RT= Retention Time
**RRT= Relative Retention Time to Hexadecanoic acid, methyl ester

Figure 4.16 GCMS analysis from *Lobophytum pauciflorum* (Hassan et al., 2016)

Table 4.9 GC/MS analysis of fatty acid methyl ester of sample

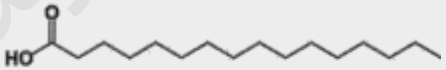
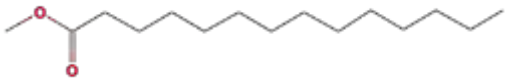
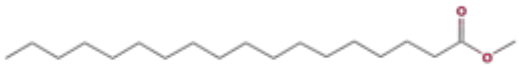
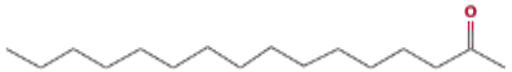
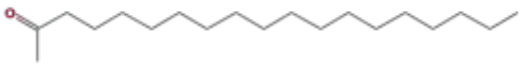
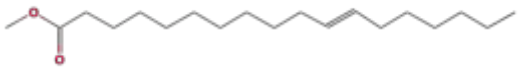
Name	SP	R.Time, min	m/z	Conc	Percentage, %	Structure
Saturated Fatty Acid (SFA) & Methyl Esters						
Palmitic acid	C16:0	40.446	74.05	29.905	29.905	
Methyl tetradecanoate	C14:0	35.067	74.05	5.058	5.058	
Methyl stearate	C19:0	45.291	74.05	4.896	4.896	
2-Hexadecanone	C16:0	37.125	58.1	2.528	2.528	
Tetradecane	C14:0	25.075	57.10	2.108	2.108	
2-Nonadecanone	C19:0	42.343	58.10	1.691	1.691	
Dodecane	C12:0	17.925	57.15	1.481	1.481	
Monounsaturated Fatty Acid (MUFA) &Methyl Esters						
11-Octadecenoic acid, methyl ester	C19:1	44.678	55.1	2.38	2.38	

Table 4.9, continued.

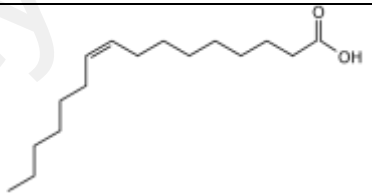
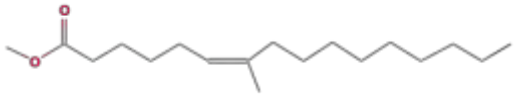
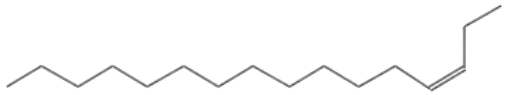
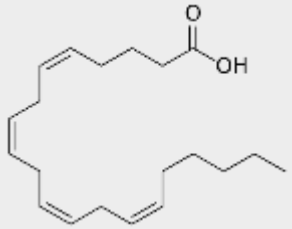
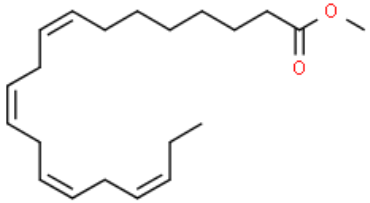
Name	SP	R.Time, min	m/z	Conc	Percentage, %	Structure
Palmitoleic acid	C16:1	39.87	55.1	2.044	2.044	
6-Hexadecenoic acid, 7-methyl, methyl ester (Z)	C18:1	41.795	55.1	1.875	1.875	
3-Hexadecene, (Z)-	C16:1	24.816	55.15	0.911	0.911	
9-Eicosene, (E)-	C20:1	31.17	55.1	0.78	0.78	
Polyunsaturated Fatty Acid (PUFA) & Methyl Esters						
Arachidonic acid	C20:4(n6)	48.349	79.1	18.891	18.891	
Methyl 8,11,14,17-eicosatetraenoate	C20:3	44.338	79.1	8.146	8.146	

Table 4.9, continued.

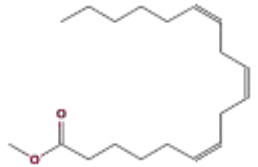
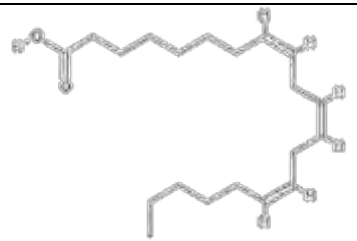
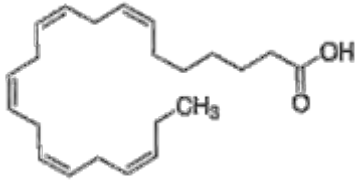
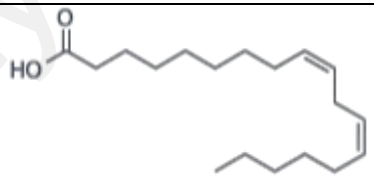
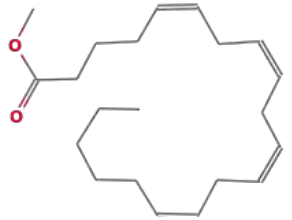
Name	SP	R.Time, min	m/z	Conc	Percentage, 100%	Structure
EPA	C20:5(n3)	48.47	79.1	5.403	5.403	
γ-Linolenic acid, methyl ester	C19:3	44.167	67.1	4.596	4.596	
DHA	C22:6(n3)	52.404	79.1	1.566	1.566	
Dihomo-γ-linoleic acid	C20:3(n6)	48.687	67.1	1.493	1.493	
cis-7,10,13,16-Docosatetraenoic acid, methyl ester	C23:4	56.154	79.1	1.485	1.485	

Table 4.9, continued.

Name	SP	R.Time, min	m/z	Conc	Percentage, 100%	Structure
Linoleic acid	C18:2	44.533	67.1	1.341	1.341	
Methyl(Z)-5,11,14,17-eicosatetraenoate	C21:4	39.572	79.1	0.955	0.955	

4.4 Antibacterial properties

Crude extracts and all six fractions of *Lobophytum* sp. does not exhibit any antibacterial properties towards Gram-negative bacteria, *E. coli* and *S. typhi* as well as Gram-positive bacteria, *S. aureus*. Weak antibacterial activity towards Gram-positive *B. subtilis* is observed in all fractions except F5.

Table 4.10 Inhibition zone diameter measured for all fractions at different concentration against *B. subtilis*.

Bacillus subtilis									
Conc, µg/ml	Inhibition zone, mm								
	Crude	F1	F2	F3	F4	F5	F6	Control	
								+ve	-ve
500.00	0	0	0	0	0	0	0	20 ±0	0
250.00	0	3.5 ±3.5	5.5 ±5.5	0	9 ±4	0	10 ±4		
125.00	0	3.5 ±3.5	0	0	0	0	2.5 ±2.5		
62.50	0	0	7 ±1	0	0	0	0		
31.25	0	0	0	6.5 ±6.5	2.5 ±2.5	0	2.5 ±2.5		

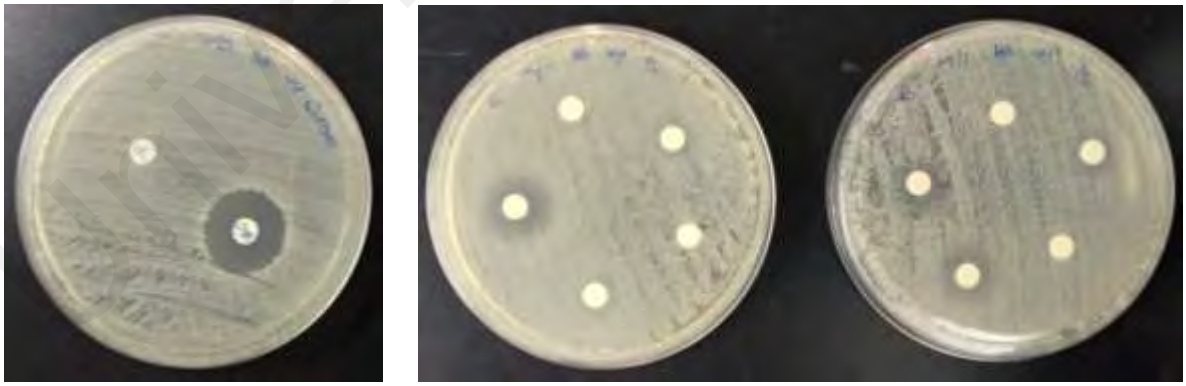


Figure 4.17 Antibacterial MHA plates for *B. subtilis*; control (left), F6 (right)

MHA test plates were observed for inhibition zone after incubation at 35°C for 24 hours. All observable inhibition zone has a diameter less than 15mm, concluded that the extracts from *Lobophytum* sp. does not have a strong antibacterial activity against *B. subtilis*. As for the other three bacterial strains no antibacterial activities can be concluded as no inhibition zone can be observed. A diameter of 15mm and above is considered susceptible (Hudzicki, 2012). As mentioned in section 2.3, secondary metabolites isolated from *Lobophytum* sp. showed antibacterial properties. The secondary metabolites reported includes cembranoids and fatty acids. Due to the lack of hard exoskeleton, soft corals such as *Lobophytum* sp. secretes secondary metabolites to protect themselves from any potential risk and this includes infections from bacteria.

In a study done on soft corals found in the Northern Straits of Malacca showed inhibition in the disc diffusion tests against *S. aureus* and MRSA (Marican et al., 2016). Another study on *Lobophytum microlobulatum* reported that hexane extract of *L. microbulatum* exhibits strong antibacterial properties against *B. cereus* and *S. aureus* (Chan et al., 2018). The data collected serves as a preliminary data for future studies on antibacterial properties of *Lobophytum* sp. found in the Straits of Malacca.

4.5 Limitations to study and recommendations

Due to limited time and financial resources, this results in insufficient sample size which makes it difficult for statistical measurements. While there are many studies done on soft corals and their secondary metabolites, there is limited research done on *Lobophytum* sp. found in Southeast Asia. To date, one study on soft corals harvested from the north of The Straits of Malacca is reported by Marican et.al. (2016) and one study on *Lobophytum* sp. reported by Chan et al. (2018). This limits the data available for comparison in terms of percentage yield of extracts, the chemical profiling of the extracts as well as molecular identification of the sample.

In future studies, purification of the fractions from crude extracts can be done using high performance liquid chromatography (HPLC) for a better separation and this opens up the possibilities in isolating pure compounds with biological activities. The study on the structure of the isolated compounds can also be done using mass spectroscopy to give a clearer understanding.

CHAPTER 5 : CONCLUSION

This investigation aimed to quantify the crude extracts of *Lobophytum* sp., to perform chemical profiling and screen antibacterial properties of these extracts. 0.396% of the crude extracts was obtained from 2kg wet weight of the samples harvested. Microscopic identification of the sample allowed us to conclude that the soft coral sample was *Lobophytum* sp., however, the species of the sample were unable to be identified using the DNA barcoding gene cytochrome c oxidase I (*COI*). No clear bands can be seen and this indicates that no comparisons can be made to proceed with further molecular work. A total of six fractions were eluted from column chromatography. These fractions were subjected to thin layer chromatography and preparative thin layer chromatography, cholesterol was isolated from F2 and were confirmed by NMR.

A lipid profile of F1 was obtained from FAME procedure. It is found that 40% of the dry mass of the sample is lipid. The major constituents of methyl esters found in F1 are saturated palmitic acid, methyl ester and a concentration of 29.905% and unsaturated arachidonic acid with a concentration of 18.891% which can be used as a reference for the species investigated. The results indicate that the samples collected are of *Lobophytum* sp. and there might be more interesting secondary metabolites which can be isolated from the extracts as compounds like cembrane-type terpenoids. These successfully isolated terpenoids exhibits potent biological activities; anti-inflammatory, anti-proliferation, cytotoxic and antibacterial. Soft coral *Lobophytum* sp. have so much potential and be part of the marine-based natural products.

The extracts obtained also showed weak antibacterial properties against *B.* subtilis. This opens up the opportunity in discovering new antibacterial compounds from local soft corals.

Further research is needed to purify and isolates any potential secondary metabolites from the extracts. In conclusion, the crude extract obtained from sample were quantified. A compound, cholesterol, were isolated after purification of the extract. FAME analysis produced the methyl esters list present in the sample, which can be used as a reference composition for this species. Antibacterial screening showed weak inhibition activity against Gram-positive *B. subtilis*. The objectives of this study have been achieved.

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