UNIVERSITI PUTRA MALAYSIA

BIOLOGICAL ACTIVITY AND METABOLITE PROFILING OF MARINE SPONGES AND CHARACTERIZATION OF BIOACTIVE COMPOUNDS ISOLATED FROM *Haliclona* sp.

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By

LEE KAH NYAN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

July 2019

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DEDICATION

This thesis is dedicated to my beloved family and friends
for their love, endless support and encouragement

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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July 2019

Chairman : Associate Professor Faridah Abas, PhD
Institute : Bioscience

Marine natural products, especially sponges, are the primary producers of secondary metabolites that of interest for investigation for clinical applications globally. However, there are limited studies being performed on sponges from Malaysia waters. Hence, the current study aimed to screen and isolate bioactive metabolites from marine sponges (*Haliclona*, *Xestospongia*, *Aaptos*, *Axinella*, *Axinyssa*, *Mycale*, *Plakortis*, and *Penares*) of Malaysia waters. Preliminary studies focused on several biological activities [α-glucosidase, cytotoxicity, antioxidant potential, nitric oxide (NO) scavenging activity, and antimicrobial] from marine sponge samples collected from Langkawi Island and Port Dickson. The most active fraction were chosen for nuclear magnetic resonance (NMR) and liquid chromatography mass spectrometry (LCMS) profiling before proceeding with various chromatographic methods for isolation. The bioassay screening results showed that marine sponges from Langkawi Island displayed better activities with DPPH inhibition ranges from 0 to 81.89%, NO scavenging ranges from 0 to 83.82%, and IC_{50} values for cytotoxicity (against MCF-7) ranging from 20.41 to 40.19 μg/mL except for α-glucosidase inhibition testing which had inhibition ranges from 0 to 0.83%. However, the Port Dickson samples showed the highest inhibition among all the tested biological activities except for the NO scavenging. However, the other samples from Port Dickson showed inhibition ranges approximately 20 to 30% for DPPH and 30 to 40% for NO scavenging testing. *Haliclona* sp. (L2) from Langkawi Island was chosen for further fractionation and isolation due to its overall performance in the screening. Further bioassay testing on the L2 fractions found that the non-polar fractions [hexane (14.05% inhibition in α-glucosidase) and dichloromethane (DCM) (45.24% inhibition of DPPH and 94.66% inhibition of NO scavenging)] were the most active fractions. NMR profiling tentatively identified 43 metabolites including amines, amides, fatty acids and carboxylic acids from the *Haliclona* sp. crude extract and nine secondary metabolites from the hexane and DCM active fractions; the main fatty acids constituents included

linolenic acid, palmitic acid and arachidic acid. Characterization of compounds such as tryptophan, indoleacrylic acid, nor-5β-cholestane-3α,7α,12α,24,25-pentol, adenylsuccinic acid, and muricatacin using LCMS were performed with supporting data from the literatures and MS database. Subsequently, compounds isolation was performed on the nonpolar fractions using various chromatographic methods, including column chromatography and high-performance liquid chromatography (HPLC). A cholestanol derivative **(1)** was isolated from the hexane fraction while 1-(phenoxyamino)undecan-4-ol **(2)** and 1-(phenoxyamino)dodecan-3-ol **(3)** were isolated from the DCM fraction. Characterization of the isolated compounds was successfully performed based on the collection of spectroscopic (IR, NMR and MS) data with comparisons to the literatures. In conclusion, the current study successfully assessed the bioactivity of 16 species from marine sponges collected from Malaysian waters and characterized isolated compounds from the nonpolar active fractions of *Haliclona* sp.. These findings are also useful and may serve as an important basis for future drug discovery and development from marine organisms.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

AKTIVITI BIOLOGI DAN PROFIL METABOLIT DARIPADA SPAN MARIN DAN PENCIRIAN SEBATIAN BIOAKTIF YANG TERPENCIL DARIPADA *Haliclona* sp.

Oleh

LEE KAH NYAN

Julai 2019

Pengerusi : Profesor Madya Faridah Abas, PhD
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Hasil semulajadi marin, terutamanya span, adalah pengeluar utama kepada metabolit sekunder yang mempunyai kepentingan untuk kajian aplikasi klinikal di seluruh dunia. Walau bagaimanapun, kajian penyelidikan terhadap span marin daripada perairan Malaysia adalah terhad. Oleh itu, kajian ini bertujuan untuk menyaring dan mengasingkan metabolit bioaktif daripada span marin (*Haliclona*, *Xestospongia*, *Aaptos*, *Axinella*, *Axinyssa*, *Mycale*, *Plakortis*, dan *Penares*) berasal dari perairan Malaysia. Kajian awal tertumpu kepada beberapa aktiviti biologi (α-glukosidase, ujian sitotoksik, potensi antioksidan, aktiviti pemerangkapan nitrik oksida (NO), and ujian antimikrob) daripada koleksi span marin yang diambil daripada Pulau Langkawi dan Port Dickson. Pecahan yang paling aktif diprofil menggunakan resonans magnetik nuklear (NMR) dan spektrometri jisim kromatografi cecair (LCMS) sebelum proses pengasingan dengan menggunakan pelbagai kaedah kromatografi. Ujian aktiviti biologi menunjukkan bahawa span marin berasal dari Pulau Langkawi menunjukkan keputusan yang lebih baik dalam pemerangkapan radikal bebas oleh DPPH yang memberikan julat perencatan antara 0 hingga 81.89%, pemerangkapan nitrik oksida (NO) dengan julat perencatan antara 0 hingga 83.82%, dan menunjukkan nilai IC_{50} yang rendah untuk ujian sitotoksik (terhadap MCF-7) yang berjulat dari 20.41 hingga 40.19 μg/mL kecuali untuk aktiviti perencatan α-glukosidase yang berjulat antara 0 hingga 0.83%. Akan tetapi, sampel dari Port Dickson yang menunjukkan nilai perencatan tertinggi dalam kalangan semua aktiviti biologi yang dijalankan kecuali untuk ujian pemerangkapan nitrik oksida (NO) di mana sampel yang lain dari Port Dickson menunjukkan nilai perencatan yang berjulat dari 20 hingga 30% untuk pemerangkapan DPPH dan 30 hingga 40% untuk pemerangkapan nitrik oksida (NO). Sampel *Haliclona* sp. (L2) yang berasal dari Pulau Langkawi dipilih untuk menjalani pecahan dan pengasingan lanjut disebabkan prestasi keseluruhannya dalam ujian aktiviti biologi yang dilakukan. Pecahan daripada pelarut tidak polar daripada L2 yang terdiri daripada heksana (nilai perencatan sebanyak 14.05% dalam ujian perencatan α-

glukosidase) dan diklorometana (DCM) (nilai perencatan sebanyak 45.24% dalam ujian pemerangkapan radikal bebas oleh DPPH dan nilai perencatan sebanyak 94.66% dalam ujian pemerangkapan nitrik oksida (NO)) merupakan pecahan yang paling aktif selepas ujian aktiviti biologi yang selanjutnya dilakukan. Pengenalpastian menerusi kajian resonans magnetik nuklear (NMR) berjaya mencirikan 43 metabolit daripada ekstrak mentah *Haliclona* sp. (L2) termasuk amina, amida, asid lemak dan asid karboksilik dan sembilan metabolit sekunder dari pecahan aktif heksana dan pecahan DCM di mana unsur-unsur asid lemak utamanya termasuk asid linolenik, asid palmitik dan asid arakidik. Pencirian sebatian seperti triptofan, asid indolakril, nor-5β-kolestan-3α,7α,12α,24,25-pentol, asid adenilokinik, dan murikatasin dilaksanakan dengan menggunakan LCMS dengan data sokongan dari penyelidikan terdahulu dan pangkalan data MS. Kemudian, pengasingan sebatian dilakukan pada pecahan daripada pelarut tidak polar dengan menggunakan pelbagai kaedah kromatografi, termasuk kromatografi kolum dan kromatografi cecair tekanan prestasi tinggi (HPLC). Terbitan kolestanol **(1)** terpencil daripada pecahan pelarut heksana dan 1-(fenoksiamino)undekan-4-ol **(2)** dan 1-(fenoksiamino)dodekan-3-ol **(3)** terpencil daripada pecahan pelarut DCM. Pencirian terhadap sebatian yang terasing telah berjaya dilakukan berdasarkan pengumpulan data-data spektroskopi (NMR dan MS) dan perbandingan dengan data daripada penyelidikan terdahulu. Kesimpulannya, kajian ini berjaya menilai bioaktiviti daripada 16 spesies span marin yang dikumpulkan dari perairan Malaysia dan mencirikan sebatian yang terasing daripada pecahan aktif iaitu pecahan pelarut tidak polar daripada *Haliclona* sp. Penemuan ini juga berguna untuk dijadikan asas penting untuk penemuan dan perkembangan ubat-ubatan daripada organisma marin pada masa depan.

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I certify that a Thesis Examination Committee has met on 15 July 2019 to conduct the final examination of Lee Kah Nyan on her thesis entitled "Biological Activity and Metabolite Profiling of Marine Sponges and Characterization of Bioactive Compounds Isolated from *Haliclona* sp." in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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1D	One-Dimensional
2D	Two-Dimensional
3-APAs	3-alkyl-pyridinium alkaloids
^{13}C-NMR	Carbon-13 Nuclear Magnetic Resonance
^{1}H-NMR	Proton Nuclear Magnetic Resonance
ACN	Acetonitrile
AchE	Acetylcholinesterase
AIDS	Acquired Immunodeficiency Syndrome
Aq	Aqueous
AsPC-1	Human Pancreatic Adenocarcinoma
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
AZT	Azidothymide
A2058	Human Melanoma Cell Line
A549	Adenocarcinoma Human Alveolar Basal Epithelial Cells
BACE-1	Beta-secretase 1 (Aspartic Protease)
BEL-7402	Hepatocellular Carcinoma Cells
BuOH	Butanol
BxPC-3	Human Pancreatic Can cer Cells
CaCO-2	Human Epithelial Colorectal Adenocarcinoma Cells
$CaCO_3$	Calcium Carbonate
CC	Column Chromatography
CD_3OD	Deuterated Methanol
CD_{50}	Median Curative Dose
CFU	Colony-Forming-Unit

$CHCl_3$	Chloroform
CNE	Human Nasopharyngeal Carcinoma
COSY	Homonuclear Correlation Spectroscopy
COX-2	Cyclooxygenase-2
CPC	Centrifugal Partitioning Chromatography
d	Doublet
DAD	Diode Array Detector
dd	Doublet of Doublet
DCM	Dichloromethane
DEPT	Distortionless Enhancement of Polarization Transfer
$CDCl_3$	Deuterated Chloroform
dH_2O	Deionized Water
DIP-MS	Direct Injection Probe Mass Spectrometer
DMNP	Dictionary of Marine Natural Products
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DPPH	2,2-Diphenyl-1-picrylhydrazyl
dt	Doublet of Triplet
EA	Ethyl Acetate
EC_{50}	Half Maximal Effective Concentration
ER (+)	Estrogen-dependent
ER (-)	Estrogen-independent
ESI	Electrospray Ionization
ESIMS/MS	Electrospray Ionization-Tandem Mass Spectrometry
EtOH	Ethanol
FA	Formic acid

FDA	Food and Drug Administration
GI_{50}	Growth Inhibition of 50% of Cells
H_2SO_4	Sulphuric Acid
HDAC	Histone Deacetylases
HCl	Hydrochloric Acid
HCT-116	Human Colon Carcinoma Cell Line
HCT-15	Human Colon Cancer Cell Line
HEK-293	Human Embryonic Kidney 293 Cell Line
HeLa	Human Cervical Cancer Cell Line
HepG2	Human Liver Hepatocellular Carcinoma Cell Line
HESI	Heated Electrospray Ionization
HIV	Human Immunodeficiency Virus
HL-60	Human Promyelocytic Leukaemia Cell
HMBC	Heteronuclear Multiple Bond Coherence
HPLC	High Performance Liquid Chromatography
HSCCC	High-Speed Counter-Current Chromatography
HSQC	Heteronuclear Single Quantum Correlation
HSV	Herpes Simplex Virus
HS 27	Human Foreskin Cell
HT-29	Human Colon Cancer Cell
Huh-7	Hepatocarcinoma Cell Line
Hz	Hertz
H460	Human Lung Cancer Cell Line
H522-T1	Human Lung Cancer Cell Line
IC_{50}	Half Maximal Inhibitory Concentration
ID_{50}	Median Infectious Dose

IDO	Indoleamine 2,3-Dioxygenase
IFO	Institute for Fermentation, Osaka, Japan
IMR-32	Human Neuroblastoma Cell Line
IMR-90	Human Fibroblast Cell
J	Coupling Constant
JB6 C141	Normal Mouse Epithelial Cell
JNK	c-Jun N-terminal kinase
J774 A1	Murine Macrophage
K-562	Human Erythroleukemia Cell Line
KB	Human Buccal Carcinoma
KB-C2	Human Buccal Carcinoma Cell Line
K_d	Distribution Coefficient
KCN	Potassium Cyanide
K^+	Potassium Ion
L929	Mouse Fibroblast Cell
LC	Liquid Chromatography
LC_{50}	Median Lethal Concentration
LC-DAD	Liquid Chromatography-Diode Array Detection-Electrospray
LC-MS	Liquid Chromatography Mass Spectrometry
LNCap	Androgen-sensitive Human Prostate Adenocarcinoma Cell
LPLC	Low-Pressure Liquid Chromatography
LS-174T	Human Colon Adenocarcinoma Cell Line
MCF-7	Human Breast Cancer Cell
MDA-MB-231	Human Breast Adenocarcinoma
MDR	Multidrug Resistance
MeOH	Methanol

MGDG	Monogalactosyl Diglyceride
MIA-PaCa-2	Pancreatic Cancer Cell
MIC	Minimum Inhibitory Concentration
MPLC	Medium-Pressure Liquid Chromatography
MS	Mass Spectrometry
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
m/z	Mass to Charge Ratio
Na^+	Sodium Ion
NCI-H23	Human Lung Cancer Cell Line
NCI-H460	Non-small Cell Lung Cancer
NMR	Nuclear Magnetic Resonance
NO	Nitric Oxide
NP	Normal Phase
NP-TLC	Normal Phase Thin Layer Chromatography
ORAC	Oxygen Radical Absorbance Activity
PANC-1	Pancreatic Cancer Cell
PC-3	Human Prostate Cancer Cell Line
PDA	Photodiode Array
PNPG	ρ-Nitrophenyl-ρ-D-glucopyranoside
ppm	Parts Per Million
PTLC	Preparative Thin Layer Chromatography
PTP1B	Target Protein of Diabetes Type II
QG56	Human Lung Carcinoma
RI	Refractive Index
RP	Reverse Phase
RP-HPLC	Reverse Phase- High Pressure Liquid Chromatography

RP-TLC	Reverse Phase Thin Layer Chromatography
RT	Reverse Transcriptase
s	Singlet
SEM	Scanning Electron Microscope
SGC-7901	Human Gastric Cancer Cell Line
SiO_2	Silica
SNP	Sodium Nitroprusside
Sub-G1	Apoptotic Cells
SKOV-3	Human Ovarian Tumor Cell Line
TIC	Total Ion Chromatogram
TLC	Thin Layer Chromatography
TMS	Tetramethly Silane
TPC	Total Phenolic Content
T47D	Human Ductal Breast Epithelial Tumor
U937	Human Myeloid Leukaemia Cell Line
UPLC-MS/MS	Ultra-Performance Liquid Chromatography-Mass Spectrometer
WEHI-164	Mouse Fibrosarcoma Cell Line
WHO	World Health Organization
WiDr	Human Colon Cancer Cell

CHAPTER 1

INTRODUCTION

1.1 Background

Mother nature serves as a source of medicine for treatments and preventions of diseases as a result of life's developing multiple solutions to recurring challenges, in response to which and to enhance their chances of survival by producing billions of diverse compounds, many of which are potentially useful to mankind (Bakuni and Rawat, 2005). Over 62% of small molecule agents approved for use as drug in the market can be traced back to its natural products origin such as aspirin from willow or birch, morphine from poppy, penicillin from fungus and adriamycin from bacterium (Newman, Cragg and Snader, 2003; Amy, 2012). However, drug discovery requires random screening of thousands of species and the procedure is not rapid. At the same time, tropical rainforests which housing 50% of earth's plants are disappearing fast, from 16% of earth's land surface in 1950 to 8% in 2002 (David, 2008). Apart from the huge inventory of flora, fauna and other forms of life, the world's biodiversity also contains all of the interactions, energy pathways, symbioses and other elements that contribute to life of all the species.

The oceans, representing 95% of the Earth's hydrosphere, are the greatest center of biodiversity that lie a treasure trove of undiscovered metabolites of novel chemical structures, elaborating diverse biological properties of potential medical interests (Jaksha, 2010). The first wave of marine-derived drug discovery in the 1950's have yielded potent cephalosporin family of antibiotics to fight a range of bacterial infections, as well as the unprecedented discovery of AZT for treating AIDS. A second wave in recent years has now yielded several clinically useful drugs and investigational drug candidates which are already in the pipeline such as dolastatin 10, ecteinascidin-743 and ziconitide. These discoveries are only the 'tip-of-the-iceberg'. A great challenge still awaits and, in this respect, the potential of the tropical Indo-Pacific oceans to yield useful natural products still remains to be sustainably tapped. With threats of drastic anthropological changes, natural disasters and pollution resulting from human activities, there is now a growing concern that we are losing many of the oceans' untold resources before we can even fully understand them. Therefore, it is imperative that natural products from these valuable bioresources be assessed and evaluated for their potential use for the benefit of mankind.

Marine sponges under phylum *Porifera* are one of the richest sources of biologically active secondary metabolites with vast chemical diversity (Rifai *et al.*, 2005). They are now among the best sources of novel compounds according to Appeltans *et al.* (2012) and Thomas *et al.* (2010), contributing the highest number of compounds per year among other marine natural products for year 2006 and 2007 (Blunt *et al.*, 2009), while in the 20th century, scientists conservatively estimate 11% of the thousands of tested sponge species may produce cytotoxic compounds that may contribute to the discovery of drugs (Garson, 1994). The chemical defenses of these sessile marine

invertebrates may possess exert a multitude of biological activities besides producing nitrogen-containing substances that usually called marine alkaloids. These compounds have been found to interact with key aspects to the cell cycle, and with enzymes or other targets, providing insights into new therapeutics, including antibacterial, anticoagulant, antiviral, antifungal, antiinflammatory, antituberculosis, antimalarial, and antiplatelet agents with the hope of new cures to important existing diseases such as cancer (Ramanjooloo *et al* 2014; Izzati *et al.,* 2011; Qaralleh *et al.,* 2010; Rao *et al.,* 2006; Coello *et al.,* 2009; Guzmán *et al.,* 2011; Takei *et al.,* 2010; Lucas *et al.*, 2003).

1.2 Problem Statement

Studies had been done on marine sponges globally on obtaining the anti-inflammatory metabolites from isolated terpenoids (Robert and Michael, 2005), anti-fungal sphingosine and its derivatives (El-Amraoui *et al*., 2013), new cytotoxic norsesterterpene peroxide obtained from Thai sponges (Phuwapraisirisan *et al*., 2003), new alkaloids obtained with anti-microbial, free redial scavenging (DPPH) and cancer growth inhibition activities (Youssef *et al.,* 2013) and more. There are few studies have been reported on the isolation of chemical compounds and bioactivities from the Malaysian sponges collected from various coastal waters including Pulau Bidong, Pulau Kapas, Pulau Perhentian and Pulau Redang in Terengganu, Pulau Kera in Penang and also Pulau Langkawi, Kedah. (Khozirah *et al.,* 2009; Habsah *et al.,* 2009a; Khamsah *et al.,* 2005*;* Izzati *et al.,* 2011; Qaralleh *et al.,* 2010). Therefore, this research aims to isolate more compounds with therapeutics effects in drug discovery field for existing diseases from marine sponges locally in view of researches done globally. Besides, current study focusses on different species of sponges available locally for a better knowledge regarding the available sponge species with the possibilities for further researches to be done based on the activities shown.

1.3 Objectives of Study

The specific objectives of current study are presented as below:

1. To screen the biological activities of selected marine sponges from West Coasts of Peninsular Malaysia.
   (From the screening activities in Objective 1, Haliclona sp. was identified as having more potential in tested activities, thus was selected for further study.)
2. To profile the chemical constituents in the *Haliclona* sp. using NMR and LCMS/MS analyses.
3. To isolate and characterize the chemical constituents from the *Haliclona* sp. using various chromatographic and spectroscopic techniques.

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