EXTRACTION AND IDENTIFICATION OF SECONDARY METABOLITES FROM MANGROVE RARE ACTINOMYCETE *Actinophytocola* sp. K4-08 WITH BIOACTIVITY POTENTIAL

BY

NUR HUDA BINTI MOHD ZIN

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Kulliyyah of Science
International Islamic University Malaysia

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ABSTRACT

Actinomycetes are aerobic filamentous Gram-positive bacteria that produce various secondary metabolites, notably antibiotics. Unfortunately, the effectiveness of this bacteria has been jeopardized in recent years due to the rise of multidrug-resistance bacteria. Hence, researchers have switched to ‘non-streptomycetes’ to gain novel metabolic compounds. The current study was designed to extract and identify the microbial compounds from mangrove rare actinomycete, *Actinophytocola* sp. K4-08 (KR902625) was previously isolated from Kuantan mangrove sediments. To date, this is the first study that demonstrates the properties of the genus *Actinophytocola* sp. concerning their biological potential. Colonies of *Actinophytocola* sp. K4-08 was subjected to morphological characterization using gram staining and scanning electron microscope (SEM). *Actinophytocola* sp. K4-08 is a Gram-positive bacterium with branched substrate mycelium fragmented into a rod-like shape and regular round chain spore formation. Moreover, this strain utilised more than 10 carbon sources and tolerated up to 10 % sodium chloride (NaCl), demonstrating its adaptation to the marine environment. Crude extracts from both supernatant and cells of *Actinophytocola* sp. K4-08 were prepared using different solvent namely, ethyl acetate, methanol, and acetone with XAD-2 resins. Extraction with ethyl acetate produced dark yellow liquid residue and brownish solid residues with the highest crude at 1.35 g. Solid acetone (AE) and liquid methanol (ME) crudes showed significant antibacterial activities against *Bacillus subtilis* with inhibition of 7.9 ± 0.1 mm and 12.0 ± 0.0 mm respectively through disc diffusion susceptibility test. Overall, liquid crude extracts exhibited higher antagonistic activity against *B. subtilis* than solid crude extracts. The antioxidant activities of crude extracts were further assessed using total phenolic content (TPC), total flavonoid content (TFC), free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH), and ferric reducing antioxidant power (FRAP) assay. Liquid ethyl acetate (EA) crude demonstrated higher TPC and TFC, while solid EA crude showed higher DPPH scavenging and FRAP assays. Both solid and liquid EA crude extracts have moderately good antioxidant potential. Liquid EA crude was further analysed for cytotoxicity assays against human non-small lung cancer cells. A549 cell was the most sensitive toward the liquid EA crude than the H1299 cell line, with a higher reduction in cell viability at 62.52 ± 0.76 % and 79.13 ± 0.90 %, respectively. The presence of O-H, C-H, and C=C bonding was identified using Fourier-transform infrared spectroscopy (FT-IR) and 2,4-bis (1,1-dimethyl ethyl)-phenol (2,4-DTP), an anticancer drug was detected in solid ME crude using gas chromatography-mass spectrometry (GC-MS). Chemical profiling using reversed-phase thin-layer chromatography (RP-TLC) and high-performance liquid chromatography (HP-LC) showed good separation of microbial compounds in liquid EA crude with acetonitrile and water (1: 1.5, v/v) solvent ratio. Ningpeisinoside, lamiophlomiol A and pseudolaric acid AO-β-D-glucopyranoside were detected using liquid chromatography-mass spectrometry (LC-MS). The present findings suggested that rare mangrove actinomycete, *Actinophytocola* sp. K4-08 is a high-potential candidate with interesting biosynthetic capabilities for the drug discovery program.

ملخص البحث

بكتيريا الشعيات هي بكتيريا هوائية، خيطية، موجبة الجرام، تنتج مستقلِبات ثانوية مختلفة، لا سيما إنتاجها للمضادات الحيوية. لكن لسوء الحظ، تأثرت فعالية هذه البكتيريا في السنوات الأخيرة بسبب ظهور بكتيريا مقاومة للعديد من الأدوية. ولهذا، تحول الباحثون إلى دراسة البكتيريا "الغير متسلسلة" للحصول على مركبات أيضية جديدة. صُممت الدراسة الحالية لاستخلاص وتحديد المركبات الميكروبية من البكتيريا الشعاوية النادرة (نوع بكتيريا شعاوية نباتية) الموجودة على أشجار المانغروف. تم عزل متسلسلة (KR902625) K4-08 سابقًا من رواسب المانغروف بمدينة كوانتان، ماليزيا. حتى الآن، تعتبر هذه الدراسة هي الأولى من نوعها التي توضح خصائص أنواع البكتيريا الشعاوية فيما يتعلق بفعاليتها البيولوجية. أُخضعت متسلسلة مستعمرات البكتيريا الشعاوية K4-08 للوصف المورفولوجي باستخدام صبغة غرام وعبر جهاز المجهر الإلكتروني الماسح (SEM). البكتيريا الشعاوية K4-08 عبارة عن بكتيريا موجبة الجرام مع طبقة فطرية متفرعة مجزأة إلى شكل يشبه القضيب وفي شكل أبواغ متسلسلة دائرية منتظمة. إضافة إلى ذلك، استعملت هذه السلالة أكثر من 10 مصادر كربون وتتحمل ما يصل إلى 10٪ من كلوريد الصوديوم، مما يدل على تكيّفها مع البيئة البحرية. المستخلصات الخامة من كل من المادة الطافية وخلايا البكتيريا الشعاوية K4-08 تم تحضيرها باستخدام مذيبات مختلفة وهي: أسيتات الإيثيل والميثانول والأسيتون مع راتنجات زاد-2 (XAD-2). أظهرت نتائج الاستخلاص باستخدام أسيتات الإيثيل بقايا سائل أصفر داكن ورواسب صلبة بنية اللون عند أعلى تركيز للخام وهو 1.35 جم. كما أظهرت مستخلصات خامات الأسيتون الصلبة والميثانول السائل نشاطاً كبيراً مضاداً للبكتيريا ضد بكتيريا العصوية الرقيقة عند تثبيط 7.9 ± 0.1 مم و 12.0 ± 0.0 مم على التوالي، من خلال قرص اختبار حساسية الانتشار. بشكل عام، فإن المستخلصات الخامة السائلة أظهرت نشاطاً مضاداً أعلى ضد بكتيريا العصوية الرقيقة من

المستخلصات الخامة الصلبة. قُيّمت الأنشطة المضادة للأكسدة للمستخلصات الخام بشكل أكبر باستخدام المحتوى الفينولي الكلي، إجمالي محتوى الفلافونويد، قياس الجذور الحرة، مسحوق 2،2-ثنائي فينيل 1-بيكريل هيدرازيل (دي بي بي ايج)، وقياسات مضادة أكسيد الحديديك. وأظهر خام أسيتات الإيثيل السائل ارتفاعًا في كلا المحتويين الفينولي والفلافونويدي، بينما أظهر خام أسيتات الإيثيل الصلب أعلى نسبة مسح دي بي بي ايج وقايسات مضادات أكسيد الحديديك. تحتوي كل من مستخلصات خام أسيتات الإيثيل الصلبة والسائلة على احتمالات جيدة لمضادات الأكسدة. تم إخضاع خام أسيتات الإيثيل السائل لفحوصات السمية الخلوية ضد خلايا سرطان الرئة البشرية غير الصغيرة. كانت الخلايا البشرية السرطانية الغدية هي الأكثر حساسية تجاه خام أسيتات الإيثيل السائل من خلايا سرطان الرئة البشرية غير الصغيرة، مع انخفاض كبير في حيوية الخلايا عند 62.52 ± 0.76٪ و 79.13 ± 0.90٪، على التوالي. تم تحديد وجود ترابط عناصر O-H و C-H و C = C باستخدام مطيافية الأشعة تحت الحمراء باستخدام تحويل فورييه و2،4-بايس (1،1-ثنائي ميثيل إيثيل)-فينول (2،4-دي تي بي)، وقد تم اكتشاف العقار المضاد للسرطان في خام الميثانول الصلب باستخدام مطياف الكتلة الكروماتوغرافي السائل. أظهر الفصل الكيميائي باستخدام كروماتوغرافيا الطبقة الرقيقة عكسي الطور وكروماتوغرافيا السائل رفيع الأداء فصلًا جيدًا للمركبات الميكروبية في مذيب خام أستات الإيثيل السائل مع الأسيتونتريل والماء (بنسبة 1.5:1). تم الكشف عن نينغبيزينوسيد وأ-لاميوفلوميول والحمض الكاذب أو-بيتا-دي-غلوكوبيرانوسايد باستخدام مطياف الكتلة الكروماتوغرافي السائل. تشير النتائج الحالية إلى أن بكتيريا المانغروف الشعاوية النادرة (نوع بكتيريا شعاوية نباتية) متسلسلة K4-08 هو مرشح ذو احتمالية عالية لاكتشاف أدوية؛ حيث يتمتع بقدرات تخليق حيوية مثيرة للاهتمام.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Master of Science (Biotechnology)

………………………………
Zaima Azira Binti Zainal Abidin
Supervisor

………………………………
Deny Susanti Binti Darnis
Co-Supervisor

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Master of Science (Biotechnology)

………………………………
Mardiana Binti Mohd Ashaari
Internal Examiner

………………………………
Japareng Bin Lalung
External Examiner

This thesis was submitted to the Department of Biotechnology and is accepted as a fulfilment of the requirement for the degree of Master of Science (Biotechnology)

……………………………….
Tengku Haziyamin Bin Tengku Abd Hamid
Head, Department of Biotechnology

This thesis was submitted to the Kulliyyah of Science and is accepted as a fulfilment of the requirement for the degree of Master of Science (Biotechnology)

……………………………….
Jesni Bin Shamsul Shaari
Dean, Kulliyyah of Science

DECLARATION

I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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To my parents and family for laying the foundation of what I turned out to be in life.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS

bar	Atmospheric pressure
cm^{-1}	Wavenumber
cells/mL	Cells per millilitre
µg	Microgram
µg/µL	Microgram per microlitre
µg/mL	Microgram per millilitre
µL	Microlitre
µm	Micrometre
µM	Micromole
cm	Centimetre
eV	Electric vehicle
g	Gram
hr	Hour
L	Litre
M	Molarity
m^2	Meter square
km^2	Kilometre square
mbar	Millibar
min	Minute
mL	Millilitre
mg/mL	Milligram per millilitre
mg/L	Milligram per litre
mL/min	Millilitre per minute
g/mL	Gram per millilitre
g/L	Gram per litre
m	Meter
mm	Millimetre
mmol/L	Millimoles per litre
ng	Nano gram
ng/µl	Nanogram per microlitre

nm	Nanometre
ºC	Degree celsius
rpm	Revolution per minute
s	Second
v/v	Volume to volume
w/v	Weight to volume
λ	Lambda
α	Alpha
hr	Hour
pH	Potential hydrogen
psi	Pounds per square inch
T	Turbidity
X	Magnification
%	Percent
>	Strict inequality greater than

LIST OF ABBREVIATIONS

A	Absorption
ACN	Acetonitrile
AE	Acetone extract
$AlCl_3$	Aluminium chloride
ATCC	American Type Culture Collection
CH_3COOH	Acetic acid
CO_2	Carbon dioxide
DCM	Dichloromethane
DMEM	Dulbecco's Modified Eagle Media
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
DMSO	Dimethyl sulfoxide
EA	Ethyl acetate extract
EtOH	Ethanol
Fe^{2+}	Ferrous iron
Fe^{3+}	Ferric iron
FBS	Fetal bovine serum
FRAP	Ferric Reducing Antioxidant Power Assay
FT-IR	Fourier-transform infrared spectroscopy
GC-MS	Gas chromatography-mass spectrometry
H_2O	Water
HCl	Hydrochloric acid
IC_{50}	Half maximal inhibitory concentration
ISP2	Yeast extract–malt extract agar
ISP3	Oatmeal agar
ISP4	Oatmeal agar
ISP5	Glycerol asparagine agar base
ISP6	Peptone yeast extract iron agar
ISP7	Tyrosine agar
YS	Yeast media

TSA	Trypticase soy agar
LC-MS QTOF	Liquid chromatography quadrupole time-of-flight mass spectrometry
ME	Methanol extract
MeOH	Methanol
MHA	Mueller hinton agar
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
N	Normality
NA	Nutrient agar
NaCl	Sodium chloride
Na_2CO_3	Sodium carbonate solution
$NaNO_2$	Sodium nitrate
NaOH	Sodium hydroxide
NP	Normal phase
OsO_4	Osmium tetroxide
PBS	Phosphate-buffered saline
R_t	Retention time
RP-HPLC	Reversed phase-high performance liquid chromatography
RP-TLC	Reversed phase-thin layer chromatography
SD	Standard deviation
S.E.M.	Standard error mean
SEM	Scanning electron microscope
SYE	Starch-yeast extract agar
TFC	Total flavonoid content
TLC	Thin layer chromatography
TPC	Total phenolic content
TPTZ	2,4,6-tri(2-pyridyl)-1,3,5-triazine
UV	Ultraviolet
i.e.	id est: that is
et al.	*et al*ia: and others
sp.	species
spp.	several species
nov.	novel

CHAPTER ONE

INTRODUCTION

1.1 RESEARCH BACKGROUND

Microbial natural products are the most reliable, well-known sources of new medicines and continue to be an incredible resource for drug development with various therapeutic agents. Infinite structural diversity and a chemical variety of compounds with a wide range of biological activities make natural microbial products the most versatile potential for the new antibiotic (Sheena & Helen, 2017). The discovery of antibiotics from microbial fermentation was practised many years ago. Since the discovery of penicillin, countless antibiotic and biologically active substances have been procured from microbial cultures. Antibiotic (Greek; anti: against, bios: life) is a chemical substance produced by microorganisms used to kill or inhibit the growth of other organisms specifically and automatically possessed antimicrobial activities (Aryal *et al*., 2019; Parham *et al*., 2020). The actinobacterial origin was reported to produce about 7000 secondary metabolite compounds and was recognized as a member of a class of Actinobacteria as the primary contributor to natural products (Rao *et al*., 2017). Therefore, actinomycetes have been gaining attention from pharmaceutical industries because of their ability to produce many bioactive secondary metabolites (Jagannathan *et al*., 2021).

Actinomycetes are ubiquitous in nature and predominantly soil inhabitant aerobic filamentous bacteria under the order of *Actinomycetales,* which are noteworthy as the antibiotic producer with known structurally varying secondary metabolites (Hotam Singh Chaudhary *et al*., 2013; Devanshi *et al*., 2021) that possessed antimicrobial, anti-parasite, antiviral, antitumor and cytotoxic properties followed by the unique chemical structures (Kekuda *et al*., 2010; Rajan & Kannabiran, 2014; Dhakal *et al*., 2019). Furthermore, around 23 000 bioactive secondary metabolites emitted by microorganisms have been reported, and over 10 000 of these compounds are produced by actinomycetes representing 45 % of all bioactive microbial metabolites discovered (Valli *et al*., 2012). Among actinomycetes, the genus *Streptomyces* is established to produce roughly 7600 bioactive secondary metabolite compounds (Chamikara, 2016). Moreover, about 75 % of metabolites and at least 5000 documented biologically active compounds belonged to the *Streptomyces* genus (Pacios-Michelena *et al*., 2021). In recent years, enduring infectious diseases and rapidly mounting multi-drug resistance (MDR) pathogen strains have alarmed the scarcity of available antibiotics. Hence, seeking novel drugs to maintain the integrity of antibiotics against pathogenic microorganisms is highly demanded (Vivas *et al*., 2019). However, finding new microbial metabolites is becoming increasingly complex, and the frequency of the rediscovery of known compounds by *Streptomyces* was pretty high. Under this situation, most researchers are changing their focus from *Streptomyces* to non-*Streptomyces*.

Rare actinomycetes, also known as 'non- *Streptomyces*'- non-taxonomic term, are typically slow-growing, challenging to isolate, and culture Actinobacteria. Therefore, they were regarded as less exploited microorganisms and might be considered the high potential producers of novel natural metabolite compounds (Baltz, 2006). Various marine rare Actinobacteria produce bioactive molecules such as *Verrucosispora* sp. AB-18-032 (abyssomicins), *Micromonospora* sp. M71-A77 (levantilides), *Nocardiopsis* sp. (nocapyrones), *Marinispora* sp. NPS12745 (lynamicinws) and *Actinomadura* sp. (Halomadurone) (Dhakal *et al*., 2017). For encountering the marine rare Actinobacteria, isolation effort has been focused on poorly studied habitats such as deep-sea, mangrove sediments, and extreme environments to obtain new marine diversities. However, many natural environments are still either unexplored or underexplored. Thus, can be considered a luxurious resource for isolating lesser studied microorganisms, including rare actinomycetes (Ouchari *et al*., 2019), with tremendous potential to produce interestingly new compounds (Hug *et al*., 2018).

One of the most favourable explored regions is the mangrove forest. The mangrove is an eccentric woody plant community of the intertidal coast in the tropical and subtropical coastal region (Selvam, 2019). Mangrove swamps occupy about 180 000 km^2 (Chen & Shih, 2019) and cover approximately 75 % of the world's tropical and subtropical coastlines (Nicholls *et al*., 2018). Mangrove forests are an important type of wetland ecosystem that has a vital role in the ecological, economic, and social