

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

BY

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A thesis submitted in fulfilment of the requirement for the
degree of Master of Science (Biotechnology)

Kulliyyah of Science

International Islamic University Malaysia

SEPTEMBER 2022

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-DTEP), 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. Ningpeisinoid, lamioflomiol 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 مستقلبات 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 الشعاعية النادرة (نوع بكتيريا شعاعية نباتية) الموجودة على أشجار المانغروف. تم عزل متسلسلة (KR902625) K4-08 سابقاً من رواسب المانغروف بمدينة كوانتان، ماليزيا. حتى الآن، تعتبر هذه الدراسة هي الأولى من نوعها التي توضح خصائص أنواع البكتيريا الشعاعية فيما يتعلق بفعاليتها البيولوجية. أُخضعت متسلسلة مستعمرات البكتيريا الشعاعية K4-08 للوصف المورفولوجي باستخدام صبغة غرام وعبر جهاز المجهر الإلكتروني الماسح (SEM). البكتيريا الشعاعية 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 تكيفها مع البيئة البحرية. المستخلصات الخامة من كل من المادة الطافية وخلايا البكتيريا الشعاعية 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 0.1 ± 7.9 مم و 0.0 ± 12.0 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 الخام الصلبة. قُيِّمت 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 ايچ)، وقياسات مضادة أكسيد الحديدك. وأظهر خام أسيتات الإيثيل السائل ارتفاعاً في كلا المحتويين الفينولي والفلافونويدي، بينما أظهر خام أسيتات الإيثيل الصلب أعلى نسبة مسح دي بي بي ايچ وقياسات مضادات أكسيد الحديدك. 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 $62.52 \pm 0.76\%$ و $79.13 \pm 0.90\%$ ، على التوالي. تم تحديد وجود ترابط عناصر O-H و C-H و C = C باستخدام مطيافية الأشعة تحت الحمراء باستخدام تحويل فورييه و 2،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 فصلاً جيداً للمركبات الميكروبية في مذيب خام أسيتات الإيثيل السائل مع الأسيتونتريل والماء (بنسبة 1:1.5). تم الكشف عن نينغيزينوسيد وأ-لاميوفلوميول والحمض الكاذب أو-بيتا-دي-غلوكوبيرانوسايد باستخدام مطياف الكتلة الكروماتوغرافي السائل. تشير النتائج الحالية إلى أن بكتيريا المانغروف الشعاعية النادرة (نوع بكتيريا شعاعية نباتية) متسلسلة K4-08 هو مرشح ذو احتمالية عالية لاكتشاف أدوية؛ حيث يتمتع بقدرات تخليق حيوية مثيرة للاهتمام.

APPROVAL PAGE

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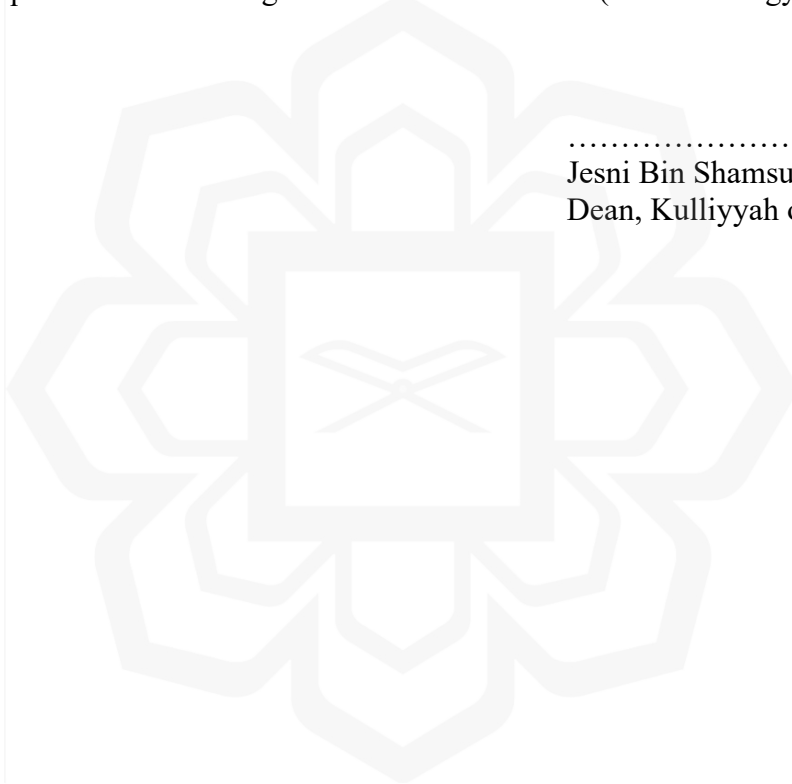
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To my parents and family for laying the foundation of what I turned out to be in life.

ACKNOWLEDGEMENTS

All glory is due to Allah, the Almighty, whose Grace and Mercies have been with me throughout the duration of my programme. Although, it has been tasking, His Mercies and Blessings on me ease the herculean task of completing this thesis.

I am most indebted to my supervisor, Assoc. Prof. Dr. Zaima Azira Binti Zainal Abidin, whose enduring disposition, kindness, promptitude, thoroughness, and friendship have facilitated the successful completion of my work. I put on record and appreciate her detailed comments, useful suggestions, and inspiring queries which have considerably improved this thesis. Her brilliant grasp of the aim and content of this work led to her insightful comments, suggestions, and queries which helped me a great deal. Despite her commitments, she took the time to listen and attend to me whenever requested. The moral support she extended to me is undoubtedly a boost that helped me build and write the draft of this research work. I am also grateful to my co-supervisor, Assoc. Prof. Dr. Deny Susanti Binti Darnis, whose support and cooperation contributed to the outcome of this work.

I would also like to convey my special gratitude to all laboratory staff in Kulliyah of Science, particularly Bro. Ahmad Muzammil, Bro. Mohamad Romizan, Bro. Mohd Azmir, Sr. Noor Izyan, and Sr. Mueizzah for their kindness and meticulous efforts to help me during my studies.

Lastly, my gratitude goes to my beloved parents and family; for their prayers, understanding, unconditional love, and endurance while away. I am also grateful to all my friends especially Nurhanisah, Nik Nurizni, Nurul Fatimah and Qusyairi who have always been there for me with their continuous moral support.

Once again, we glorify Allah for His endless mercy on us one of which is enabling us to successfully round off the efforts of writing this thesis. Alhamdulillah.

TABLE OF CONTENTS

Abstract.....	ii
ملخص البحث.....	iii
Approval Page.....	v
Declaration.....	vii
Acknowledgements.....	x
List of Tables.....	xiv
List of Figures.....	xvi
List of Symbols.....	xix
List of Abbreviations.....	xxi
CHAPTER ONE: INTRODUCTION.....	1
1.1 Research Background.....	1
1.2 Research Objectives.....	4
1.3 Research Hypothesis.....	4
CHAPTER TWO: LITERATURE REVIEW.....	5
2.1 Actinomycete.....	5
2.1.1 Classification of Actinomycetes.....	6
2.1.2 General Morphology.....	8
2.1.3 Ecology and Distribution of Actinomycetes in Nature.....	10
2.1.4 Importance of Actinomycetes.....	12
2.1.5 Actinomycetes: Unparallel Potential As The Bioactive Secondary Metabolite Producers.....	13
2.2 Rare Actinomycete.....	15
2.2.1 <i>Actinophytocola</i> sp.....	16
2.3 Rare Actinomycete As A Notable Producer Of Novel Secondary Metabolites.....	25
2.4 Mangrove Forest: A Microbial Paradise.....	26
2.4.1 Characteristics of Mangrove Forest.....	26
2.4.2 Importance of Mangrove Ecosystem.....	27
2.4.3 Mangrove Actinomycete.....	28
2.5 Solvent Extraction of Bioactive Secondary Metabolites From Mangrove Actinomycete.....	29
CHAPTER THREE: MATERIAL AND METHOD.....	33
3.1 Research Flow Chart.....	33
3.2 Rare Actinomycete <i>Actinophytocola</i> sp. K4-08.....	34
3.3 Morphological Characterization.....	34
3.3.1 Gram Staining.....	34
3.3.2 Scanning Electron Microscope (SEM).....	35
3.4 Physiological and Biochemical Characteristics.....	37
3.4.1 Phenotypic Fingerprint: Biolog Microbial Identification and Characterization.....	37
3.4.2 Salt Tolerance Test.....	39
3.5 Preparation of Crude Extract From <i>Actinophytocola</i> sp. K4-08.....	39

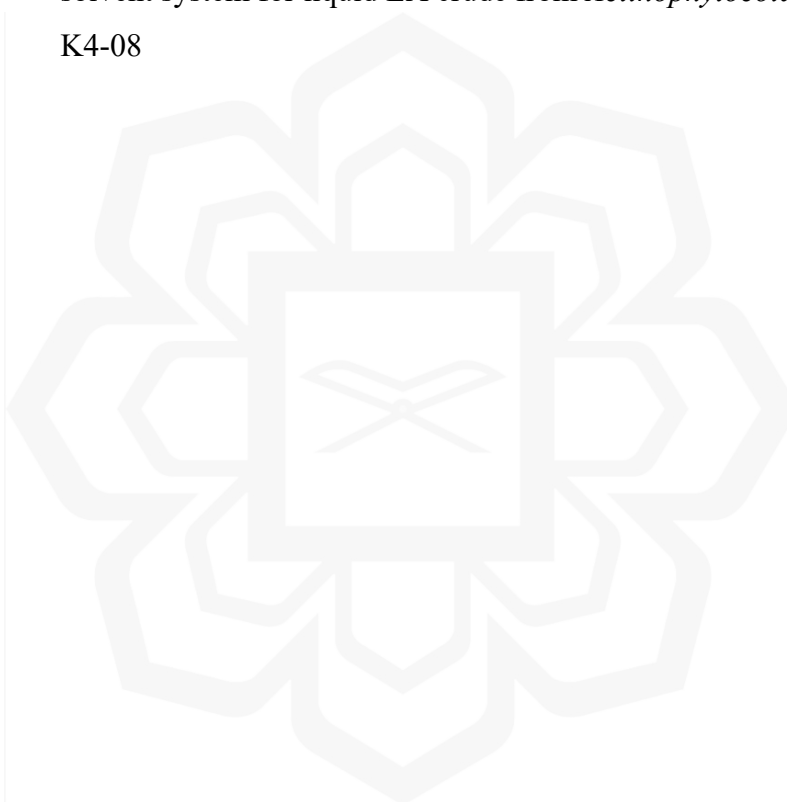
3.5.1 Microbial Submerged Fermentation and Cell-free Supernatant Preparation.....	39
3.5.2 Crude Extract Preparation From Different Part of <i>Actinophytocola</i> sp. K4-08 That Using Different Solvent Extraction	40
3.5.3 Chemical Solubility Test of Crude Sample	41
3.6 Evaluation of Antibacterial Activity	41
3.6.1 Preparation of Test Organism	41
3.6.2 Disc Diffusion Susceptibility Test	41
3.7 Antioxidant Potential of <i>Actinophytocola</i> sp. K4-08	42
3.7.1 Screening Antioxidant Activities of Crude Extracts.....	42
3.7.1.1 Total Phenolic Content (TPC) Assay.....	43
3.7.1.2 Total Flavonoid Content (TFC) Assay	43
3.7.1.3 Free Radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) Scavenging Assay	44
3.7.1.4 Ferric Reducing Antioxidant Power (FRAP) Assay	45
3.8 Cytotoxicity Bioassay	46
3.8.1 Cell Lines Maintenance and Growth Condition	46
3.8.2 Cell Treatment and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) Cell Viability Assay	46
3.9 Statistical Analysis	47
3.10 Structural Identification Techniques For Secondary Metabolite Compounds From <i>Actinophytocola</i> sp. K4-08	48
3.10.1 Fourier-Transform Infrared (FT-IR) Spectroscopy Analysis....	48
3.10.2 Gas Chromatography-Mass Spectrometry (GC-MS) Analysis.	48
3.10.3 Reversed Phase-Thin Layer Chromatography (RP-TLC) Analysis	49
3.10.4 Reversed Phase-High Performance Liquid Chromatography (RP-HPLC) Analysis	50
3.10.5 Liquid Chromatography Quadrupole Time-of-Flight Mass Spectrometry (LC-QTOF-MS) Analysis	50
CHAPTER FOUR: RESULT AND DISCUSSION	51
4.1 Morphological Characterization	52
4.1.1 Characterisation of <i>Actinophytocola</i> sp. K4-08	53
4.2 Physiological And Biochemical Characteristics of <i>Actinophytocola</i> sp. K4-08.....	57
4.2.1 Biolog Phenotypic Fingerprint.....	58
4.2.2 Salt Tolerance Test.....	60
4.3 Extraction of Secondary Metabolites Compounds Using Different Solvent Extraction	62
4.4 Screening For Antibacterial Activity	67
4.5 Antioxidant Potential of <i>Actinophytocola</i> sp. K4-08	71
4.5.1 Screening Antioxidant Activities of Bacterial Ethyl Acetate Crude Extracts	71
4.5.1.1 Total Phenolic Content (TPC) Assay.....	72
4.5.1.2 Total Flavonoid Content (TFC) Assay	74
4.5.1.3 Free Radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) Scavenging Assay	76
4.5.1.4 Ferric Reducing Antioxidant Power (FRAP) Assay	80

4.6 <i>In Vitro</i> Cytotoxicity Properties of <i>Actinophytocola</i> sp. K4-08 Against Human Lung Cancer Cell Lines.....	80
4.6.1 Cytotoxicity Effect of Liquid Ethyl Acetate Crude Extract against H1299 and A549 Human Cancer Cell Lines.....	84
4.7 Chemical Analysis For The Identification of Secondary Metabolite Compounds.....	88
4.7.1 Fourier Transform Infrared (FT-IR) Spectroscopy Analysis.....	88
4.7.2 Gas Chromatography-Mass Spectrometry (GC-MS) Analysis...	91
4.7.3 Reversed Phase- Thin Layer Chromatography (RP-TLC) Analysis	96
4.7.4 Reversed Phase- High Performance Liquid Chromatography (RP-HPLC) Analysis.....	102
4.7.5 Liquid Chromatography Quadrupole Time-of-Flight Mass Spectrometry (LC-QTOF-MS) Analysis.....	108
CHAPTER FIVE: CONCLUSION	111
5.1 Conclusion	111
5.2 Future Work.....	112
REFERENCES.....	113
APPENDIX A	145
APPENDIX B	146
APPENDIX C	146
APPENDIX D	169

LIST OF TABLES

Table 2.1	The classification of Actinobacteria	6
Table 2.2	The classification of Actinobacteria based on cell wall constituents	8
Table 2.3	The morphological, physiological and chemotaxonomic characterisation of <i>Actinophytocola</i> sp	21
Table 2.4	The list of bioactive secondary metabolites using solvent extraction from mangrove actinomycetes from year 2020 until 2022	30
Table 4.1	Overview result of 96 wells Biolog Gen III MicroPlate on 7 th day culture of <i>Actinophytocola</i> sp. (K4-08)	58
Table 4.2	Positive phenotypic test of Biolog Gen III MicroPlate on 7 th day culture of <i>Actinophytocola</i> sp. (K4-08) for carbon sources utilization assay and chemical sensitivity assay	59
Table 4.3	Weight of ethyl acetate (supernatant), methanol (mycelia cell), and acetone (mother liquor) crude extracts <i>Actinophytocola</i> sp. K4-08	63
Table 4.4	Chemical solubility of solid ethyl acetate and methanol crude extracts from <i>Actinophytocola</i> sp. K4-08	65
Table 4.5	Antibacterial activity of <i>Actinophytocola</i> sp. K4-08 solid and liquid crude extract diluted in DMSO solvent against <i>B. subtilis</i> using disc diffusion assay	67
Table 4.6	Concentration of total phenolic content (TPC) in solid and liquid ethyl acetate crude extract of <i>Actinophytocola</i> sp. K4-08	72
Table 4.7	Concentration of total flavonoid content (TFC) in solid and liquid ethyl acetate crude extract of <i>Actinophytocola</i> sp. K4-08	75
Table 4.8	DPPH-scavenging activity of solid and liquid ethyl acetate crude extract from <i>Actinophytocola</i> sp. K4-08	77
Table 4.9	Ferric-reducing antioxidant power (FRAP) activity in solid and liquid ethyl acetate crude extract of <i>Actinophytocola</i> sp. K4-08	81

Table 4.10	Percentage of cell viability value of H1299 and A549 cancer cells with different concentrations of liquid EA crude extract <i>Actinophytocola</i> sp. K4-08	84
Table 4.11	FT-IR analysis wavenumber of EA and ME crude extract by submerged state fermentation of <i>Actinophytocola</i> sp. (K4-08)	90
Table 4.12	List of chemical constituents detected compounds from solid ME crude of <i>Actinophytocola</i> sp. K4-08 based on GC-MS chromatogram	94
Table 4.13	Gradient condition using acetonitrile: water (0.1 % acetic acid) solvent system for liquid EA crude from <i>Actinophytocola</i> sp. K4-08	102



LIST OF FIGURES

Figure 2.1	Hierarchic classification of Actinomycetes	7
Figure 2.2	General morphology of actinomycete colony growing on agar	9
Figure 3.1	Post fixation <i>Actinophytocola</i> sp. K4-08 colony with osmium tetroxide, a heavy metal stain. The sample was mounted on the SEM sample stub and sputtered with gold before viewing under SEM	36
Figure 3.2	A 96 wells of Biolog Gen III microplate, inoculating fluid (IF-A), and a fresh culture of <i>Actinophytocola</i> sp. K4-08	38
Figure 4.1	Colony morphology of mangrove rare actinomycete, <i>Actinophytocola</i> sp. K4-08 strain on SYE agar media at 30 °C after 10 days	52
Figure 4.2	There was no production of any diffusible pigment formed by isolate strain <i>Actinophytocola</i> sp. K4-08 on SYE agar and broth media (300 mL)	53
Figure 4.3	The image arrangement of vegetative hyphae of rare actinomycete, <i>Actinophytocola</i> sp. K4-08 using compound microscopic under 400X magnification	55
Figure 4.4	Scanning electron micrograph of strain <i>Actinophytocola</i> sp. K4-08 on SYE agar on the 10 th day culture showing abundant substrate mycelium and no spore chains, branching spore-chain-like formation, and spore chains with regular round spores. Bar, 10 µm	55
Figure 4.5	<i>Actinophytocola</i> sp. K4-08 growth on SYE agar with different salt content without seawater with 0 %, 5 %, and 10 % sodium chloride (NaCl)	60
Figure 4.6	Crude extract of <i>Actinophytocola</i> sp. K4-08 from different solvent extraction after concentration using a rotary evaporator. (A) solid and liquid ethyl acetate (B) solid methanol (C) solid and liquid acetone with the presence of XAD-2 resins	64

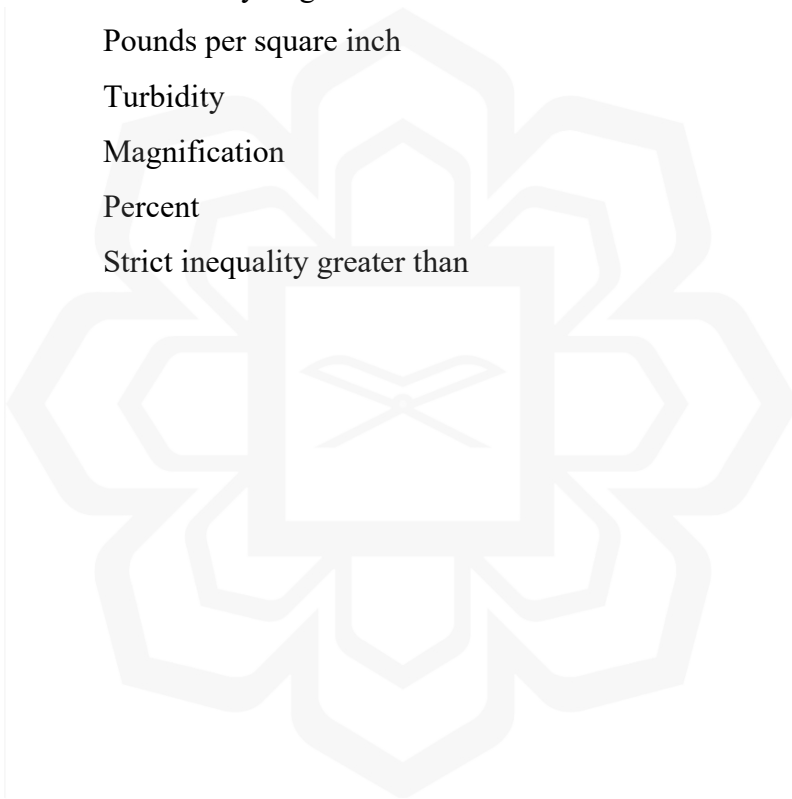
Figure 4.7	Antibacterial activity of solid crude extract from <i>Actinophytocola</i> sp. K4-08 using different solvent extraction, ethyl acetate (EA), methanol (ME), and acetone (EA) against <i>B. subtilis</i> with a concentration range from 20 μ L to 100 μ L.	68
Figure 4.8	Antibacterial activity of liquid crude extract from <i>Actinophytocola</i> sp. K4-08 using different solvent extraction, ethyl acetate (EA), methanol (ME), and acetone (EA) against <i>B. subtilis</i> with a concentration range from 20 μ L to 100 μ L.	68
Figure 4.9	The inhibition activity of crude extract of <i>Actinophytocola</i> sp. K4-08 with concentration at 40 μ L against <i>B. subtilis</i> bacteria	69
Figure 4.10	Concentration of gallic acid in mg/L against the absorbance of phenolic content (TPC) at measurement at 750 nm	72
Figure 4.11	The colour of EA crude extracts on 96 well microplates in triplicate and absorbed at 750 nm using the microplate reader (A) solid crude (B) liquid crude	73
Figure 4.12	Concentration of standard catechin (μ g/mL) against the absorbance of total flavonoid content (TFC) at wavelength 510 nm	74
Figure 4.13	Concentration of standard ascorbic acid (μ g/mL) against percentage inhibition of DPPH assay	76
Figure 4.14	Comparison antioxidant activity between solid and liquid ethyl acetate crude extract from <i>Actinophytocola</i> sp. K4-08 with standard ascorbic acid (positive control) in the DPPH method	79
Figure 4.15	Concentration of trolox (μ g/mL) against the absorbance of ferric-reducing antioxidant power at 593 nm	80
Figure 4.16	The formation of light navy-blue colour of reducing ferrous-TPTZ complex by solid and liquid EA crude in triplicate	81
Figure 4.17	Comparison between the percentage of cell viability of H1299, human non-small lung carcinoma cells and A549, lung carcinoma cell line measured by MTT assay treated with liquid EA crude extract at the concentration of 3.125 to 100 μ g/mL for 24 hours	85

Figure 4.18	FT-IR spectrum of EA (solid and liquid) and ME (solid only) crude extracted from <i>Actinophytocola</i> sp. K4-08 ranging from 600 cm ⁻¹ to 4000 cm ⁻¹	89
Figure 4.19	Chromatogram of solid ME crude diluted with DCM using GC-MS analysis	93
Figure 4.20	Solvent development for isolation of EA (solid and liquid) and ME (solid) crude by using RP-TLC technique	98
Figure 4.21	Solvent system development for isolation of compound in solid and liquid EA crude only using RP-TLC technique	99
Figure 4.22	Separation of compounds in solid ME crude that diluted with DCM and water using RP-TLC with MeOH: DCM solvent system	100
Figure 4.23	Chromatogram of liquid EA crude using method 1, ACN: H ₂ O at ratio (10:90), flow rate 1.0 mL/min, and injection volume 10 μL	103
Figure 4.24	Chromatogram of liquid EA crude using method 2, ACN: H ₂ O at ratio (2:98), flow rate 1.0 mL/min, and injection volume 10 μL	103
Figure 4.25	Chromatogram of liquid EA crude using method 3, ACN: H ₂ O at ratio (10:90) at minute 5.0, flow rate 1.0 mL/min, and injection volume 10 μL	104
Figure 4.26	Chromatogram of liquid EA crude using method 4, ACN: H ₂ O at ratio (2:98), flow rate 0.94 mL/min, and injection volume 10 μL	104
Figure 4.27	Chromatogram of liquid EA using prep-HPLC with prep method 4 at ratio (2:98), flow rate 20.0 mL/min, and injection volume 1000 μL	105
Figure 4.28	Solvent development of liquid EA crude purified using analytical HPLC and prep HPLC	106
Figure 4.29	The identified compound in liquid EA crude from <i>Actinophytocola</i> sp. K4-08 using LC-MS QTOF	107

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
°C	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 ₃ COOH	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 ₂ O	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 ₂ CO 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
<i>et al.</i>	<i>et alia</i> : 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 *Verrucosipora* 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 km² (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