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# Antioxidant and antimicrobial activities of crude extracts of actinomycetes from soils

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# ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF CRUDE EXTRACTS OF ACTINOMYCETES FROM SOILS

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# ฤทธิ์ต้านออกซิเดชันและฤทธิ์ต้านจุลชีพของสารสกัดหยาบแอคติโนมัยซีตีสจากดิน

น.ส.คาง ซาร์ ไว



วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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คาง ชาร์ ไว : ฤทธิ์ต้านออกซิเดชันและฤทธิ์ต้านจุลชีพของสารสกัดหยาบแอคติโนมัยซีตีสจากดิน. ( ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF CRUDE EXTRACTS OF ACTINOMYCETES FROM SOILS)
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แอคติโนมัยซีตีสจำนวน 17 สายพันธุ์ที่คัดแยกจากดินซึ่งเก็บจากจังหวัดหนองคาย นครราชสีมา ขอนแก่น พิจิตร ฉะเชิงเทราและอุดรธานี ได้ถูกประเมินฤทธิ์การยับยั้งจุลินทรีย์และต้านอนุมูลอิสระ จากผลการศึกษาลักษณะทางฟีโนไทป์ ลักษณะอนุกรมวิธานเคมีรวมทั้งผลการวิเคราะห์ลำดับเบสบนยีน 16S rRNA สามารถพิสูจน์เอกลักษณ์สายพันธุ์ได้เป็น *Streptomyces* 16 สายพันธุ์ และ *Micromonospora* 1 สายพันธุ์ จากผลการวิเคราะห์ลำดับเบสบนยีน 16S rRNA ของสายพันธุ์ JA03 มีความคล้ายคลึง 98.95% กับ *S. puniciscabiei* DSM 41929T และจากผลการวิเคราะห์ค่าเฉลี่ยความเหมือนของลำดับเบสนิวคลีโอไทด์ของจีโนม (ANI) จึงจัดได้ว่าสายพันธุ์ JA03 เป็น *Streptomyces* สปีชีส์ใหม่ ทุกสายพันธุ์ที่คัดแยกได้มีการศึกษาฤทธิ์ต้านจุลชีพด้วยวิธี disk diffusion พบว่าสายพันธุ์ NE2-6 ซึ่งใกล้เคียงกับ *Streptomyces alboniger* NRRL B 1832$^T$ มีฤทธิ์ต้านจุลชีพมากที่สุดต่อ *K. rhizophila* ATCC 9341 และมีฤทธิ์ยับยั้งน้อยต่อ *S. aureus* ATCC 25923 สายพันธุ์ ET1-12 มีฤทธิ์ยับยั้ง *C. albicans* ATCC 10231, *K. rhizophila* ATCC 9341 และ *B. subtilis* ATCC 6633 สายพันธุ์ ET2-2 ซึ่งใกล้เคียงกับ *Streptomyces diastaticus* subsp. *ardesiacus* NRRL B 1773$^T$ มีฤทธิ์ยับยั้งปานกลางต่อ *B. subtilis* ATCC 6633 และ *K. rhizophila* ATCC 9341 สายพันธุ์ CT2-4 ซึ่งใกล้เคียงกับ *Streptomyces capoamus* JCM 4734$^T$ มีฤทธิ์ยับยั้งปานกลางต่อ *S. aureus* ATCC 25923 สายพันธุ์ JA03 ซึ่งใกล้เคียง *Streptomyces puniciscabiei* DSM 41929$^T$ มีปริมาณสารประกอบฟีนอลิกสูงสุด (33.4 µg GAE/ mg of extract) จากผลการวิเคราะห์ TPC สายพันธุ์ MKP33 ซึ่งใกล้เคียง *Micromonospora yasonensis* DS 3186$^T$ แสดงค่า FRAP สูงสุด (135 µg AAE/mg of extract) สายพันธุ์ CT1-17 ซึ่งใกล้เคียง *Streptomyces olivaceus* NRRB L 3009$^T$ แสดงฤทธิ์แรงในการต้านอนุมูลอิสระ (80.21±0.04 %) ที่ 1,000 µg/ml โดยมีค่า $IC_{50}$ เท่ากับ 218.6±27.4 µg/ml จากการวิเคราะห์ด้วย DPPH สายพันธุ์ MKP33 ที่ 100 µg/ml ยังแสดงฤทธิ์ในการขจัดไนตริกออกไซด์ (NO) ได้ 49.3±1.96 % ซึ่งมีฤทธิ์แรงมากกว่าสายพันธุ์ NE2-6 (49.6±1.34 %) ที่ 500 µg/ml จากการประเมินผลความเป็นพิษต่อเซลล์ของสายพันธุ์ NE1-12, ET3-23, CT1-17, NE2-6, CT2-4, CT2-10, JA03 และ MKP33 ต่อ RAW264.7 macrophage cells พบว่าสายพันธุ์ NE1-12, CT1-17, JA03 และ MKP33 ไม่มีความเป็นพิษต่อเซลล์ที่ 200µg/ml ขณะที่สายพันธุ์ CT2-4, CT2-10, ET3-23 และ NE2-6 ไม่มีความเป็นพิษต่อเซลล์ที่ 10 µg/ml สายพันธุ์ ET3-23 มีฤทธิ์ยับยั้งการสร้างอนุมูลอิสระภายในเซลล์ (33±6.56 %) ที่เหนี่ยวนำด้วย $H_2O_2$ (100 µM) ใน RAW 264.7 cells สายพันธุ์ CT2-10 ที่ 10 µg/mL แสดงฤทธิ์ยับยั้งอย่างมีนัยสำคัญต่อการสร้าง NO ซึ่งชักนำโดย LPS (100 ng/ml) ใน RAW 264.7 cells เมื่อเปรียบเทียบกับสายพันธุ์ MKP33 ที่ 200 µg/ml (13.87±1.78 µM และ2.9±1.02 µM ตามลำดับ) โดยสรุปการศึกษานี้พบสายพันธุ์ JA03 เป็น *Streptomyces* สปีชีส์ใหม่ซึ่งมีฤทธิ์ต้านออกซิเดชันและแอคติโนมัยซีตีสที่คัดแยกได้จากดินสามารถพัฒนาต่อไปเพื่อให้ได้สารที่ฤทธิ์ต้านออกซิเดชันและฤทธิ์ต้านจุลชีพ

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| สาขาวิชา | เภสัชศาสตร์และเทคโนโลยี | ลายมือชื่อนิสิต ..... |
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Khaing Zar Wai : ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF CRUDE EXTRACTS OF ACTINOMYCETES FROM SOILS. Advisor: Assoc. Prof. RATAYA LUECHAPUDIPORN, Ph.D. Co-advisor: Prof. SOMBOON TANASUPAWAT, Ph.D.

A total of 17 actinomycetes isolated from soil collected from Nong Khai, Nakhon Ratchasima, Khon Kaen, Phichit, Chachoengsao and Udonthani Province, were evaluated on antimicrobial and antioxidant activities. They were identified as *Streptomyces* (16 strains) and *Micromonospora* (1 strain) based on their phenotypic and chemotaxonomic characteristics including 16S rRNA gene sequences analysis. Strain JA03 showed 98.95% similarity of 16S rRNA gene sequence to *S. puniciscabiei* DSM 41929T. Strain JA03 was found to be the novel species of genus *Streptomyces* based on whole genome average nucleotide identity (ANI). All these strains were evaluated on antimicrobial and antioxidant activities. Based on agar disk diffusion method, strain NE2-6 closely related to *Streptomyces alboniger* NRRL B 1832$^T$, has the most potent antimicrobial activity against *K.rhizophila* ATCC 9341 and mild activity against *S.aureus* ATCC 25923. ET1-12 was effective on *C. albicans* ATCC 10231 and on *K. rhizophila* ATCC 934 and on *B.subtilis* ATCC 6633. ET2-2 closely related to *Streptomyces diastaticus subsp. ardesiacus* NRRL B 1773$^T$ exhibited moderate activity against *B.subtilis* ATCC 6633 and *K. rhizophila* ATCC 9341. CT2-4 closely related to *Streptomyces capoamus* JCM 4734$^T$ showed moderate effect on *S.aureus* ATCC 25923. Strain JA03 closely related to *Streptomyces puniciscabiei* DSM 41929$^T$ showed the highest phenolic content (33.4 µg GAE/ mg of extract) based on TPC assay and MKP33 closely related to *Micromonospora yasonensis* DS 3186$^T$ showed the highest FRAP value (135 µg AAE/ mg of extract) based on FRAP assay. Strain CT1-17 closely related to *Streptomyces olivaceus* NRRB L 3009$^T$, showed potent scavenging activity (80.21±0.04%) at 1,000 µg/ml with $IC_{50}$ value of 218.6±27.4 µg/ml based on DPPH assay. Strain MKP33 showed more potent in nitric oxide scavenging (49.3±1.96%) at 100 µg/ml than NE2-6 (49.6±1.34%) at 500 µg/ml. Cytotoxic effects of strains NE1-12, ET3-23, CT1-17, NE2-6, CT2-4, CT2-10, JA03 and MKP33 on RAW264.7 macrophage cells were evaluated by % cell viability. Strains NE1-12, CT1-17, JA03 and MKP33 showed no cytotoxic effect at 200µg/mL and strains CT2-4, CT2-10, ET3-23 and NE2-6 at 10 µg/ml on RAW 264.7 macrophage cells. Strain ET3-23 inhibited intracellular ROS generation (33±6.56%) in $H_2O_2$ (100µM) induced RAW 264.7 cells. CT2-10 at 10 µg/mL significantly inhibited NO production induced by LPS (100ng/ml) in RAW264.7 macrophage cells when compared with MKP33 at 200 µg/ml (13.87± 1.78µM and 2.9±1.02µM, respectively). In conclusion, these findings confirmed that Strain JA03 is the novel species of genus *Streptomyces* and possesses antioxidant activity. The actinomycetes isolated from soil provided the activity information in the development of good candidates for antibiotics and antioxidants.



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Khaing Zar Wai

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| | |
|---|---|
| AAE | Ascorbic Acid Equivalent |
| ABTS | 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) |
| ATCC | American Type Culture Collection |
| AST | Antimicrobial Susceptibility Testing |
| β | Beta |
| BLAST | Basic Local Alignment Search Tool |
| DAP | Diaminopimelic acid |
| DCFH-DA | 2′,7′-dichlorofluorescein diacetate |
| DCF | 2'-7'dichlorofluorescein |
| DNA | Deoxyribose Nucleic acis |
| DPPH | 1, 1-diphenyl-2-picrylhydrazyl |
| DMEM | Dulbecco's modified eagle's medium |
| DMSO | Dimethyl sulfoxide |
| EPS | Exopolysaccharides |
| FRAP | Ferric Reducing Antioxidant Power |
| FCR | Folin-Ciocalteu reagent |
| $\gamma$ | Gamma |
| GAE | Gallic Acid Equivalents |
| $H_2O_2$ | Hydrogen peroxide |

| | |
|---|---|
| $OH^{\circ}$ | Hydroxyl radicals |
| ISP-2 | International *Streptomyces* Project-2 |
| IL | Interleukin |
| IFN | Interferon |
| LPS | Lipopolysaccharide |
| MTT | (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) |
| NaCl | Sodium Chloride |
| NAG | N-acetyl-d-glucosamine |
| NAM | N-acetyl-d-muramic acid |
| NO | Nitric oxide |
| NOS | Nitric oxide synthases |
| eNOS | Endothelium NO synthase |
| iNOS | Inducible NO synthase |
| nNOS | Neural NO synthase |
| NADPH | Nicotinamide Adenine Dinucleotide Phosphate |
| NCBI | National Center for Biotechnology Information |
| $O_2^{\circ-}$ | Superoxide anions |
| ORAC | Oxygen Radical Absorbance Capacity |
| $ONOO^{\circ}$ | Peroxynitrite |
| ROS | Reactive Oxygen Species |

| | |
|---|---|
| RNS | Reactive Nitrogen Species |
| ROS | Reactive Oxygen Species |
| SOD | Superoxide dismutase |
| TPC | Total Phenolic Content |
| TPTZ | Tripyridyltriazine |
| TNF | Tumor Necrosis Factor |
| TH1 | T helper cells |
| TLC | Thin Layer Chromatography |
| PBS | Phosphate Buffer Saline |
| PAMPs | Pathogen-Associated Molecular Patterns |
| PDK | Pyruvate Dehydrogenase Kinase |
| PCR | Polymerase Chain Reaction |

# CHAPTER I

## INTRODUCTION

Nowadays, bacterial infections suddenly become unresponsive to the existing antibiotics and the increasing incidence of antibiotic resistance is a major concern. Consequently, there are many approaches to the sighting new bioactive compounds from actinomycetes that produced about two-thirds of all known antibiotics of microbial origin. In the soil micro-ecosystem, many actinomycetes are isolated from soil and 75% of antibiotics are produced from actinomycetes. Among them, nearly 80% from the genus *Streptomyces* (1). Despite discovery in thousands of antibiotics, in search of more effective antibiotics and less toxic side effects are needed. Moreover, the rapid drug resistance emergence among multidrug resistant bacteria, leads to an urgent search for new antibiotics (1). Due to the presence of environmental pollution, resistance of antibiotics and emerging diseases, natural microbial products with novel structures and innovative enrichments persist the most abundant and adaptable encouraging antibiotics source with interesting biological activities.



The members of actinomycetes such as *Streptomyces* strains, have widely spread in the manufacturing of secondary metabolites among microorganisms for medical care system and nutritional value. The bioactive secondary metabolites almost 23,000 from microorganisms are described today, in which 45% of discovery are originated from actinomycetes. Among actinomycetes, *Streptomyces* strains produce approximately 7,600 compounds (2). Conventionally actinomycetes are the most

abundant and advertised antibiotic production group. Actinomycetes are the beneficial antibiotics sources including streptomycin, gentamicin, rifamycin and erythromycin (3).

In biological systems, reactive oxygen species (ROS) and free radicals are generated both oxygen reduction by-products and xenobiotic catabolism in biological systems (4). Unstable ROS such as hydroxyl radicals, superoxide anion, peroxy radicals and nitric oxide (NO) can damage proteins, nucleic acids and lipids (5). The consequences of oxidation in biomolecules are related to various kinds of human disorders such as cancer, disease of the nervous system and atherosclerosis (6).

Antioxidants are molecules that can prevent oxidative damage degenerative diseases and other pathologies. Antioxidants can inhibit and scavenge free radicals, thus protecting different kinds of infectious diseases and degenerative diseases to humans. Instead of synthetic antioxidants, the usage of natural antioxidants has been employed as an effective and safe source (7). Natural microbial products have both the potent therapeutic activities and the desirable pharmacokinetic properties for clinical development (6).



In the process of searching for natural antioxidants with pharmacological activities, microbial secondary metabolites produce the major compounds having inventive structures and potential biological activities. Because of the drawbacks of synthetic antioxidants (8), beneficial antioxidants from natural microorganisms' sources were applied for health care in recent years. Some major antioxidants are actinomycin C2, benthocyanins and carquinostatin A isolated from *Streptomyces lavendulae* strain

SCA5, *Streptomyces prunicolor* and *Streptomyces exfoliates* respectively (9). Recently, modern research is focused on safe therapeutics and natural product based on microorganisms. Nowadays, more potent, safer and less expensive natural compounds are extremely expected, more interesting and meaningful (6).

Actinomycetes are filamentous Gram-positive bacteria owing to the phylum Actinobacteria which are extensively distributed in not only terrestrial for example in soil, but also aquatic ecosystems, fungal materials, plants and animals. They are also critical in the biodegradation of soil and the development of humus causing from geosmin, the compound with the characteristic of "wet earth odor". Actinomycetes also show various physiological and metabolic characterization and extracellular enzymes production (2).

Modern research is focused on antibiotics and antioxidants from natural sources such as plants and microorganisms and served as safe therapeutics. Principally due to the risks of synthetic antioxidants, the recent investigation was exposed those microbial resources from the soil can be manufactured for natural antibiotics and antioxidants. Therefore, it is interesting in isolation and identification of actinomycetes from soil and potential search for new secondary metabolites of actinomycetes with the antimicrobial and antioxidant activities.

## OBJECTIVES

The objectives of this present study are

1. To isolate and identify actinomycetes from soils based on phenotypic and genotypic characteristics
2. To evaluate antimicrobial activity of actinomycetes based on disk diffusion method
3. To evaluate antioxidant activity of crude extracts of actinomycetes based on total phenolic content (TPC) assay, DPPH radical scavenging assay, ferric reducing antioxidant power (FRAP) assay and nitric oxide (NO) scavenging assay
4. To investigate inhibitory effect of crude extracts on intracellular radical generation in RAW264.7 macrophage cells

## HYPOTHESIS

1. Actinomycetes exhibit the antimicrobial activity on based on agar disk diffusion method.
2. Crude extracts of actinomycetes exhibit antioxidant activity based on total phenolic content (TPC) assay, ferric reducing antioxidant potential (FRAP) assay, DPPH radical scavenging assay and nitric oxide (NO) scavenging assay.
3. Crude extracts of actinomycetes inhibit intracellular radical generation in RAW264.7 macrophage cell.

**EXPECTED OUTCOMES**

These *in vitro* assays demonstrate that the actinomycetes extracts can be an essential microbial source of natural antioxidants, which might be used to prevent against oxidative stress. The isolated strain will be genetically characterized as a novel actinomycete species.

These findings also observe that the selected isolates which potentially inhibit bacterial pathogenic strains will be further study for the purification of compounds used for infectious diseases.

# CHAPTER II

# LITERATURE REVIEWS

## 2.1 Reactive Oxygen Species

ROS are reactive oxidant molecules and free radicals originated from oxygen molecule which are active in oxidation of cellular macromolecules including proteins, lipids, and polynucleotides. The cellular metabolism and environmental factors such as air impurities or cigarette smoking are one of the causing effects of ROS production.

The major ROS of physiological significance are superoxide anions ($O_2$), hydroxyl radicals ($OH^{\circ}$), and hydrogen peroxide ($H_2O_2$). Superoxide radical ($O_2^{\circ-}$) is the principal ROS species consequently converted into hydrogen peroxide ($H_2O_2$) by the help of superoxide dismutase (SOD)-catalyzed dismutation. The reaction response of $O_2^{\circ-}$ and nitric oxide (NO) produces peroxynitrite ($ONOO^{\circ}$), reactive oxygen and nitrogen species (RNS) (10). Low to moderate ROS intensities perform a significant character in physiological conditions including vascular tone regulation, oxygen sensing, immunological reaction, regulation of translation, and signal transduction. Due to high ROS concentrations, modifications of carbohydrates, lipids, proteins, and DNA have been linked to diseases including atherosclerosis, diabetes, cancer, and neurological disorders. As a result, regulation of reducing and oxidizing (redox) state is serious and attractive therapeutic target both for cell health and for many diseases (11).

## 2.2 Oxidative Stress

Oxidative stress refers to the imbalance between the enhancement of reactive oxygen species and impairment of antioxidant enzymes in the body. Free radicals and reactive oxygen species are generated in oxygen reduction by-products and xenobiotic catabolism.


**Figure 1. Free radicals causing oxidative stress (12).**

ROS or free radicals including hydroxyl radicals (OH°), superoxide anion ($O_2^{\circ-}$) nitric oxide ($NO^{\circ}$) and peroxy radicals ($ROO^{\circ}$) can be produced by cellular metabolism and reaction with protein, lipid and DNA causing cellular damage and degenerative changes. At low level, free radicals are an important role in the regulation of physiological and cell signaling processes, but the deleterious cellular changes can be caused by the high level of ROS (13).



Despite ROS and RNS having an essential role in both biological processes and host defense, excessive production of free radicals contributes to the immunopathologic conditions including diabetes mellitus, cardiovascular diseases such as hypertension and atherosclerosis, inflammatory diseases, cancer, AIDS and aging

process and also deteriorate the food. Nevertheless, antioxidants can interrupt the lipids oxidation by inhibiting the oxidative chain initiation or propagation (11).

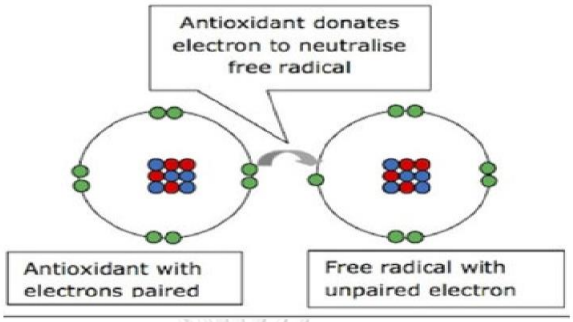



**Figure 2. Role of free radical and antioxidant (14).**

### 2.2.1 Role of hydrogen peroxide ($H_2O_2$)

The first ROS, superoxide anion, is produced from nicotinamide adenine dinucleotide phosphate (NADPH) by NADPH oxidase, termed "respiratory burst". which is a phenomenon by which invading microorganism are attacked by macrophages in the human body. Superoxide anion ($O_2^{\bullet -}$) is then converted to $H_2O_2$ by superoxide dismutase, and $H_2O_2$ is subsequently altered to water by catalase. In evidence, exposure to exogenous hydrogen peroxide induces NF-**K**B translocation. This property of hydrogen peroxide as a second messenger is termed "redox-sensitive" signal transduction. Hydrogen peroxide can act as the intracellular and extracellular signaling molecule throughout the cell membranes and transfer pro-inflammatory mediators (15).

**2.3 Current approaches to exploit actinomycetes as a source of novel natural products**

Empirical utilization of the most productive microbial assemblies: actinomycetes and filamentous fungi have conventionally concentrated on drug discovery from natural compounds in manufacturing and educational research laboratories. In history, actinomycetes have been the main source of the enormous amount of new antibiotic candidates and lead molecules with treatments in the numerous curative regions. Nowadays, traditional methodologies created entirely on screening of vast numerals of strains have been used and proven to be incompetent for distribution of novel molecules in a profitable way.

Modern approaches have proceeded forward for qualities to the deficiency of new molecules as sources of novel benefitted frameworks and advancement in the discovery of lead molecules for the improvement of drug candidates. Novel genomic approaches intended at discovering the biosynthetic potential of actinomycetes exposed in molecular genomics analyses and concentrated on novel bioengineering and many research units have appreciated extraordinary development in metagenomic instruments for the manufacture of new candidates from microbial natural products (16).

**2.4 Actinomycetes**

Taxonomic classification

| | |
|---|---|
| Kingdom: | Bacteria |
| Phylum: | Actinobacteria (Older name: Actinomycetes) |
| Class: | Actinobacteria |
| Order: | Actinomycetales |
| Family: | Streptomycetaceae |
| Genus: | *Streptomyces* |
| Species: | *Streptomyces coelicolor,* |
| | *Streptomyces venezuelae* |
| | *Streptomyces aureofaciens* |
| | *Streptomyces kanamyceticus* |
| | *Streptomyces griseus* |



"Actinomycetes" originated from Greek term "atkis" means a ray and "mykes" means fungus, have characteristics of both bacteria and fungi (12). Actinomycetes, gram-positive bacteria forming aerobic spore and filamentous like fungi, contain high guanine (G) and cytosine (C) content (57-75%) in genomic type and possess the order Actinomycetales having aerial and substrate mycelium. Actinomycetes cell wall comprises of a vast variety of composite compounds along with peptidoglycan, teichoic and teichuronic acid and polysaccharides. The peptidoglycan consists of glycan (polysaccharides) chains of alternating N-acetyl-d-glucosamine (NAG) and N-

acetyl-d-muramic acid (NAM) and diaminopimelic acid (DAP), which is inimitable in prokaryotic cell walls. Actinomycetes own true aerial hyphae and are abundant in various kind of environments such as mountains, forests and river's mud and mainly occur in dry alkaline soil. Moreover, these actinomycetes are identified for the secondary metabolites production having medical principles such as antibiotics, antiprotozoal, antifungal, anticholesterol, antiviral and anticancer (3).

### 2.4.1 Morphology of actinomycetes

Actinomycetes are traditionally the ray fungi and are associated to the true fungi such as bread molds because they formed mats (mycelia) of branching filaments (hyphae). However, dissimilar with the true fungi, the actinomycetes have thin hyphae (0.5-1.5 mm in diameter) with genetic material twisted inside as free DNA. The cell wall of the hyphae is consisted of a cross linked polymer comprising amino acids short chains and amino sugars long chains (3).



Actinomycetes are the branching unicellular filamentous bacteria and replicate either by separation or by special spores or conidia and are described by the branching threads or rods formation (3). The filamentous forms of Actinomycetes are aerobic and may provide spore individually or in chains forms. The appearance of colony displays as a powdery mass and are pigmented by the aerial spore’s formation.

The spores are circular, cylinder-shaped, or oval-shaped and generate original microcolonies comprised the diverging system strands that after 24-48 h disintegrate

into diphtheroid, short chain and coccobacillary forms. The sporulating mycelium may be branching or non-branching, straight or spiral shape (3).

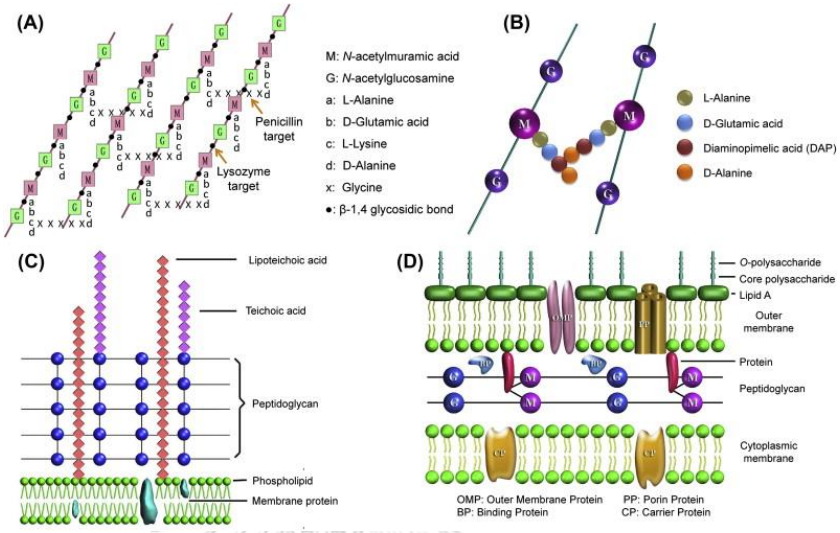



**Figure 3. Schematic representation of cell wall peptidoglycan (17).**

The cell wall is a stiff structure which maintains the cell shape and inhibits the cell exploding due to the high osmotic pressure. The cell wall comprises peptidoglycan consisting of glycan (polysaccharides) chains of alternating N acetyl-d-glucosamine (NAG) and N-acetyl-d-muramic acid (NAM) and diaminopimelic acid (DAP), teichoic and teichuronic acid. These features of peptidoglycan composition and structure are (i) diaminopimelic acid isomer on tetrapeptide side chain position 3, (ii) sugar content of peptidoglycan, and (iii) the presence of glycine in interpeptide bridges. In evidence, sugar patterns characteristic are present only in cell wall types II-IV of those actinomycetes with meso-diaminopimelic acid (3).

### 2.4.2 Life cycle of actinomycetes

Life cycle of actinomycetes originates the spore's germination as a result in filaments dispersion into the solid medium to form a vegetative mycelium. The developed vegetative mycelium appears sporophores which spread upright in the direction of the surface to occur aerial mycelium (18).

**Germination**: The spores remain as a "Free spore" remaining latent until the germination and germinates a "Germ tube" (18).

**Vegetative Growth**: The germ tube encourages a vegetative growth providing the substrate and aerial mycelium. Firstly, a germ tube generates a primary mycelium, so-called substrate hyphae under the media. After growing, it forms secondary mycelium, termed aerial hyphae above the substratum. During unfavorable conditions, the aerial hyphae develop spirally coiled (18).

**Septation**: At this step, septa configures between the vegetative hyphae and generally divide into 20 mm long cells and owing many bacterial chromosomes (nucleoids) (18).

**Spore maturation**: The septa occur within the vegetative hyphae maturation and forms a chain of spores. The spore, therefore, appears by fragmentation or swelling of the hyphae. Each spore comprises a single genome copy. Subsequent reproductive growth often proceeds with the development of filamentous aerial hyphae undergoing variation into unigenomic spores chains (18).

**Release of spore**: During the unfavorable condition, the spores get disconnected from the vegetative hyphae and are free in the environment. The resulting sheaths come to be spores and then another life cycle initiates (18).

Microbial secondary metabolites are organic compounds including antibiotics, pigments, toxins, enzymes, and antitumor agents are not directly involved in the normal growth, development or breeding of the producing organism. Microorganisms generate secondary metabolites throughout the stationary period of the growth cycle (19).

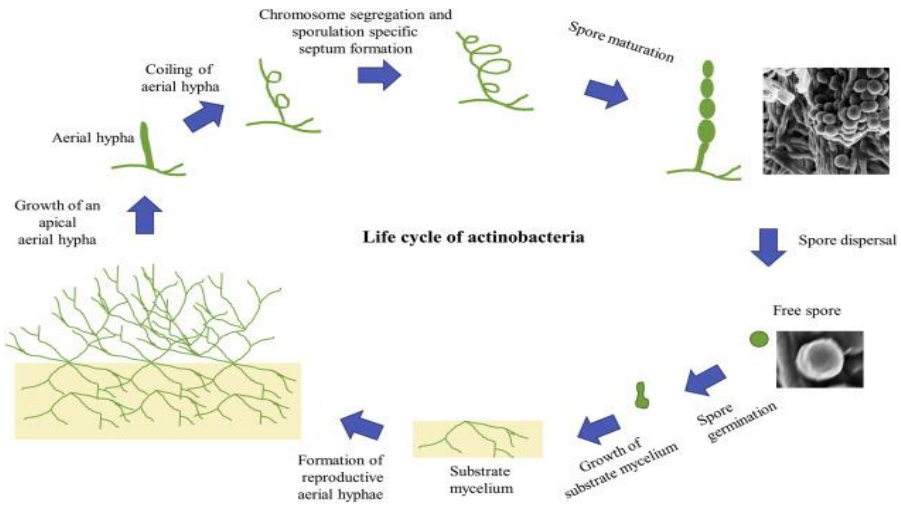



**Figure 4. Life cycle of actinomycetes (20).**

### 2.4.3 Habitats of actinomycetes

In the soil micro-ecosystem, many actinomycetes are isolated and screened from soil. Actinomycetes can be produced 75% of all known antibiotics, and nearly 80% of antibiotics are produced from *Streptomyces* genus. Population of actinomycetes are commonly distributed in upper soils layer and declines gradually depending on the depth of the soil; actinomycetes are existing in all soil layers. Actinomycetes are sensitive to waterlogged soil conditions and acidity/low pH (optimum pH 6.5-8.0) and are mesophilic organisms (25°C-30°C) and some species are thermophilic (55°C-65°C) in fertilizer (3).

## 2.5 Secondary metabolites from actinomycetes

Actinobacteria are an importance role in the biotechnological field, as an overabundance of bioactive secondary metabolites producers with extensive manufacturing, therapeutic, and farming applications. In certain, actinobacteria produce the conventional of the biologically antibiotics (21).



The first antibiotics in actinobacteria discovered by Waksman and coworkers existed as actinomycin from *Streptomyces antibioticus* in 1940, streptothricin from *Streptomyces lavendulae* in 1942, and streptomycin from *Streptomyces griseus* in 1944. Streptomycetes have been the major source of clinical antibiotics and are responsible for over 80% of all antibiotics of actinobacterial origin (21).

Actinomycetes produce aminoglycosides (neomycin, kanamycin, streptomycin, angucyclines (auricin; also, antitumor agents like landomycin and moromycin, ansamycins (rifamycin, geldanamycin), anthracyclines (primarily antitumor agents, e.g., daunorubicin), β -lactams (cephamycins) and β-lactamase inhibitor clavulanic acid, chloramphenicol, glutarimides (cycloheximide), glycopeptides (vancomycin, teichoplanin), lipopeptides (daptomycin), lantibiotics (mersacidin, actagardine), macrolides (clarythromycin, erythromycin, tylosin, clarithromycin), oxazolidinones (cycloserine), streptogramins (streptogramin), and tetracyclines (21).

The producing capacity of individual actinomycetes can also vary enormously. Some *Streptomyces* species produce a single antibiotic, while others yield a variety of diverse compounds. Moreover, Actinobacteria can isolate herbicides, antifungals, antitumor or immunosuppressant drugs, and anthelmintic agents (21).



**2.5.1 Phenolic compounds**

Natural phenolic phytochemicals have been increasing interest from the scientists for their benefits on health owing to their antioxidant activity (22). Phenolics are the most extensive secondary metabolite in both plants and microorganisms.

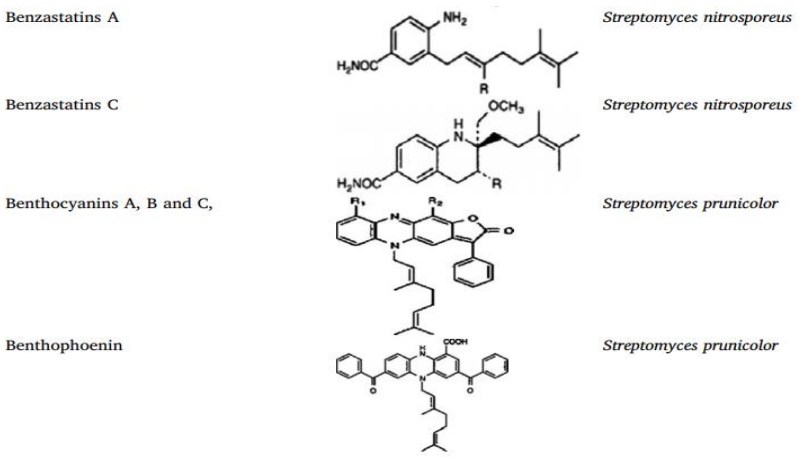



**Figure 5. Phenolic compounds from *Streptomyces* strains (23).**

Microbial fermentation has a potent effect on the production of phenolic compounds. This diverse group of compounds are designated as the resourceful natural antioxidants acting as either efficient radical scavengers or metal chelator. The radical scavenging activity may partially be due to the extensive diversity of phenolics, flavonoids, carotenoids, and ascorbate (13). Natural antioxidants, the only alternative to synthetic antioxidants, are safe and bioactive in stabilizing the free radicals related to the diseases. Due to redox function, the antioxidant activities of phenolics serve as the reducing agents, the hydrogen donors, and the singlet oxygen quenchers and serve an imperative character in the counteraction or adsorption of free radicals (24).

### 2.5.2 Antioxidant compounds

The natural antioxidants from the promising and safe source as instead of conventional synthetic antioxidants have been employed. Natural microbial products have both the effective therapeutics and the desirable pharmacokinetics for clinical

development; evidence is that microorganisms can produce drug-like small molecules. Actinomycetes are an essential source for production of bioactive secondary metabolites in industrial applications (13).

In the screening database for antioxidants from actinomycetes, primary metabolites with good antioxidants contain benthocyanins A to C and benthophoenin from *S. prunicolor* DSM 40335. Carazostatin and carbazomycin B from actinomycetes had antioxidant activity more than synthetic products (25). Terpenoids and naphterpins B and C from *Streptomyces* sp possess antioxidant activity (23).

Carbazole was obtained from *Streptomyces* sp. CMU-JT005 and phenazine hetero-cycles (N-H groups) have been established antioxidant activity. Moreover, 5-hydroxymaitol isolated from *S. melanogenes* JCM 4398 (26), naphtherin obtained from *S. aeriouvifer* CL190 (26) and 7-dimethyl naphtherpin produced from *S. violaceus* DSM 40082 (26) have been hindered peroxidation of lipid in mice liver microsome comparable with vitamin E. Derivatives of phenazine as the class of antioxidants were isolated from *Streptomyces* sp UICC B-92 (26). Benthophoenin and Carquinostatin A were obtained from *S. exfoliates* LP10 and *S. prunicolor* DSM 40335 particularly (27). Isoflavonoids derivative; 4′, 7, 8 trihydroxyisoflavone having antitumor and antioxidant activity was isolated from *Streptomyces* sp. (23).

In addition, stealthins A and B from *S. viridochromogenes* DSM 40110 show antioxidant activity better than vitamin E (28). Neocarazostatins A to C from *Streptomyces* sp. mycelium was also proved antioxidant potential and regarded as a

new class of therapeutic agents (28). Benthocyanins A to C from *S. prunicolor* DSM 40335 delayed lipid peroxidation is comparable to vitamin E. Moreover, these by-products retained functional group (O-H group) reacting with free radicals (23).

Actinomycetes from different environments possess antioxidant effects. *S. lydicus* DSM 40461 (29), *Streptomyces* sp. SRDP-H03 (28) and BI244 from coastal region and soil were also exhibited DPPH radical scavenging (27). *S. misionensis* INTA 3944 from forest soil in the mountain displayed strong antioxidant ability on NO scavenging, DPPH radical scavenging and hydrogen peroxide radical scavenging (30). (Z)-1-((1-hydroxypenta-2,4-dien-1-yl) oxy) anthracene-9,10-dione was obtained by *Nocardiopsis alba* CDCW 2536 from the mangrove soil yield (23).

*Streptomyces* sp VITTK3 from marine owned 96% DPPH free radical scavenging ability at the concentration of 5mg/mL. Similarly, marine *Streptomyces* sp VITSVK5 proved 5-(2, 4-dimethylbenzyl) pyrrolidine-2-one with 59.32% DPPH radical scavenging capability. JBIR-94 and JBIR-125 containing phenolic compounds and (O-H and N-H group) from *Streptomyces* sp R56-07, has been established DPPH radical scavenging ($IC_{50}$ 11.4 M and 35.1 M) (23).

Phenazoviridin from *Streptomyces* sp. MUSC 14, the derivative of glycosylated phenazine, inhibit the peroxidation of lipid (31). Further, thiazostatins A and B (N-H group) from *S. tolurosus* 1368-MT1 (31) and benzastatins A to D were manufactured from *S. nitrosporeus* DSM 40023 (culture broth) possessing both a rare p-aminobenzamide unit and a tetrahydroquinolone ring (32).

A diketopiperazine having antioxidant activity with 72% activity at 10mmol and 51% activity at 1mM using DPPH free-radical scavenging assay was isolated from *Pseudoalteromonas haloplanktis* TAC 125 compared to hydroquinone with 100% activity. The diketopiperazine derivative; pyrrolo pyrazine-1, 4-dione, hexahydro-3-(2-methylpropyl) from *Streptomyces* sp. VITMK1 is a nontoxic potential scavenging free radicals (33).

Phenol,2,4-bis(1,1-dimethylethyl)-(1) having antioxidant and cytotoxicity against Hela cells was isolated from *S. cavouresis* KUV 39 from vermicompost samples in India. Phenolic compounds from *Streptomyces* sp. MUM 256 can be used for prevention of oxidative-stress related diseases and chemotherapeutics drugs (34). Besides the detection of pyrrolopyrazine in *Streptomyces sp* MUSC 5, pyrrolo [1,2a] pyrazine-1,4-dione, hexahydro isolated from *Bacillus* sp. in sponge can be reduced oxidative damages by free radicals. (33)



Probiotic food wirh millions of bacteria are essential in the human diet. These bacteria can colonize the gastrointestinal tract and yield exopolysaccharides (EPS) with antioxidant effect (35). EPS from *Lactobacillus paracasei* subsp. *paracasei* and *Lactobacillus plantarum* had antioxidant activity (DPPH radical scavenging, chelation of ferrous ions, inhibition of linoleic acid peroxidation and reduction power) (36).

*Lactobacillus casei, Lactobacillus acidophilus* and *Lactococcus lactis* in fermented milk exhibited antioxidant and cholesterol reducing activities and potent DPPH, malonaldialdehyde and $H_2O_2$ radical scavenging together with inhibition of

linoleic acid peroxidation. Moreover, *L. casei* have maximum Trolox equivalents (48.7 mM) followed by *L. acidophilus* (46.3 mM) and *L. lactis* (23.4 mM) (36).

*Bifidobacterium* sp. *Leuconostoc* sp., *Lactobacillus* sp. *Lactococcus* sp., *Kluyveromyces* sp. together with yeast *Saccharomyces* were evaluated for radical scavenging activity (3-53%) (37). *Lactobacillus acidophilus* exhibited (42-53%) radical scavenging activity. Low inhibition rate (3-10%) has been occurred in *Lactobacillus lactis* strains, *Lactobacillus casei*. The inhibition rate (12-42%) was exhibited in *Lactobacillus* strains and *Lactococcus* strains (3-22%). The lipid peroxidation inhibition during the fermentation process showed 90% inhibition and in whey parts with (20-24%) by ABTS assay. EPS are produced during fermentation process and improved antioxidant activity beside organic acids (38).

Resorstatin with anti-lipoperoxidative activity and xanthin with inhibition of linoleic acid peroxidation were produced from the Gram-negative *Pseudomonas* bacteria. (39). *Bifurcaria bifurcate* were isolated; almost a few-part of this microbial group contains *Vibrio* sp. (48.72%) and *Alteromonas* sp. (12.82%) and *Shewanella* sp. (12.26%). Methanol and dichloromethane extracts exhibited natural sources of antioxidant with DPPH radical scavenging, oxygen radical absorbance capacity (ORAC), and total phenolic content (TPC) (40).

## 2.6 General description of RAW264.7 macrophage cell

| | |
|---|---|
| Organism: | Mouse (Adult, Male) |
| Strain: | BALB/c |
| Tissue: | Abelson murine leukemia virus-induced tumor; ascites |
| Cell Type: | Macrophage; Abelson murine leukemia virus transformed |
| Culture Properties: | Adherent |
| Storage Conditions: | - 80℃ / Liquid nitrogen vapor phase |

### 2.6.1 Macrophage

Macrophages are the primary responders to initiation and propagation of inflammatory particles (41) and are divided into classically activated (M1) and alternatively activated (M2) subclasses. Classically activated M1 macrophages are categorized by proinflammatory cytokines such as interleukin-1$\beta$, IL-12, tumor necrosis factor-$\alpha$ (TNF-$\alpha$), and superoxide anions. The M2 type can produce anti-inflammatory mediators IL-10 and tumor growth factor-$\beta$ and express cell signals for tissue remodeling and inflammation (42).



#### (i) Classically activated macrophage (M1)

In response to dangerous endogenous signals, macrophage can produce proinflammatory mediators and modify the surface markers. Classically activated type could be triggered by either interferon-$\gamma$ (IFN$\gamma$) produced by NK cells and adaptive T helper 1 (TH1) cells or pathogen-associated molecular patterns (PAMPs) distributed

through Toll like receptor (TLRs) via MyD88 dependent manner. For the meantime, TLR3 and TLR4 stimulus can stimulate MyD88 independent pathway with the induction of IFN$\beta$ secretion which is facilitated by Toll/IL-1 receptor (TIR) area with connector and knockdown of TIR leading to inflammation. The classically activated macrophages induce secretion of inflammatory mediators facilitating the antigen release and employment of TH1 reaction for ensuing pathogen destroying impact. The classically activated macrophages are associated with the upregulation of ferritin and the ferroportin reduction, causing the iron retention and inflammation. The glycolytic flux is satisfactory in classical M1 types involving the extraction variations of 6-phospho-2-kinase isoforms from liver-type to abundant isoenzyme, thus sustaining the fructose-2,6- bisphosphate.

Moreover, classically activated macrophages are related with the aerobic glycolysis and extracellular acidification rate; increasing HIF-1$\alpha$ links with IL1$\beta$ promoter and sustains IL1$\beta$ production in M1 macrophages. Through regulating glycolytic flux, inhibition of carbohydrate kinase like protein CARKL activates M1 polarization whereas pyruvate dehydrogenase kinase 1 PDK1 encourages aerobic glycolysis in M1 macrophages. At a distance from glucose metabolic rate, activated M1 type is supplemented by increment of COX-2 and decrement of COX-1, thromboxane A synthase 1, arachidonate 5-lipoxygenase, and leukotriene A4 hydrolase (42).

**(ii) Alternative activated macrophage M2**

In difference to classical M1 type, innate immune cells including basophils and mast cells and other adaptive cells create IL-4 and IL-13 priming M2 alternative phenotype. IL-4 stimulated M2 type stated elevated intensity of IL-10, decoy receptor IL-1R, IL-1R antagonist, chemokines CCL22 and CCL17, and intracellular enzyme arginase-1 (42).

**2.6.2 Morphology of RAW264.7 macrophage cells**

RAW 264.7 macrophage cell is designated as an adherent line and is handled as a monolayer. In the initial stages, the cells attach and appear cuboidal similar with thin extensions. While the compact culture becomes, another layer of rounded cells is attached to the original monolayer. In extremely heavy cultures, cells into the media are usually both attached and suspended cells.

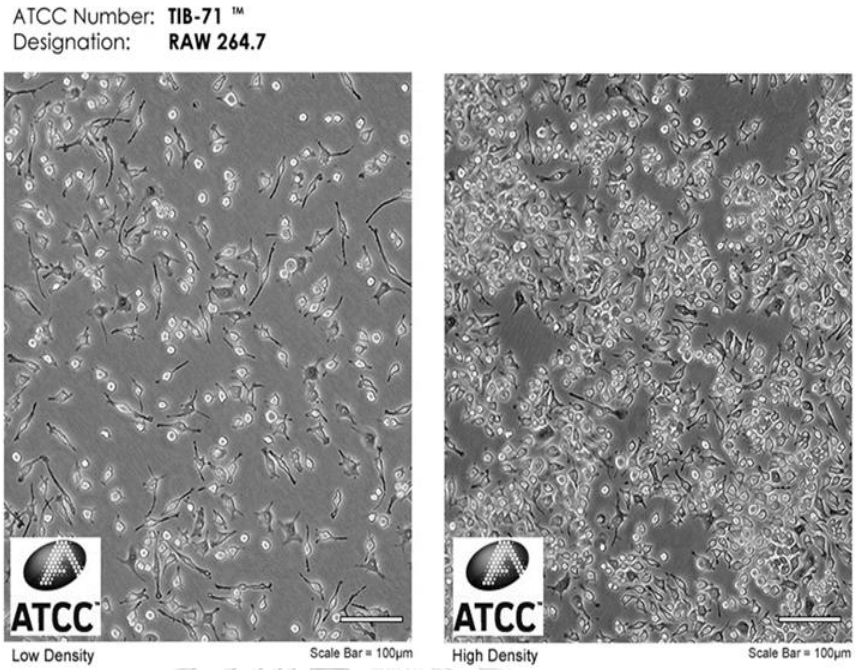



**Figure 6. Morphology of raw 264.7 macrophage cell.**

(https://www.atcc.org/products/tib-71)

### 2.6.3 Macrophages as the major source of oxidative stress

In macrophages, NO is synthesized by iNOS; while NADPH oxidase produces superoxide anion which interacts with NO leading to peroxynitrite. This iNOS-derived peroxynitrite forms nitrotyrosine having antibacterial and cytotoxicity on macrophages. These peroxynitrite interacts with lipids, DNA, and proteins causing cellular responses ranging from signaling cell modulations to overwhelming oxidative injury. In vivo, peroxynitrite generation has been related to inflammatory diseases, chronic heart failure, diabetes, cancer, and neurodegenerative disorders (43).

NO is an intercellular mediator synthesized by three forms of nitric oxide synthases (NOS) namely endothelium NO synthase (eNOS), neural NO synthase (nNOS)

and inducible NO synthase (iNOS). Macrophages are important components of the mammalian immune system and provide an instantaneous protection against foreign mediators before the leukocyte migration and the pro-inflammatory mediators release such as nitric oxide (NO). Lipopolysaccharide (LPS) produced from the cell walls of Gram-negative bacteria, is the most powerful macrophages activator and entails the pro-inflammatory cytokines release (43).

# CHAPTER III

# MATERIALS AND METHODS

## 3.1 Collection of soil samples

Soil samples were collected from Nong Khai Province (North East; NE-1), Nakhon Ratchasima Province (North East; NE-2), Khon Kaen Province (North East; NE-3), Phichit Province (Central Thailand; CT-1 and CT-2), Chachoengsao Province (Eastern Thailand; ET-1, ET-2 and ET-3) and Udonthani Province (JA03 and MKP33). The surface layer of the soil samples was dug up into 5-10 cm depth and the samples were placed in the Ziploc plastic bags for prevention of external contamination and stored in room temperature (44).

## 3.2 Isolation of actinomycetes from soil

In general methods for bacterial isolation, serial dilution for reduction of overcrowding, pouring plate, streaking and centrifugation techniques are also relevant for isolation of actinomycetes. (45).



Soil samples were taken from the ground surface area and kept in the Ziploc plastics bags to prevent environmental contamination and stored at room temperature. For the isolation of actinomycetes, the soil samples were pretreated by using air-dried, 100°C heated and treated with 1.5% phenol. These soil samples were approved by shifting 1 ml of suspension until serial dilution factor ($10^{-1}$-$10^{-4}$) and these suspensions were seeded by spread plate method on humic acid vitamin agar

accompanied with nalidixic acid to prevent Gram-negative bacteria and cycloheximide to prevent fungal growth and then incubated at 30°C, for 14 days. The pigmented powdery colonies were selected from the culture and these isolates were purified and preserved on the ISP 2 slant at 4 °C for maintenance until use in this study.

### 3.3 Identification methods of actinomycetes

The identification methods were used such as: i) Phenotypic characteristics based on morphological, cultural, physiological, and biochemical characteristics, ii) Chemotaxonomic characteristics based on the diamino acids component in cell wall in peptidoglycan, iii) Genotypic characteristics based on the 16S rRNA sequences and genome analysis of the selected isolates compared with the type strain sequences.

#### 3.3.1 Phenotypic characteristics



The inoculum of all isolates was cultured in ISP 2 broth in a shaking condition at 180 rpm 30 ℃ for 3-5 days. These culture broths were washed with 0.85% normal sterile saline solution 3 times. These inoculums were used for all phenotypic studies. The phenotypic characteristics comprise morphological, cultural, physiological and biochemical features according to Shirling and Gottlieb (46).

**Morphological characteristics**

The selected actinomycete isolates were observed by using scanning electron microscopy after cultivation on yeast extract-malt extract agar (ISP-2 medium) at 30°C for 7-14 days.

**Cultural characteristics**

Actinomycete isolates were cultivated at 30°C for 14 days on ISP2 agar and the colonial appearance and color, aerial and substrate mycelium and diffusible pigment production were compared by using the colour of designation with the NBS/ISCC color system (47).

**Biochemical characteristics**

Decomposition of various compounds was examined using the basal medium (48). Carbon source utilization was examined using ISP-9 medium supplemented with a final concentration of 1% the tested carbon sources (49). Gelatin liquefaction, peptonization of milk, nitrite production and starch hydrolysis were tested on the various media (50).



**Physiological characteristics**

Actinomycete isolates were incubated on ISP-2 medium at different temperatures (28 and 45 °C), NaCl concentrations (2, 4 and 6 % w/v) and pH range (5, 7 and 10) for 7-14 days.

### 3.3.2 Chemotaxonomic characteristics

Chemotaxonomy is the variation of chemical compounds in the microbial cell and the chemical characteristics to classify and identify bacetria according to Shirling and Gottlieb method has been recommended for applying to the species, genus, and taxa level. Therefore, it is related to the chemicals distribution in cell envelope including amino acid, sugar, polar lipids, menaquinones, mycolic acid and fatty acid by the extraction, the fractionation, the purification and the resolution of the target products (46).

The diaminopimelic acids (DAP) were examined by thin layer chromatography (TLC) according to Staneck and Robert method (1974). In brief, the cells were hydrolyzed with 6N HCl at 121°C for 15 mins. The residual cell was analyzed by using cellulose TLC plate (20 x 20 cm) with twice the developing solvent system as MeOH: $H_2O$:6NHCl:pyridine (80:26:4:10, v/v). The DAP isomer spots were detected as dark-green spots by spraying with 0.2% ninhydrin solution and heating at 110°C for 5 min and compared with DAP standard solution.



### 3.3.3 Genotypic characteristics

#### 16S rRNA gene sequence

Pure culture of each strain was inoculated in freshly ISP 2 broth and incubated at 28°C, 200 rpm for 2 days. The genomic DNA was extracted by physical and chemical combination methods (51). The 16S rRNA gene was amplified from the extracted

genomic DNA by using the universal primers 1492R (5′ACGGCTACC TTGTTACGACTT 3′) and 27F (5′AGTTTGATCCTGGCTCAG 3′) directions. Master mixer for PCR reaction (final volume 50μL) contains 20 F and 1530 R primers (10 pmol $\mu L^{-1}$), dNTP (10 mM), 10X Taq buffer (Buffer A), $MgCl_2$ (50 mM), Taq DNA polymerase, ultrapure water and DNA template. The thermal cycling of three processes is described: denaturation of target DNA at 94°C (3 mins) followed by 29 cycles at 94°C (1 min), and then annealing of the primer at 50 °C (2 mins), and elongation of primer at 72°C (2 mins). In the end of the cycle, the reaction mixture was held at 72°C for 2 mins and then cooled to 4°C. The amplified PCR product was visualized by gel electrophoresis. The amplified product was purified, and the obtained sequences were sent to Macrogen (Seoul, Korea). The amplified PCR product was sequenced by sequencing service (Macrogen-Seoul, Korea). The sequences were analyzed using EzTaxon-e server BLASTn searching program; (http://www.ezbiocloud.net/eztaxon) and aligned against the selected type strains sequences, which received from the GenBank using Bioedit software.



**3.3.4 Whole genome sequencing analysis**

Genomic DNAs were extracted using a PureLink™ genomic extraction kit (Invitrogen; USA). The genomic DNA were sequenced using the MiSeq sequencing system (Illumina; USA). The bioinformatics data of the genome were analyzed using the bacterial bioinformatics database and analysis resource (PATRIC) (52). The biosynthetic gene cluster in the genome were determined using the antiSMASH

database (53). The phylogenetic tree based on genome data was constructed using TYGS (54).

### 3.4 Fermentation and extraction of secondary metabolites from actinomycetes

The pure culture of the selected strains was cultivated in the seed medium (100ml) at 30°C for 3 days, 180rpm. Additionally, 2% of each inoculum was transferred into ISP-2 broth (1000ml) and incubated at 180 rpm, 30°C for 14 days. These fermentation broths were partitioned with ethyl acetate (2:1) using separatory funnel. These ethyl acetate extracts were evaporated on a rotary evaporator at 45°C to get the dry extract. After evaporation, the ethyl acetate extracts were evaluated for the determination of antioxidant assay.

### 3.5 Evaluation of antimicrobial activity by disk diffusion method



1 -2 loops of actinomycetes isolates were inoculated in ISP-2 broth and incubated at 30°C for 3 days. Then, the culture broth of each isolate was transferred to four production media (10ml) including 30 medium, 54 medium, 57 medium and yeast-dextrose (YD) medium and incubated at 180-200 rpm for 14 days. After that, equal volume of 95% ethanol (1:1 v/v) was added into the tubes and then centrifuged at 3400 rpm for 25 minutes and then the supernatant was collected and kept at -20℃. Mueller-Hinton Agar (MHA) was used to for antimicrobial activity by the disk diffusion method. Tested bacteria, including *Staphylococcus aureus* ATCC 25923, *Bacillus*

*subtilis* ATCC 6633, *Kocuria rhizophila* ATCC 9341, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and the tested fungi, *Candida albicans* ATCC 10231, were cultivated on Mueller Hinton Agar (MHA) at 37°C for 24 h before antimicrobial susceptibility testing. One loopful of tested bacteria were added into the tubes containing 0.85% sodium chloride (NaCl) compared with 0.5 McFarland to estimate the bacterial growth turbidity. The microbial suspension was taken with the sterile swab and the sterile swab was spread all over the MHA surface at an angle of 60°. The supernatant was placed onto the sterilized filter paper discs (8mm) and then air-dried for 30 mins and placed onto the inoculated plates with sterile forceps. The plates were put at 4°C for 30 mins and incubated at 30°C overnight. The inhibition zone (mm) was recorded by measuring the Vernier caliper (55).

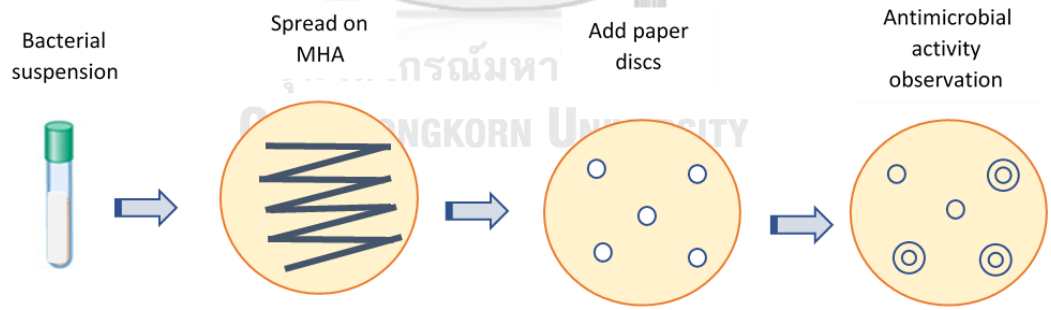



**Figure 7. Antimicrobial activity based on disk diffusion method.**

### 3.6 Evaluation of antioxidant activity

#### 3.6.1 Determination of total phenolic content assay

**Principle**

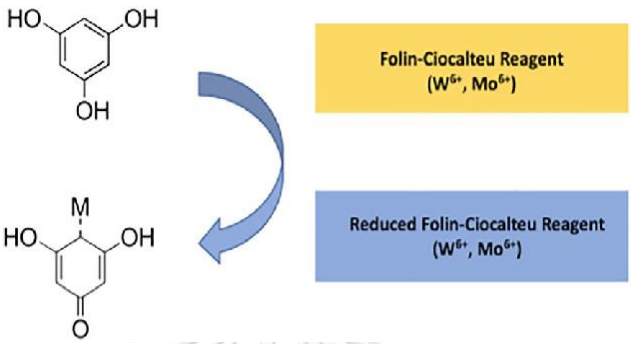



**Figure 8. Principle of Folin-Ciocalteau method (56).**

**Preparation of Folin-Ciocalteu reagent**

Folin-Ciocalteu reagent (FCR) (1ml) in DI water (9ml) (1:10 v/v) and sodium bicarbonate ($NaHCO_3$) (1g) (10% v/v) in DI water (10ml) were prepared (57).

**Procedure**

The Folin-Ciocalteau method was assessed for estimation of total phenolic content (TPC) and revealed minimum quantity of phenolics. 10µl ethyl acetate extract, 40µl FCR followed by 40µl sodium bicarbonate and 160µl DI water were adjusted into each well (250µl) and incubated for 15 mins at RT. Gallic acid (standard) and ethanol (negative control) were used and then the absorbance was measured spectrophotometrically at 765nm. The total phenolic content of extracts was obtained

from an absorbance–gallic acid standard curve and expressed as gallic acid equivalents (GAE)/mg of extract.

### 3.6.2 Determination of Ferric-Reducing Antioxidant Power (FRAP) Assay

**Principle**

FRAP assay is done by using Benzie and Strain method (1999). FRAP assay measures the antioxidants' ability to reduce the ferric iron and the antioxidant activity is measured by the reduction of the ferric complex ($Fe^{3+}$tripyridyltriazine (TPTZ) 2,3,5-triphenyl-1,3,4-triaza-2-azoniacyclopenta-1,4- diene chloride which is colorless to the blue-colored ferrous complex ($Fe^{2+}$tripyridyltriazine). This action is due to the electron donating antioxidants at low pH. This reduction is measured spectrophotometrically at 593 nm (4).

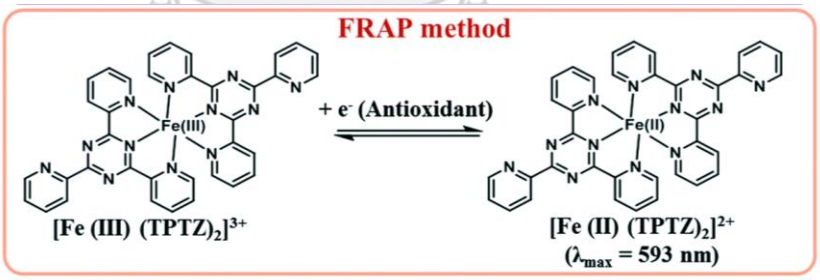



**Figure 9. Principle of FRAP assay.**

**Preparation of FRAP reagent for FRAP assay**

**Reagent A: 300 mM sodium acetate buffer solution** (pH 3.6): glacial acetic acid was added to sodium acetate trihydrate. This solution was made up to volume with distilled deionized water.

**Reagent B: 10 mM TPTZ solution:** TPTZ was added in 40mM HCl and dissolved at 50°C. 40mM HCl were prepared by mixing with 6M HCl and distilled deionized water make up to a final volume with distilled deionized water.

**Reagent C: 20mM $FeCL_3.6H_2O$ solution:** ferric chloride was dissolved in distilled deionized water.

The ferric reducing antioxidant power (FRAP) reagent (pale yellow orange in color) was prepared by mixing 300mM sodium acetate buffer, 10mM TPTZ in 40mM HCl and 20mM $FeCl_3.6H_2O$ (10:1:1) at 37°C.

**Procedure**

Antioxidant activity was measured by FRAP method characterized by intense, blue-colored due to electron donating antioxidants. FRAP reagent with pale yellow in color was obtained by mixing 300mM sodium acetate buffer, 10mM TPTZ in 40mM HCl and 20mM $FeCl_3.6H_2O$ (10:1:1). Ethyl acetate extracts (20µl) and DI water (20µl) followed by FRAP reagent (180µl)) were incubated at room temperature for 15 mins. Different concentration of ascorbic acid (10 µg/ml - 160 µ g/ml) was used to set up a standard curve. The reducing power of ethyl acetate extracts is directly related to the absorbance of the reaction mixture at 593 nm spectrophotometrically. FRAP values were expressed as µg of ascorbic acid equivalent/mg of extracts (µg AAE/mg of the extract).

### 3.6.3 Determination of DPPH radical scavenging assay

**Principle**

1, 1-diphenyl-2-picrylhydrazyl (DPPH), a stable free radical, delocalizes the spare electron over the molecule. The electron delocalization shows the deep violet, spectrophotometrically measured by the absorbance at 517 nm. DPPH solution is mixed with that of a substrate (AH) donating hydrogen atom, and occurs to the reduced form having no appearance in violet color This discoloration indicates the antioxidant compounds scavenging potential in terms of hydrogen donating ability (4).

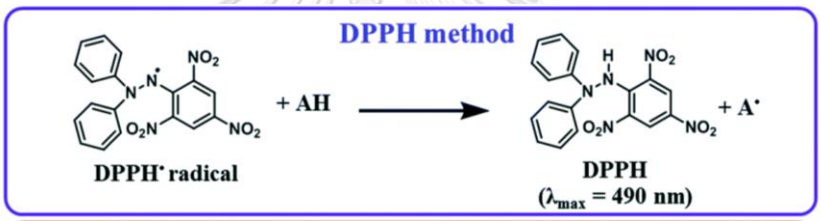



**Figure 10. Principle of DPPH assay.**

**Preparation of DPPH (50µM) solution**



DPPH (1,1-diphenyl-2-picrylhydraxyl) was dissolved in ethanol (V= mg/MW) (1mM). From this stock solution, DPPH solution was diluted with ethanol (50µM DPPH solution).

**Procedure**

Antioxidant activity was shown by DPPH radical scavenging method, described by intense violet due to the free electrons. Ethyl acetate extract was dispersed in ethanol for serial concentration (50-1000µg/mL). 100 µl each dilution was mixed with

100 µl DPPH solution and incubated in the dark at 37°C for 20 mins. Ethanol (the negative control) and vitamin C (the positive control) were used. Absorbance was measured spectrophotometrically at 517 nm. The percent scavenging of DPPH was calculated from the equation below.

% DPPH radical scavenging = $[(A_0 - A_1) / A_0] \times 100$

$A_0$ = Absorbance of the control, $A_1$ = Absorbance of the tested sample

Due to the darker color of extract, then DPPH activity may get affected. In such cases, lower concentration of ethyl acetate extract must get proper results.

**3.6.4 Determination of nitric oxide (NO) scavenging assay**

**Principle**

Sodium nitroprusside in the aqueous solution at physiological pH (7.2) spontaneously generates NO interacting with $O_2$ for nitrite ions production estimated by Griess reagent. Scavengers of NO compete with $O_2$ leading to reduction in nitrite ions production (4).



**Preparation of Griess reagent and sodium nitroprusside**

Griess reagent (1g) was dissolved in ultrapure water(25mL) (58) and sodium nitroprusside dihydrate was dissolved in 10 mM phosphate buffer solution (PBS).

**Procedure**

The tested extract (50µl) was incubated with sodium nitroprusside (10mM) (50µl) in phosphate buffer saline (PBS) (pH 7.4) at room temperature for 2 hours. The same procedure was followed for the standard without the test samples in the same

amount of sodium nitroprusside. 100 µl Griess reagent was added into the tested samples after incubation for 2 hours. The absorbance at which the color (a purple azo dye) changed was measured at 542 nm on spectrophotometer. This was due to the diazotization of the nitrite with the sulphanilamide and coupling with the napthyl ethylene diamine hydrochloride. The procedure was carried out similarly with ascorbic acid as the standard.

**Calculation**

% inhibition = (OD of the control – OD of the tested sample) / OD of the control x 100

**3.7 Evaluation of intracellular ROS generation**

**3.7.1 Cells culture**

RAW 264.7 macrophage cells were obtained from the American Type Culture Collection (ATCC) and re-suspended in supplemented culture medium (DMEM with 10% fetal bovine serum (FBS), 1% of streptomycin and penicillin to prevent the contamination and 3.7 g of sodium bicarbonate) and in a humidified incubator (5% $CO_2$ at 37 °C). Cells confluent (approximately 80%) was sub-cultured every 3-5 days and was standardized to 2 x$10^5$cells/ml. (59).

### 3.7.2 Cell cytotoxicity assay

**Principle of MTT assay**

MTT assay, a colorimetric assay for measuring cell metabolism, is one of the most widely used methods to analyze the cell proliferation and the viability and based on the ability of nicotinamide adenine dinucleotide phosphate (NADPH)-dependent cellular oxidoreductase enzymes to reduce the tetrazolium dye to its insoluble formazan with a purple color. This assay measures viability of cell in terms of enzymatic reduction of the tetrazolium compound to the insoluble formazan crystals by dehydrogenases occurring in the mitochondria of the living cells. In MTT assay, dimethyl sulfoxide (DMSO) is used to dissolve insoluble purple formazan into a colored solution. The absorbance can be measured spectrophotometrically at 570 nm.

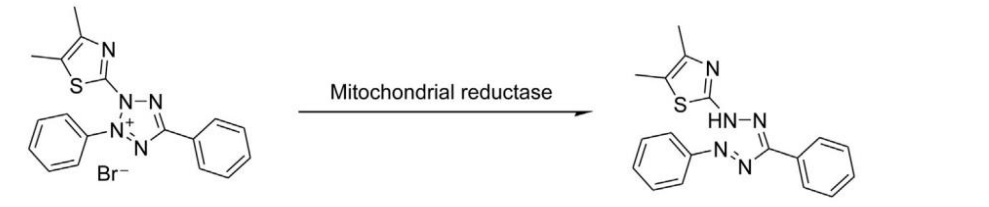



**Figure 11. Enzymatic reduction of MTT to formazan (60).**

**Procedure**

The cell viability was measured using MTT-based assay. The cells were seeded at a concentration of 2x $10^5$cells/well in a 96-well plate and incubated for 18-24 hours. The macrophage cells were treated with different concentration of extracts and incubated for 18-24 h. After 24 hrs. incubation, MTT solution was added to the wells

and incubated for 3 h and then removed. After media aspiration, dimethyl sulfoxide (100% DMSO) was added to each well in dissolving the crystalline deposits on the cells. The optical density of the cells at 540 nm was measured using microplate reader.

% Cell viability = OD of tested sample/ OD of the control x 100

**3.7.3 Cell pretreatment and induction of oxidative stress**

RAW 264.7 macrophage cell was cultured in a 96-well plate at a density of $2 \times 10^5$ cells/well and cultured with 5% $CO_2$ at 37 °C for 18-24 h. Cells confluence (80%) was pretreated with ethyl acetate extract in serum-free media with 0.5% DMSO at 37 °C for 24 h. Then, the pretreated cells were exposed to $H_2O_2$ in serum-free media for 30 mins. Untreated cells and cells treated with $H_2O_2$ alone were used as control, respectively (61).

**3.7.4 Determination of intracellular ROS generation**



**Principle of DCFH-DA assay**

2′,7′-dichlorofluorescein diacetate (DCFH-DA) is used to detect the intracellular radical generation. This probe can be permeable within the cell and hydrolyze intracellularly to the DCFH non-fluorescence probe in the cell. DCFH-DA can measure intracellular generation of $H_2O_2$ and other oxidants or monitor redox signaling variations in cells by the intra- or extracellular activation of the oxidative stimulus (62). After cell uptake, DCFH-DA is deacetylated by the cellular esterase to a nonfluorescent

compound, oxidized by ROS into 2'-7'dichlorofluorescein (DCF), a fluorescent probe detected by a maximum excitation and emission spectra of 485 nm and 535 nm respectively.

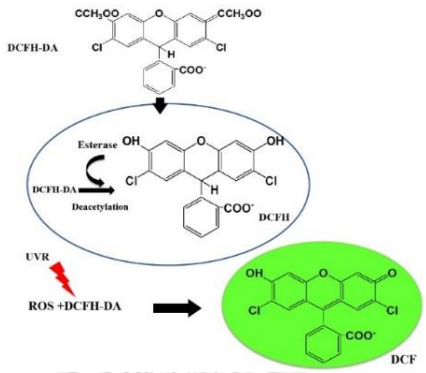



**Figure 12. Principle of DCFH-DA assay (63).**

**Procedure**

Intracellular reactive oxygen species (ROS) level was evaluated by monitoring fluorescent signal generated from oxidized DCFH-DA. The fluorescence intensity is generally considered to reflect the level of intracellular ROS. In brief, RAW 264.7 macrophage cell ($2 \times 10^5$ cells/well) were seeded in a 96-well plate for 18-24 h incubation and pretreated with a variety of concentrations of the crude extract for 24 h. After the incubation treatment, cells were incubated with DCFH-DA at 37 °C for 30 min before incubation with $H_2O_2$ for 30 mins. After incubation, the intracellular ROS were detected by observing DCF using fluorescence microplate reader at excitation 485 nm and emission 525 nm, respectively (61).

### 3.7.5 Determination of nitric oxide (NO) production

**Principle**

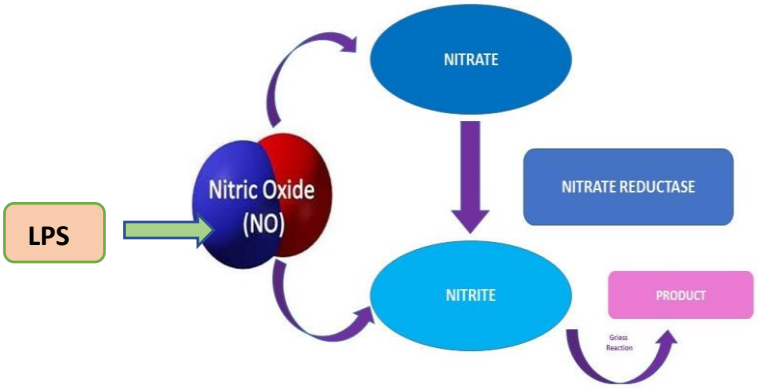



**Figure 13. Principle of NO assay.**

**Procedure**

Nitrite was measured as an indicator of NO production based on the Griess reaction (43). RAW 264.7 cells were seeded at a density of ($2\times10^5$ cells/well) in 96 well plates and incubated for 24 h at 37 °C and 5% $CO_2$. Different concentrations of ethyl acetate extract in serum free medium and lipopolysaccharide (LPS) were pretreated for 24 h. Briefly, an equal volume of supernatant and Griess reagent in 96-well plate were incubated at room temperature for 10-15 min. Then the absorbance was measured at 540 nm using the fluorescence microplate reader. A standard curve was prepared using sodium nitrite as a standard solution to calculate the concentration of NO.

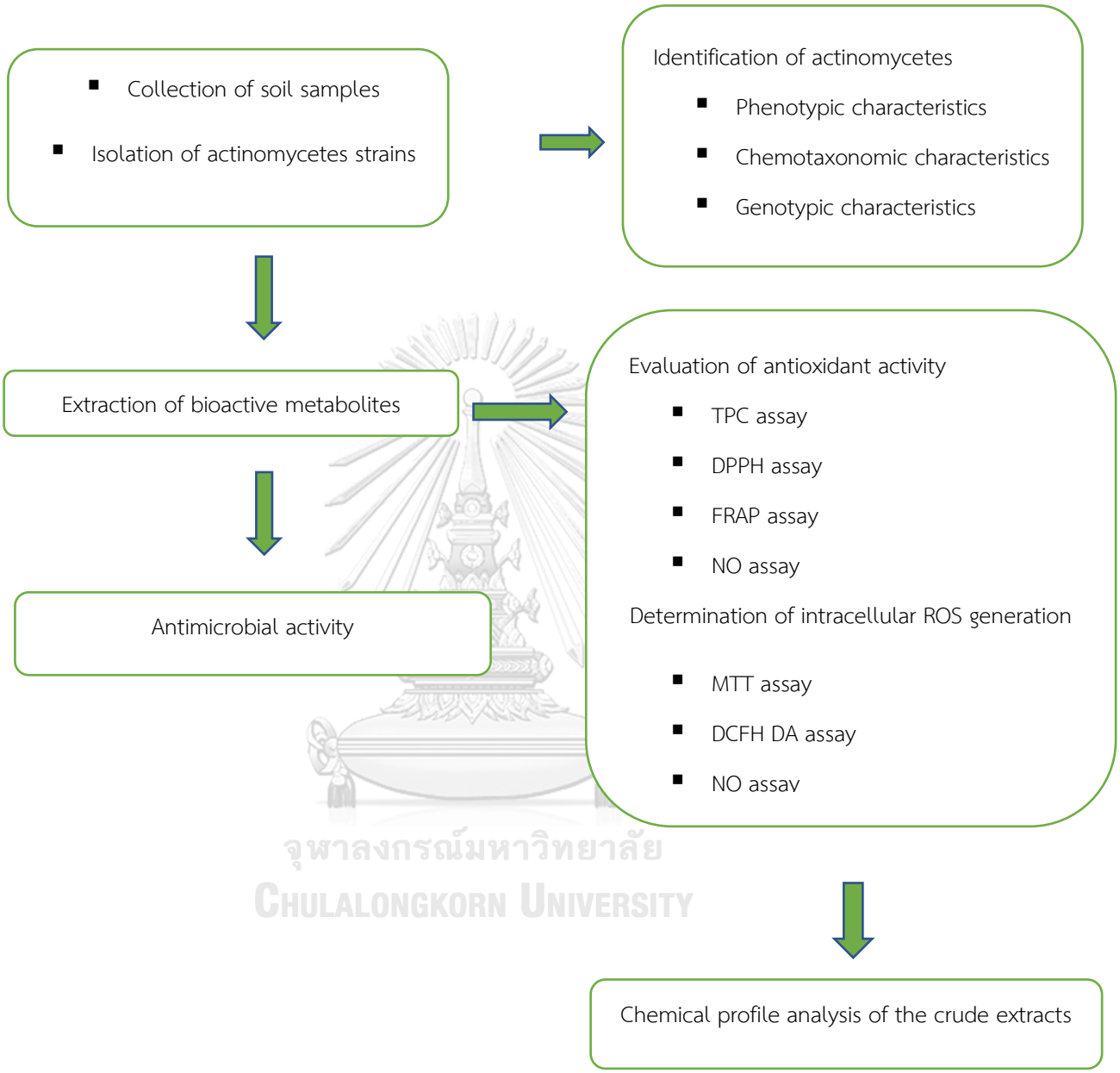
### 3.8 Chemical profile analysis of the crude extracts

The chemical profile (UV and retention time) of the crude extracts that have potential antimicrobial or antioxidant activity was analyzed by using HPLC equipped with a C-18 column, with the linear gradient system. The UV/UVVIS was used as a detector. The HPLC chromatograms was compared with the in-house database. The LC/MS/MS system equipped with Inertsil ODS-4 column was used for measuring the LC-ESI-MS spectra. The chemical profile (retention time, UV absorbance and pseudomolecular ion) was compared with the reported chemical profile of in-house database and the Dictionary of Natural Product database.

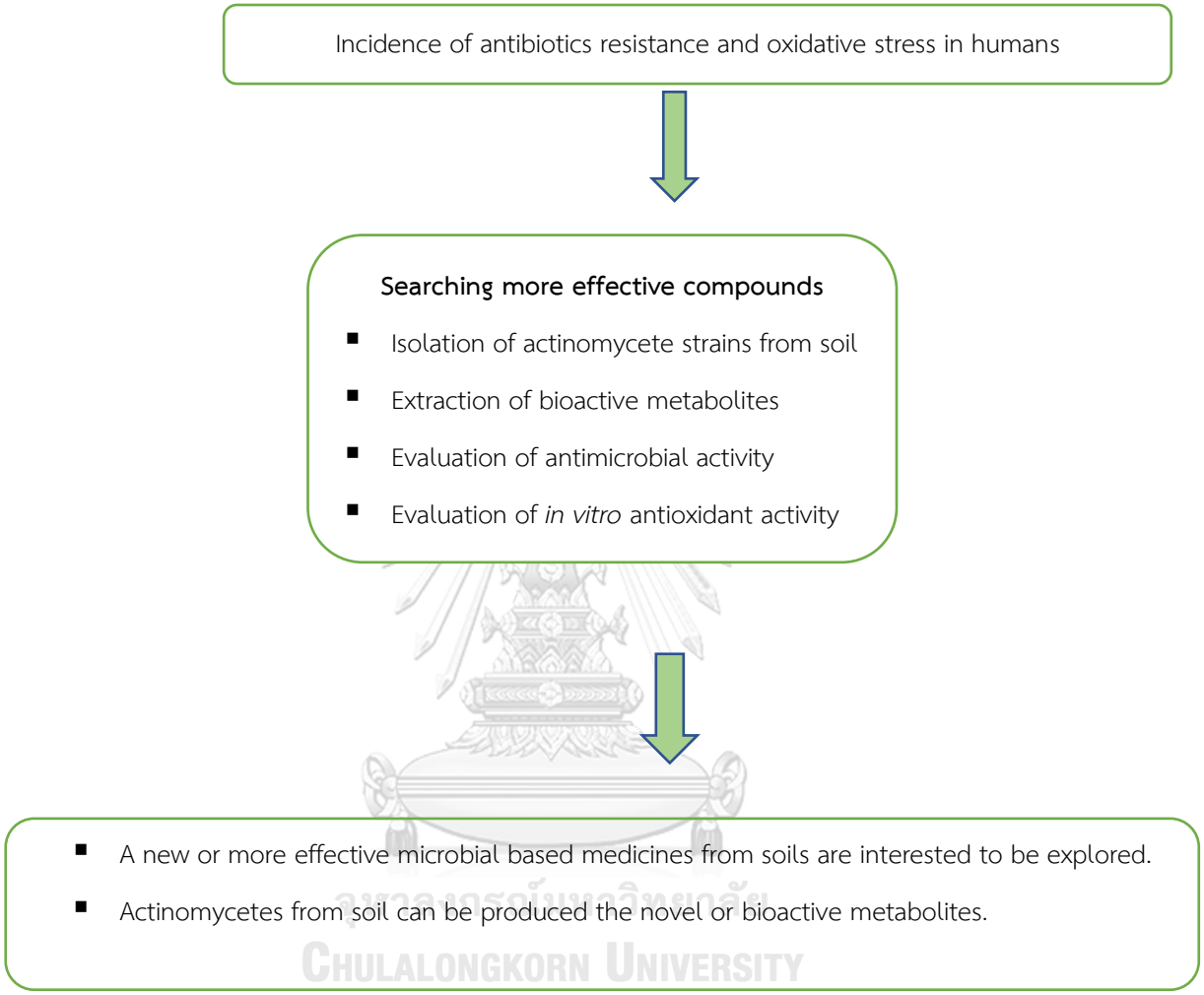
### 3.9 Statistical analysis

Statistical analysis was performed using SPSS Statistics 22 (22.0.0.0) Software. All parameters were performed in three independent experiments. Data were reported as mean ± standard errors of mean (SEM) and analyzed statistically by one way analysis of variance (ANOVA) followed by Fisher LSD test. Values with $p < 0.05$ was considered statistically significant.

## EXPERIMENTAL DESIGN



## CONCEPTUAL FRAMEWORK



# CHAPTER IV

# RESULTS

## 4.1 Identification of actinomycetes

### Phenotypic and chemotaxonomic characteristics

Actinomycete isolates were characterized by cultural characteristics including colors of aerial and substrate mycelia. The isolate examined were in the series of blue, green, white, purple, yellow and grey and reverse side pigmentation green, yellow, brown, grey and orange on ISP2 medium compared with NBS/ISCC color system, physiological features affecting the pH range (5-10) of medium, different concentration of NaCl (4-6 %) and incubation temperature (28°C and 45°C) observed optimal growth in ISP 2 media with 28 °C and pH 7 and except at 2 % NaCl, biochemical features including the coagulation and peptonization of milk, the gelatin liquefaction, the reduction of nitrite and the starch hydrolysis and on acid production from carbon sources and chemical composition in cell wall peptidoglycan in which it is the primary chemotaxonomic data for identification of a strain to the genus level and separation of diaminopimelic acid (DAP) isomers (LL-DAP and *meso*-DAP) from peptidoglycan analyzed by Thin layer chromatography shown in Table 1 and 2.

**Table 1. Isolate number, location of soil samples, and cultural characteristics of isolates.**

| | | Cultural characteristics | |
|---|---|---|---|
| **Isolate no** | **Location and Province** | **Upper surface color** | **Reverse surface color** |
| NE1-3 | Mueang District, Nong Khai | Pale Blue | Strong Greenish Yellow |
| NE1-4 | Mueang District, Nong Khai | Pinkish White | Moderate Reddish Brown |
| NE1-6 | Mueang District, Nong Khai | Very Pale Blue | Pale Greenish Yellow |
| NE1-12 | Mueang District, Nong Khai | Greyish Greenish Yellow | Moderate Greenish Yellow |
| NE2-4 | Nakhon Ratchasima | Very Pale Blue | Deep Greenish Yellow |
| NE2-6 | Nakhon Ratchasima | Brilliant Blue, Celestial Blue | Greyish Reddish Brown |
| NE3-2 | Sumran Municipal Office, Khon Kaen | Light Yellowish Brown | Dark Orange Yellow |
| ET1-7 | Bamboos, Chachoengsao | Yellowish Grey | Light Yellow |
| ET1-12 | Bamboos, Chachoengsao | Blackish Red | Dark Reddish Brown |
| ET1-13 | Bamboos, Chachoengsao | Greenish White | Yellowish White |
| ET2-2 | Soil under the Santol, Chachoengsao | Purplish Gray | Greyish Red |
| ET3-23 | Pond, Chachoengsao | Light Yellowish Brown | Pale Yellow |
| CT1-17 | Rice field, Phichit | Light Purplish Grey | Greyish Yellow |
| CT2-4 | Rice field, Phichit | Greyish White | Light Orange Yellow |
| CT2-10 | Rice field, Phichit | Greyish White | Moderate Yellow |
| JA03 | Rhizosphere soil of plant, Udothani | Bluish White | Moderate Orange Yellow |
| MKP33 | Rhizosphere soil of plant, Udothani | Dark Olive Green | Deep Olive Green |

Table 2. Physiological, biochemical, and chemotaxonomic characteristics of *Streptomyces* strains.

| Characteristics | NE 1-3 | NE 1-4 | NE 1-6 | NE 1-12 | NE 2-4 | NE 2-6 | NE 3-2 | CT 1-17 | CT 2-4 | CT 2-10 | ET 1-7 | ET 1-12 | ET 1-13 | ET 2-2 | ET 3-23 | JA03 | MKP33 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Maximun NaCl tolerance | + | + | - | + | + | - | + | + | + | + | + | + | + | + | + | + | - |
| Growth at 28 °C | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Growth at 45 °C | + | - | + | + | + | - | - | - | - | - | + | - | - | + | - | + | + |
| pH 5-7 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| pH 10 | + | + | - | + | + | - | + | + | + | + | - | + | - | + | + | + | + |
| Nitrate reduction | + | - | + | + | + | + | - | + | + | + | - | + | + | + | + | - | - |
| Hydrolysis of Starch | - | + | - | - | + | + | + | + | + | - | + | + | + | + | + | + | + |
| Gelatinization | + | - | - | - | - | - | + | - | - | - | - | + | + | - | + | - | + |
| Milk coagulation | - | - | - | + | + | + | + | - | - | + | + | + | - | - | - | + | + |
| Milk peptonization | + | _ | + | - | - | + | + | + | + | + | - | - | - | - | - | - | + |
| Acid production from: | | | | | | | | | | | | | | | | | |
| L-arabinose | + | + | + | + | + | + | + | + | + | + | + | + | + | + | w | + | w |
| D-cellobiose | + | + | + | + | + | + | + | + | + | + | + | + | + | + | w | + | w |
| D-galactose | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | w |
| Lactose | + | + | + | + | + | + | + | + | + | + | + | + | + | w | w | + | w |
| myo-inositol | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - |
| D-mannitol | + | + | + | w | + | + | + | + | + | + | + | + | + | + | + | + | - |
| D-maltose | + | + | + | + | + | + | + | + | + | + | + | w | + | + | + | + | w |
| D-raffinose | + | + | + | w | + | + | w | + | + | + | + | w | + | + | - | + | - |
| D-sorbitol | + | w | - | - | + | w | - | - | + | + | + | w | - | w | - | + | - |
| D-xylose | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - |
| **Diaminopimelic acid in cell wall** | LL-DAP | LL-DAP | LL-DAP | LL-DAP | LL-DAP | LL-DAP | LL-DAP | LL-DAP | LL-DAP | LL-DAP | LL-DAP | LL-DAP | LL-DAP | LL-DAP | LL-DAP | LL-DAP | *meso* -DAP |

+, positive reaction; w, weak reaction; -, negative reaction

**4.2 Genotypic characteristics based on 16S rRNA gene sequences analysis**

The isolates were identified using primers targeting its 16S rRNA sequence compared to sequences in the NCBI gene bank database with the Basic Alignment Search Tool (BLAST) and the results showed high levels of sequence similarity (98.98%-100%) with the genus *Streptomyces* including NE1-3, NE1-4, NE1-6, NE1-12, NE2-4, NE2-6, NE3-2, CT1-17, CT2-4, CT2-10, ET1-7, ET1-12, ET1-13, ET2-2, ET3-23 and JA03 and *Micromonospora* including MKP33 shown in Table (3).

BLASTn analysis of 16S rRNA sequences revealed that strains NE1-3, NE1-4, NE1-6, NE1-12, NE2-4, NE2-6 and NE3-2 were closely related to *Streptomyces geysiriensis* NBRC 15413$^{T}$ (99.93%), *Streptomyces violarus* NBRC 13104$^{T}$ (99.48%), *Streptomyces geysiriensis* NBRC 15413$^{T}$ (99.93%), *Streptomyces nigra* 452$^{T}$ (99.78%), *Streptomyces nigra* 452$^{T}$ (99.49%), *Streptomyces alboniger* NRRL B1832$^{T}$ (99.20%), and *Streptomyces iakyrus* NRRL ISP-5482$^{T}$ (100%).

Strains CT1-17, CT2-4 and CT2-10 were the nearest similar to *Streptomyces olivaceus* NRRB L 3009$^{T}$ (99.64%), *Streptomyces capoamus* JCM 4734$^{T}$ (99.04%), and *Streptomyces capoamus* JCM 4734$^{T}$ (98.84%), individually. Strains ET1-7, ET1-12, ET1-13, ET2-2 and ET3-23 were adjacent to *Streptomyces lannensis* TA 4.8$^{T}$ (100%), *Streptomyces carpinensis* NRRL B16921$^{T}$ (99.04%), *Streptomyces diastaticus* subsp. *ardesiacus* NRRL B 1773$^{T}$ (99.63%), and *Streptomyces morookaense* LMG 20074$^{T}$ (99.49%), respectively. These strains were identified as *Streptomyces.*

Strain JA03 was similarly related to *Streptomyces puniciscabiei* DSM 41929$^{T}$ and showed 16S rRNA sequences similarities lower than 99% (98.98%) in BLASTn analysis and confirmed the novel species. MKP33 was closely related to *Micromonospora yasonensis* DS 3186$^{T}$ (99.56%). This strain was identified as *Micromonospora*.

**Table 3. Isolate number, base pair length, similarity (%) of 16S rRNA gene sequence, accession numbers and and nearestives type strain of isolates.**

| Isolate no | Base pair length | Similarity (%) | Accession no | Nearest type strain |
|---|---|---|---|---|
| NE1-3 | 1374 | 99.93 | LC635725 | *Streptomyces geysiriensis* NBRC 15413$^{T}$ |
| NE1-4 | 1353 | 99.48 | LC635726 | *Streptomyces violarus* NBRC 13104 $^{T}$ |
| NE1-6 | 1356 | 99.93 | LC635727 | *Streptomyces geysiriensis* NBRC 15413 $^{T}$ |
| NE1-12 | 1417 | 99.78 | LC635728 | *Streptomyces nigra* 452 $^{T}$ |
| NE2-4 | 1403 | 99.49 | LC635729 | *Streptomyces nigra* 452 $^{T}$ |
| NE2-6 | 1400 | 99.20 | LC635730 | *Streptomyces alboniger* NRRL B 1832 $^{T}$ |
| NE3-2 | 1381 | 100 | LC635731 | *Streptomyces iakyrus* NRRL ISP-5482 $^{T}$ |
| ET1-7 | 1427 | 100 | LC635736 | *Streptomyces lannensis* TA 48 $^{T}$ |
| ET1-12 | 1408 | 99.85 | LC635732 | *Streptomyces wuyuanensis* CGMCC 4.7042 $^{T}$ |
| ET1-13 | 1377 | 99.04 | LC635737 | *Streptomyces carpinensis* NRRL B16921 $^{T}$ |
| ET2-2 | 1345 | 99.63 | LC635738 | *Streptomyces diastaticus subsp. ardesiacus* NRRL B 1773 $^{T}$ |
| ET3-23 | 1389 | 99.49 | LC635739 | *Streptomyces morookaense* LMG 20074 $^{T}$ |
| CT1-17 | 1382 | 99.64 | LC635733 | *Streptomyces olivaceus NRRB L 3009* $^{T}$ |
| CT2-4 | 1361 | 99.04 | LC635734 | *Streptomyces capoamus* JCM 4734 $^{T}$ |
| CT2-10 | 1574 | 98.94 | LC635735 | *Streptomyces capoamus* JCM 4734 $^{T}$ |
| JA03 | 1369 | 98.98 | LC635741 | *Streptomyces puniciscabiei* DSM 41929$^{T}$ |
| MKP33 | 1367 | 99.56 | LC635740 | *Micromonospora yasonensis* DS 3186 $^{T}$ |

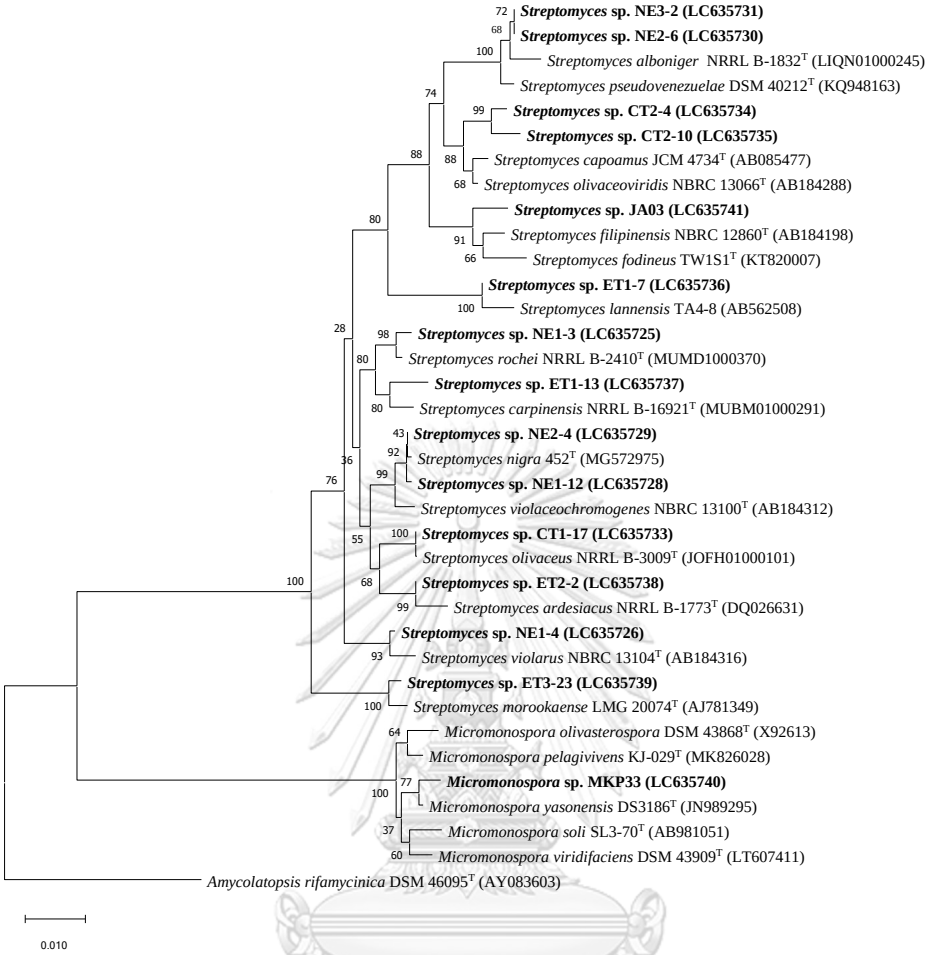





Figure 14. Neighbor-joining phylogenetic tree based on the 16S rRNA gene sequences of *Streptomyces* strains and *Micromonospora* strain.

**4.3 Genome analysis of *Streptomyces* strains CT1-17, ET3-23 and JA03**

The assembled genome of strain CT1-17 had 92 contigs, with a total length of 8,015,389 bp with an average G+C content of 72.59%. The genome had 7,241 protein coding sequences (CDS), 65 transfer RNA (tRNA) and 3 ribosomal RNA (rRNA) genes.

The assembled genome of strain ET3-23 had 106 contigs, with a total length of 8121874 bp with an average G+C content of 71.46 %. The genome had 7718 protein coding sequences (CDS), 79 transfer RNA (tRNA) and 3 ribosomal RNA (rRNA) genes.

This assembled genome of strain JA03 had 197 contigs, with the total length of 9,092,851 bp and an average G+C content of 71.28%. This genome has 8,687 protein coding sequences (CDS), 68 transfer RNA (tRNA) genes, and 6 ribosomal RNA (rRNA) genes (Table 4 and 5).

**Table 4. ANIb, ANIm and dDDH values among the draft genomes of strain JA03$^T$ and *Streptomyces puniciscabiei* DSM 41929$^T$.**



| Query genome | Reference genome | ANIb (%) | ANIm (%) | % dDDH (Formula 2a) | Model C.I. | Distance | Prob. DDH > = 70% | G + C differenc e |
|---|---|---|---|---|---|---|---|---|
| JA03$^T$ | DSM 41929$^T$ | 90.38 | 92.35 | 45.30 % | 42.8 - 47.9 % | 0.3342 | 6.14 % | 0.10 |

**Table 5. Genomic statistics of *Streptomyces* strains CT1-17, ET3-23 and JA03 and *Streptomyces puniciscabiei* DSM 41929$^T$.**

| Attribute | CT1-17 | ET3-23 | JA03 | DSM 41929$^T$ |
|---|---|---|---|---|
| Genome size (bp) | 8015389 | 8121874 | 9092851 | 9146138 |
| G+C content (%) | 72.59 | 71.46 | 71.28 | 71.4 |
| N50 | 315560 | 362883 | 503457 | 6617564 |
| L50 | 8 | 8 | 7 | 1 |
| No of contigs | 92 | 106 | 197 | 9 |

Based on the analysis of the biosynthesis gene clusters of secondary metabolites using antiSMASH, strains CT1-17, ET3-23 and JA03 include terpene, polyketide, saccharide, lanthipeptide, melanin and thiopeptide. Among these biosynthetic secondary metabolites gene clusters, two clusters (geosmin and neocarazostatin A) of ET3-23, six clusters (informatipeptin, albaflavenone, geosmin, ectoine, citrulassin and pristinol) of JA03 and four clusters (divergolide, geosmin, coelichelin and germicidin) of CT1-17 had a similarity value of 100% (Table 6). These compounds are commonly found in various *Streptomyces* species.

**Table 6. Distribution of biosynthetic gene cluster in *Streptomyces* strains CT1-17, ET3-23 and JA03 showing only biosynthetic gene clusters with similarity values greater than 20%**

| | | Similarity (%) | | |
|---|---|---|---|---|
| **Type** | **Most similar known cluster** | **CT1-17** | **ET3-23** | **JA03** |
| T2PKS | Polyketide | 66 | - | - |
| NRPS, T1PKS | Divergolide | 100 | - | - |
| Ecotine | Ecotine | 50 | - | - |
| NRP | Deimino-antipain | - | 66 | - |
| Lanthipeptide | SBI-06990 A1, SBI-06989 A2, Cinnamycin, Informapeptin | 75 | 47 | 100 |
| Siderophore | Desferrioxamine | 83 | - | 83 |
| NRPS | Coelichelin | 100 | - | - |
| RiPP | Citrulassin | - | 75 | 100 |
| RiPP | Berninamycin | - | 66 | - |
| NRPS, Nucleoside | Nogalamycin | 40 | - | - |
| T3PKS | Germicidin | 100 | - | - |
| T1PKS, NRPS, Oligosaccharide | Lobophorin A | 73 | - | - |
| Terpene | Geosmin | - | 100 | 100 |
| Terpene | Isorenieratene | 75 | - | - |
| Terpene | Cyclooctatin | 100 | 50 | - |
| Terpene | Hopene | 92 | 53 | 92 |
| Terpene | Albaflavenone | - | - | 100 |
| Terpene | Pristinol | - | - | 100 |
| Alkaloids | Marinacarboline | - | - | - |
| Polyketide | Butyrolactone | - | 34 | - |
| Polyketide | Formicarmycin | - | 34 | - |
| Polyketide | Pentamycin | - | 80 | - |
| NRP, Polyketide | Althiomycin | - | 83 | - |
| Polyketide | Lungdunomycin | - | 85 | - |
| NRP, Polyketide | Virginiamycin | - | 27 | - |
| NRP, Polyketide | Aurantimycin | - | - | 57 |
| Polyketide | Halstoctacosanolide | - | - | 77 |
| Polyketide | Spirangien | - | - | 40 |
| Polyketide | ECO-02301 | - | - | 32 |
| Polyketide | Pellasoren | - | - | 33 |
| Polyketide | Oligomycin | - | - | 38 |
| Other | Neocarazostatin | - | 100 | - |
| Other | Melanin | - | - | 71 |
| Other | Ectonine | - | - | 100 |

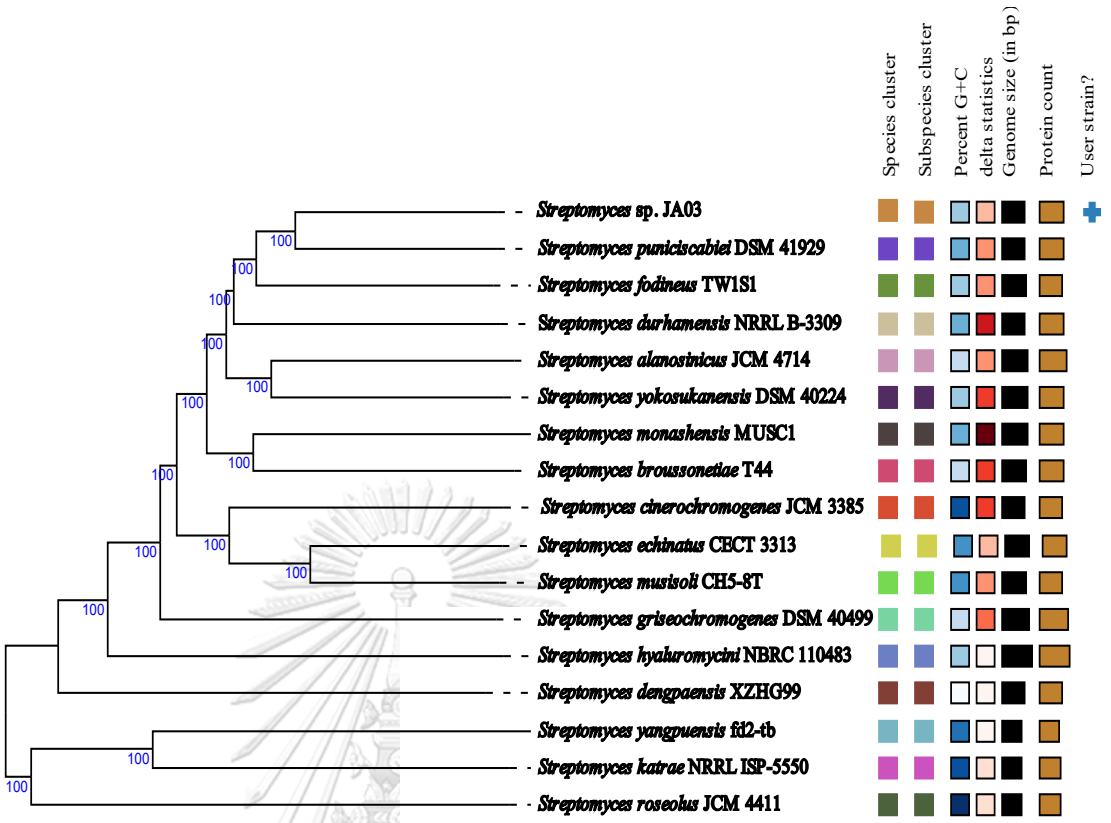



Figure 15. Phylogenetic tree of *Streptomyces* sp. JA03 and related type strains.

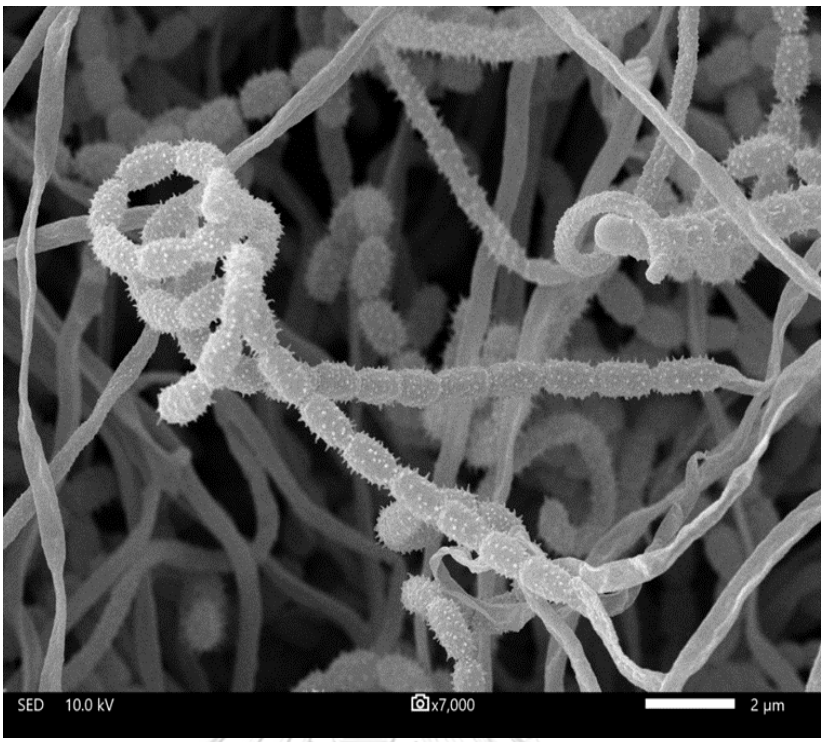



Figure 16. Scanning electron micrograph of *Streptomyces* sp. JA03 grown on ISP 2 agar plates at 30°C for 14 days.

**4.4 Antimicrobial activity**

Screening of antimicrobial activity of actinomycetes isolates were assessed by agar disk diffusion method. The culture supernatants (50µl) isolated from the production media such as 30 media (30), 54 media (54) and 57 media (57) and the tested microorganisms including *Staphylococcus aureus* ATCC 25923*, Candida albicans* ATCC 10231*, Escherichia coli* ATCC 25922*, Bacillus subtilis* ATCC 6633, *Pseudomonas aeruginosa* ATCC 27853 and *Kocuria rhizophila* ATCC 9341 were the main factor that exhibited the antimicrobial activity.

In this study, all of the actinomycetes isolates were not effective on *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853. Actinomycetes isolates NE1-3, NE2-4, NE2-6, CT2-4, ET2-2 and ET1-12 showed antimicrobial activity against *Staphylococcus aureus* ATCC 25923*, Candida albicans* ATCC 10231*, Bacillus subtilis* ATCC 6633 and *Kocuria rhizophila* ATCC 9341. Among these isolates, NE2-6 has the most potent antimicrobial activity on *K. rhizophila* with a diameter of inhibition zone at 30.6mm and also mild effect on *S.aureus* at 14.1mm. ET1-12 showed the most potent effect on *C. albicans* with an inhibition zone at 28.7mm and moderate effect on *K. rhizophila* with an inhibition zone at 26.7mm and also mild effect on *B.subtilis* at 15.9mm. ET2-2 displayed moderate activity against *B.subtilis* with a zone of inhibition (16.2mm) and also on *K. rhizophila* (20.4mm). CT2-4 showed moderate effect on *S.aureus* with an inhibition zone (19.2mm) The standard antibiotics such as ciprofloxacin (30µg/disc) for *B.subtilis* (26.1mm)*, S.aureus* (23.4mm) and ceftriaxone (30µg/disc) *K. rhizophila*

(35.3mm) were used. (Susceptible ≥ 19 mm; Intermediate ≥15-18 mm; Resistant ≤ 14 mm). These isolates could be the potential effectiveness of antibiotics for further study.

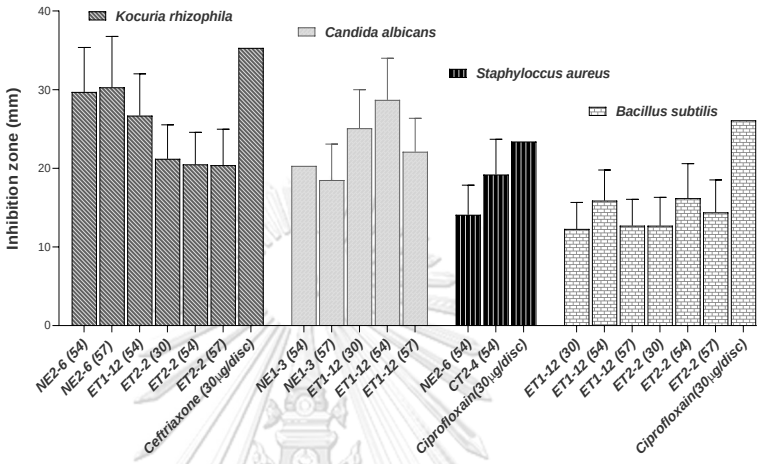



**Figure 17. Antimicrobial activity of *Streptomyces* strains on tested microorganisms (inhibition zone, mm).**

### 4.5 Determination of antioxidant activity

#### 4.5.1 Total phenolic content



Total phenolic contents (TPC) were analyzed to quantify its potential antioxidant activity by using the Folin-Ciocalteu (FC) method. Under the basic reaction conditions, a phenol loses an $H^+$ion to produce a phenolate ion, which reduces Folic-Ciocalteu reagent. The occurrence of blue coloration has a maximum absorption at 765 nm and increment in absorbance was proportional to the higher total phenolic quantity of tested extracts. The quantity of phenolics were interpreted from the equation of the calibration curve of gallic acid and expressed as µg gallic acid equivalent/mg of extracts (µg GAE/mg of extract). It could be estimated that the

phenolic contents of ethyl acetate extracts showed in ranging from 3.1µg GAE/mg to 33.4µg GAE/mg (Figure 18). JA03 showed the higher phenolic content 33.4±0.66µgGAE/mg extract than all the other extracts followed by MKP33 (31.2±0.60µgGAE/mg) while NE1-4 and ET1-13 presented the low phenolic content levels (3.3±0.56µgGAE/mg and 3.1±1.07µgGAE/mg) followed by CT1-17 (7.9±0.98µgGAE/mg of extract) and ET3-23 (9.1±0.54µgGAE/mg). Antioxidant potentials of ethyl acetate extracts (NE1-12, NE2-4 and NE2-6) were found to be moderate phenolic levels (21.2±1.13µgGAE/mg, 20.2±0.44µgGAE/mg and 19.3±0.75µgGAE/mg) followed by NE1-6 with 15.6±1.41µgGAE/mg and CT2-10 with 16.8±0.66µgGAE/mg. Phenolic compounds were related with antioxidant activity and other biological functions and may prevent the development of aging and diseases.

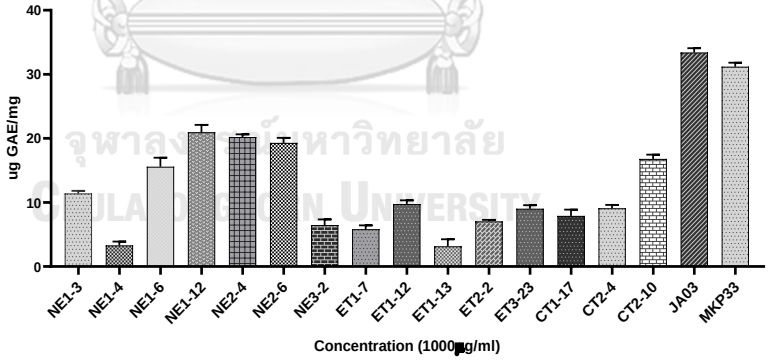



**Figure 18. Total phenolic contents of ethyl acetate crude extracts of actinomycetes.**

**4.5.2 Determination of ferric reducing antioxidant potency (FRAP)**

Antioxidant ability of extract was determined by using FRAP assay. Ferric reducing antioxidant potency were carried out to evaluate the reduction of ferric ions complex (TPTZ-$Fe^{3+}$) to a blue-colored chromophore ferrous tripyridyl triazine complex (TPTZ-$Fe^{2+}$) at pH 3.6 in the presence of an antioxidant as a reductant and TPTZ is used as a prooxidant based on the electron-transfer reactions. Increasing the absorbance indicated in response with the higher antioxidant capacity and expressed as µg ascorbic acid equivalent/mg of extract (Benzie & Strain, 1996). The antioxidant activity was calculated from calibration curve in the range of concentration (10µg/mL -160µg/mL) of ascorbic acid and with linearity. Results are expressed in µg ascorbic acid equivalents/mg of extracts (µg AAE/mg of extract) shown in Figure 19.

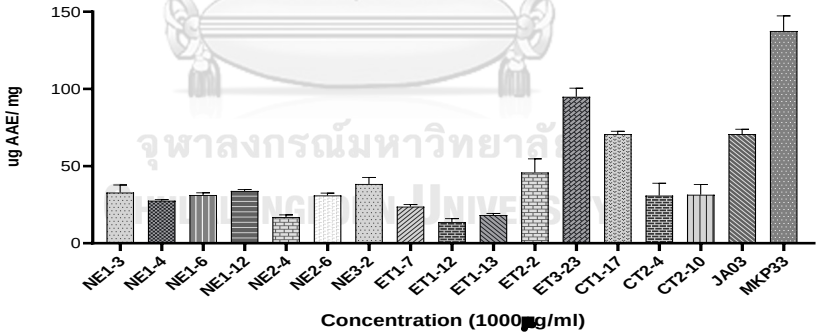



**Figure 19. FRAP values of ethyl acetate crude extracts of actinomycetes.**

FRAP values were estimated from reduction of TPTZ-Fe (III) to TPTZ-Fe (II). Based on the results, MKP33 showed the highest antioxidant capacity (137.5±5.66µgAAE/mg) followed by ET3-23 (95±3.14µgAAE/mg), CT1-17 and JA03 with a higher reductant capacity (70.8±1.38 and 70.8±1.75µgAAE/mg of extract) and ET2-2

showed moderate FRAP value (45.9±5.10μgAAE/mg) followed by and CT2-10 with 31.4±3.84μgAAE/mg and NE1-12 with 33.9±0.55μgAAE/mg. NE2-4, ET1-12 and ET1-13 displayed lower reductant potential (17±0.79μgAAE/mg, 13.8±1.26μgAAE/mg and 18.3±0.61μgAAE/mg). Accordingly, the antioxidant activity of extracts could reflect the higher electron donating activity of extract relative to DPPH radical scavenging capacity and exhibited considerably higher antioxidant potential in FRAP assay in comparison to TPC assay. There was an evidence association between total phenolic contents and FRAP values signaling the point that phenolics are highly potent antioxidants.

**4.5.3 Estimation of radical scavenging activity using DPPH assay.**

Scavenging activity was measured by DPPH assay based on the reduction of purple colored DPPH in the presence of hydrogen donating antioxidants, to form yellow colored non-DPPH radical. DPPH radical has absorption maxima at 517 nm and the decrement in the absorbance was related to the increment in DPPH radical scavenging activity. Different concentrations (50-1000μg/ml) of ethyl acetate crude extracts were evaluated for antioxidant potency.

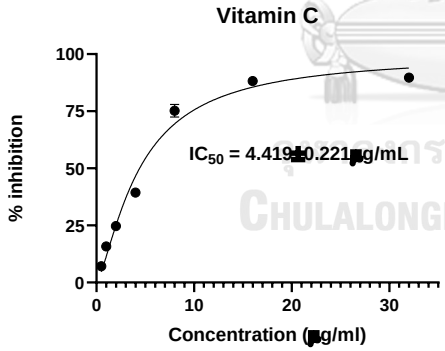


In this present study, vitamin C as positive control has potent DPPH radical scavenging activity (88.061 ± 1.60%) at 32μg/ml with an $IC_{50}$ value of 4.419±0.22μg/mL. Strain CT1-17 showed potent scavenging activity with $IC_{50}$ value 218.6±27.4μg/ml as follows: (24.47±0.34%) at 50μg/mL, (31.87±0.42%) at 100μg/mL, (47.53±0.50%) at 200μg/mL, (64.52±0.625%) at 300μg/mL and (80.21±0.04%) at 400μg/mL and whereas

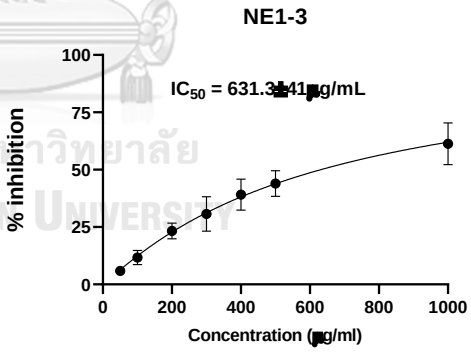
increasing the concentration, it exhibited (72.09±0.05%) and (58.43±0.32%) scavenging activity at 500µg/mL and 1000µg/mL. Strains CT2-4 and CT2-10 showed potent DPPH radical scavenging activity (77.84±1.59%) and (74±1.12%) at 1000µg/ml.

**Table 7. Expression of $IC_{50}$ values of ethyl acetate crude extracts.**

| Strains | $IC_{50}$ values (µg/mL) | Strains | $IC_{50}$ values (µg/mL) | Strains | $IC_{50}$ values (µg/mL) |
|---|---|---|---|---|---|
| NE1-3 | 631.3±41 | CT1-17 | 218.6±27.4 | ET2-2 | 255.3±14.6 |
| NE1-6 | 505.7±30.7 | CT2-4 | 240±10.7 | ET3-23 | 67.8±4.6 |
| NE1-12 | 457.7±8.3 | CT2-10 | 527.6±15.6 | JA03 | 67±21.1 |
| NE2-6 | 600.1±14.1 | NE3-2 | 468.9±15.4 | MKP33 | 131.1±3.1 |




(A)

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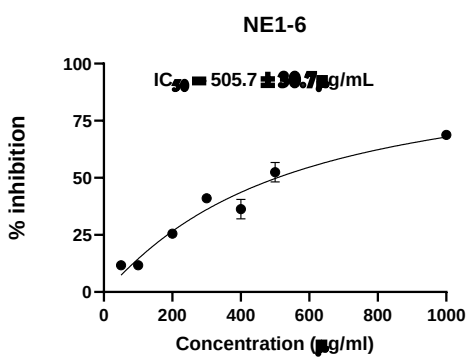


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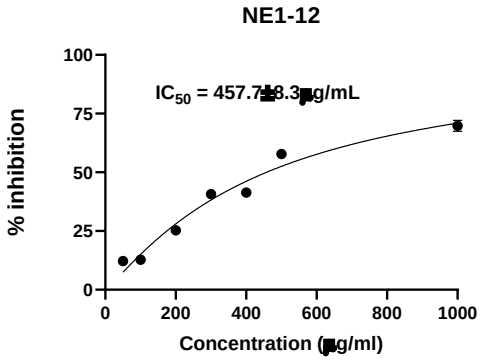


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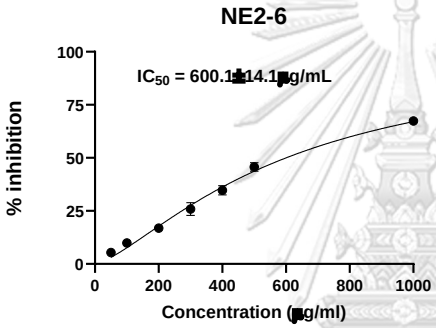
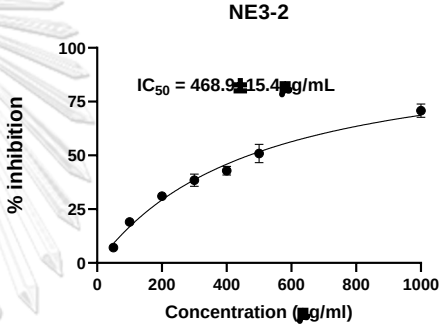



(E)

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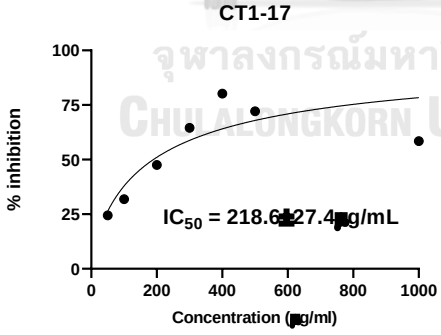


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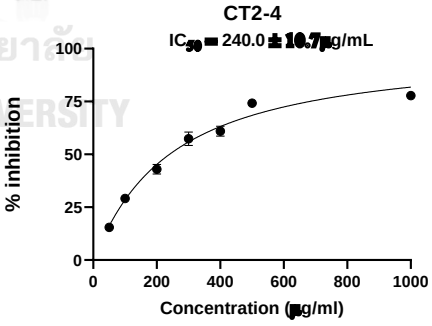


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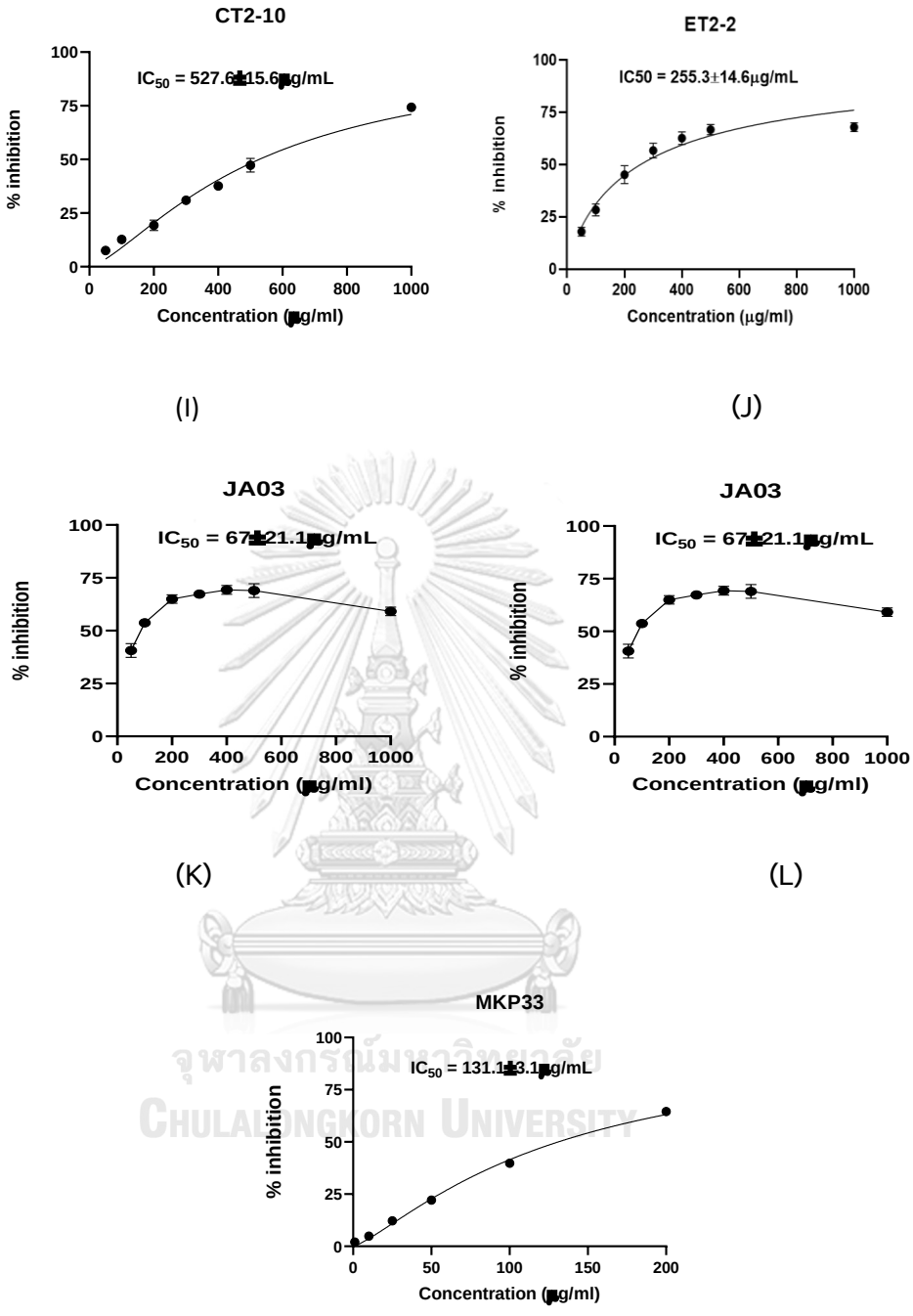
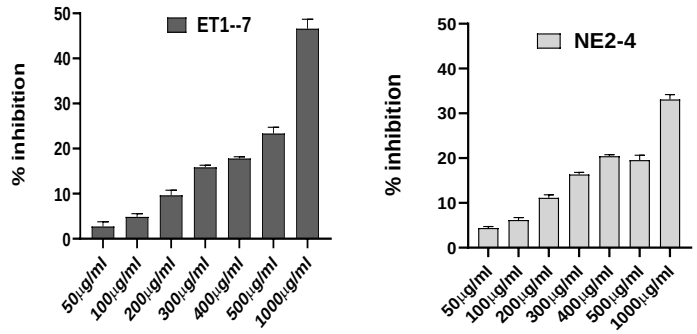





Figure 20 (A-M). Expression of $IC_{50}$ values of vitamin C and ethyl acetate crude extracts on DPPH radical scavenging activity.

The low $IC_{50}$ value of ET3-23 (67.8±4.6µg/mL) and JA03 (67±21.1µg/mL) indicated significantly potent DPPH radical scavenging activity (77.7±0.98%) at 200µg/mL and (69.3±2.13%) at 400µg/mL than other ethyl acetate crude extracts and increasing the concentration to 1000µg/mL, these crude extracts exhibited decline in DPPH radicals scavenging activity. By determining the antioxidant effect, DPPH radical scavenging of ethyl acetate crude extracts were reported to be correlated with the levels of phenolic content and FRAP value. By determining the antioxidant effect, strong correlation between TPC and antiradical activities indicated phenolic compounds and FRAP value underlined the activities.

Among these crude extracts, MKP33 and NE1-4 were evaluated in lower concentration due to the dark color. Different concentrations (1-200 µg/mL) of NE1-4 and MKP33 showed 46.4±1.16% and 64.04±1.36% DPPH radical scavenging at 200µg/mL. NE3-2 showed moderate DPPH radical scavenging activity (70.3±3.13%) in concentration dependent manner, followed by NE1-12 (69±2.32%), NE1-6 (68.73±1.31%), ET2-2 (67.973 ± 2.09%) and NE2-6 (67.38±0.82%) at 1000µg/ml.




(A)

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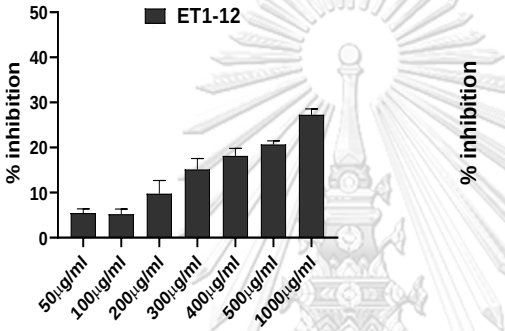


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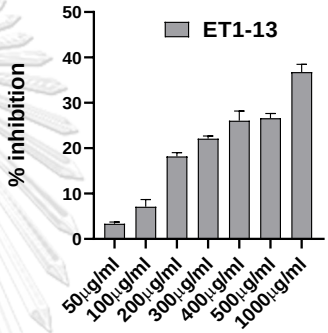


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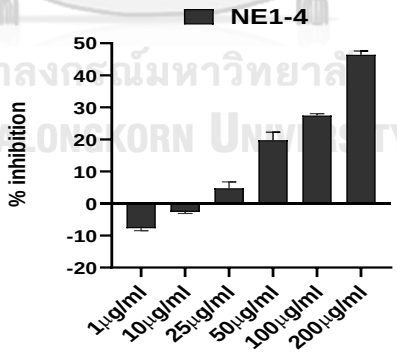



(E)

Figure 21 (A-E). Effect of different concentration of ethyl acetate crude extracts on DPPH radical scavenging assay.

ET1-7 exhibited 46.6±2.069% inhibition followed by NE2-4 (33.2±1.00%), ET1-13 (36.71±1.70%) and ET1-12 (27.288±1.27%) at 1000µg/mL indicating the weak DPPH radical scavenging.

**4.5.4 Determiation of nitric oxide radical scavenging assay**

Nitric oxide generated from sodium nitroprusside reacts with oxygen to form nitrite. Incubation of sodium nitroprusside in phosphate buffer solution at 27℃ for 2hrs resulted in linear time-dependent nitrite production, which is reduced by ethyl acetate crude extracts. In nitric oxide scavenging assay, a pink colored chromophore was occurred, and the absorbance of these solutions was measured at 540 nm against the corresponding blank. Based on the results, vitamin C (10005µg/mL) showed potent NO scavenging activity (87.3±0.40%) with $IC_{50}$ value of 206.5µg/mL in a concentration-dependent manner. NE2-6, NE1-12, JA03 and MKP33 exhibited NO radical scavenging activity, whereas other extracts had no NO scavenging activity. Based on this results, NE2-6 showed 49.6±1.34% nitric oxide radical scavenging activity at 500µg/ml in a concentration dependent manner and MKP33 (49.3±1.96%) at 100 µg/ml and JA03 (48.1±4.13%) at 500µg/ml, ET3-23 (45±0.55%) and NE1-12 (44.1±3.68%) at 400µg/mL shown in Figure 22.

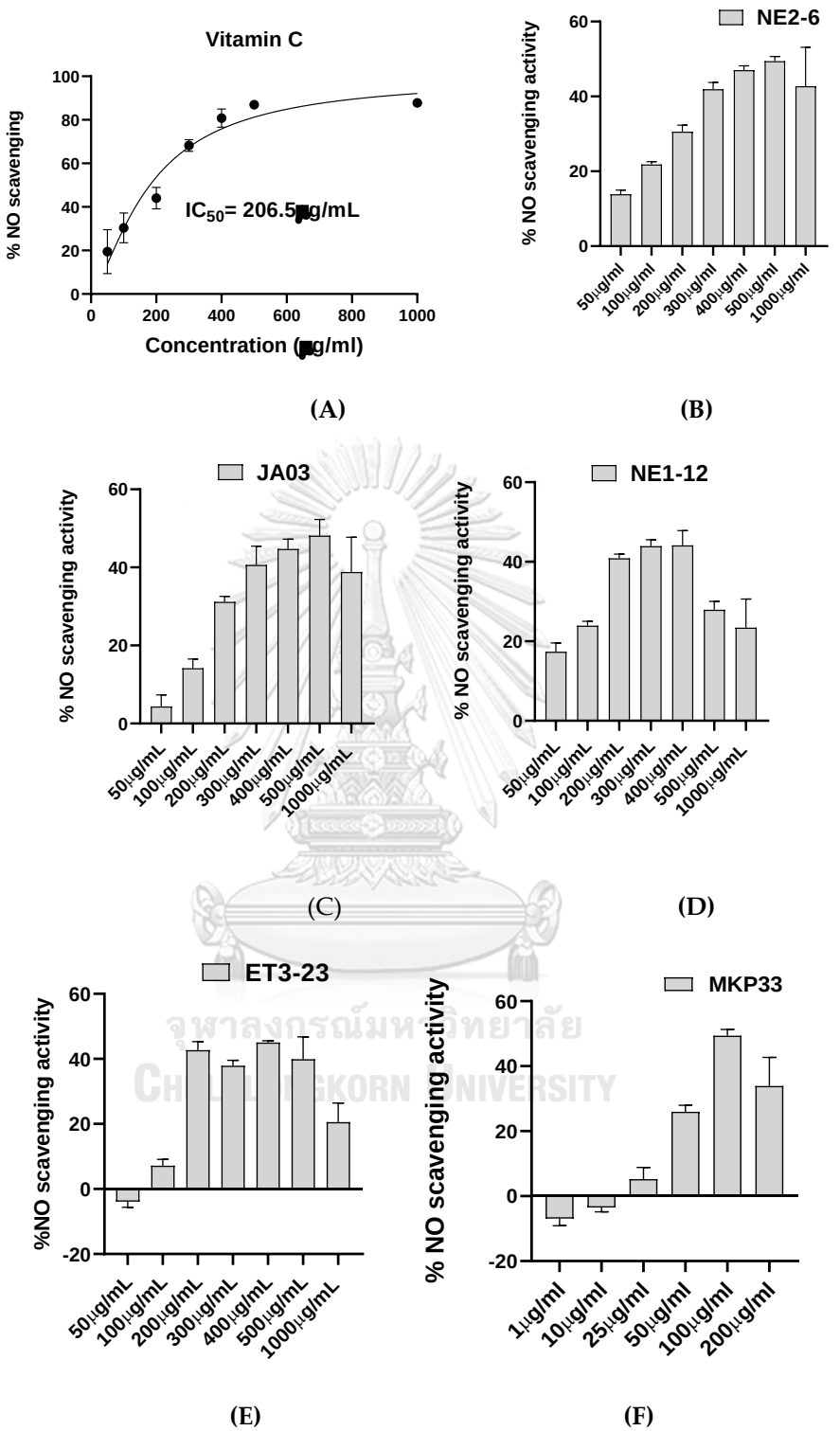



Figure 22 (A-F). $IC_{50}$ value of vitamin C and NO radical scavenging activity of ethyl acetate crude extracts of actinomycetes

Strain NE2-6 and JA03 showed nitric oxide scavenging at 500 µg/ml while increasing the concentration to 1000 µg/ml, they exhibited (42.992 ± 10.48) and (38.8±8.87%) NO scavenging activity, NE1-12 (27.9±2.10% and 23.4±7.19% at 500 and 1000µg/ml) and MKP33 (33.8±8.79% at 200µg/ml) also showed nitric oxide scavenging activity. Nevertheless, MKP33 also showed more potent in nitric oxide scavenging (49.3±1.96%) at 100 µg/ml than NE2-6 (49.6±1.34% at 500 µg/ml). Moreover, owing to the higher components of phenolic contents and FRAP values of MKP33, this ethyl acetate crude extract could be attributed to scavenge nitric oxide.

**4.6 Cell viability assay**

MTT assay is based on the mitochondrial activity in viable cells of mitochondrial dehydrogenase enzyme that reduces the yellow tetrazolium MTT into purple formazan crystal indicating the number of metabolically active viable cells.



Based on the higher DPPH radical and NO scavenging activity, eight ethyl acetate crude extracts were selected for cell metabolic activity on RAW 264.7 macrophage cells. Cytotoxic effects of NE1-12, ET3-23, CT1-17, NE2-6, CT2-4, CT2-10, JA03 and MKP33 on RAW264.7 macrophage cells were evaluated by exposing the cells to various concentrations (10-1000µg/mL) of extracts for 24h. The ethyl acetate crude extracts in low concentration had no cytotoxic effect on RAW264.7 macrophage cells. The results showed that there was no cytotoxic effect (nearly 80-100% cell viability) at the tested concentration (200µg/mL) having non-toxic concentration on

macrophage with retaining cell viability. Therefore, ethyl acetate crude extracts in the range of non-toxic concentration (10-200µg/mL) were chosen for further experiments.

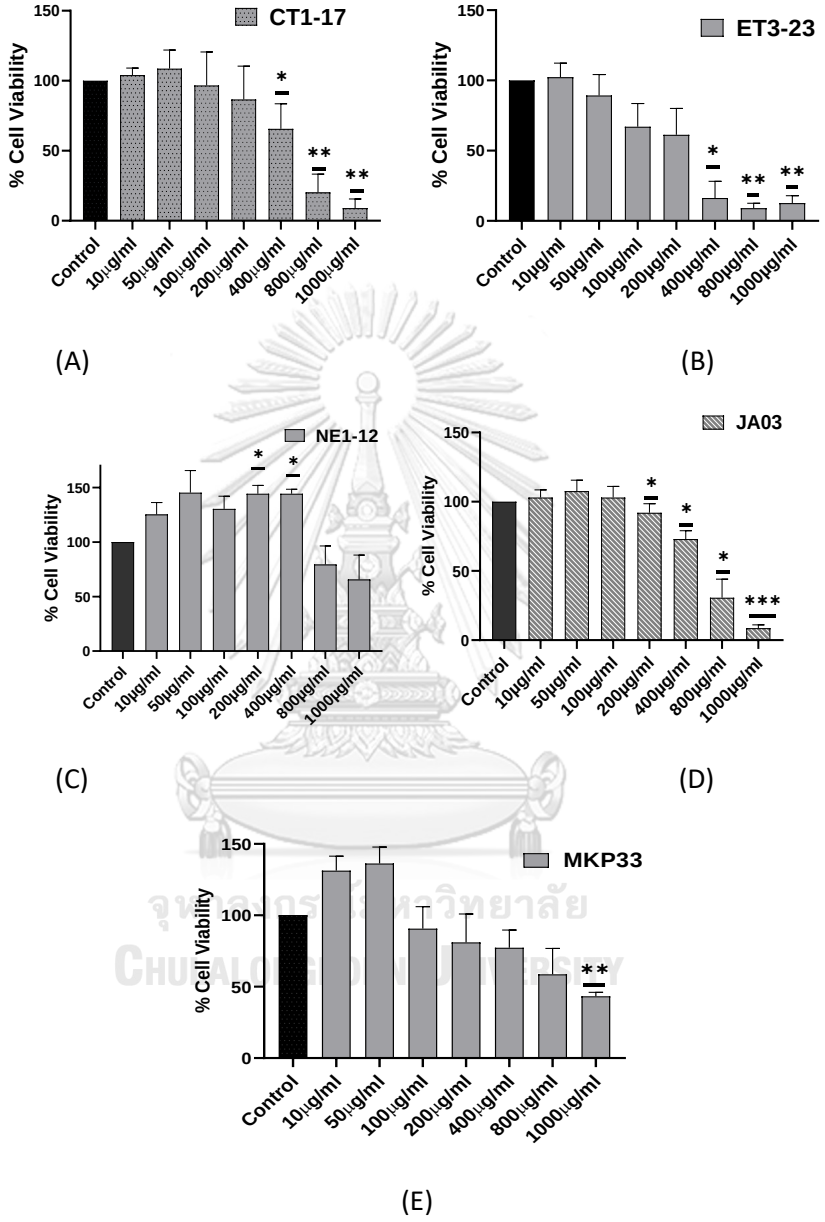



**Figure 23 (A-E). Effect of ethyl acetate crude extracts on cell viability on RAW 264.7 macrophage cells, *p<0.05, **p<0.005, ***p<0.0001 compared to the control.**

MTT assay revealed that cytotoxicity of CT2-4, CT2-10 and NE2-6 observed nearly 90% cell viability at 10μg/ml on RAW 264.7 macrophage cells shown in Figure 24. Therefore, ethyl acetate crude extracts in the range of non-toxic concentration (1-20μg/mL) were chosen for further investigation of intracellular ROS and NO production on RAW264.7 macrophage cells induced by hydrogen peroxide and lipopolysaccharide.

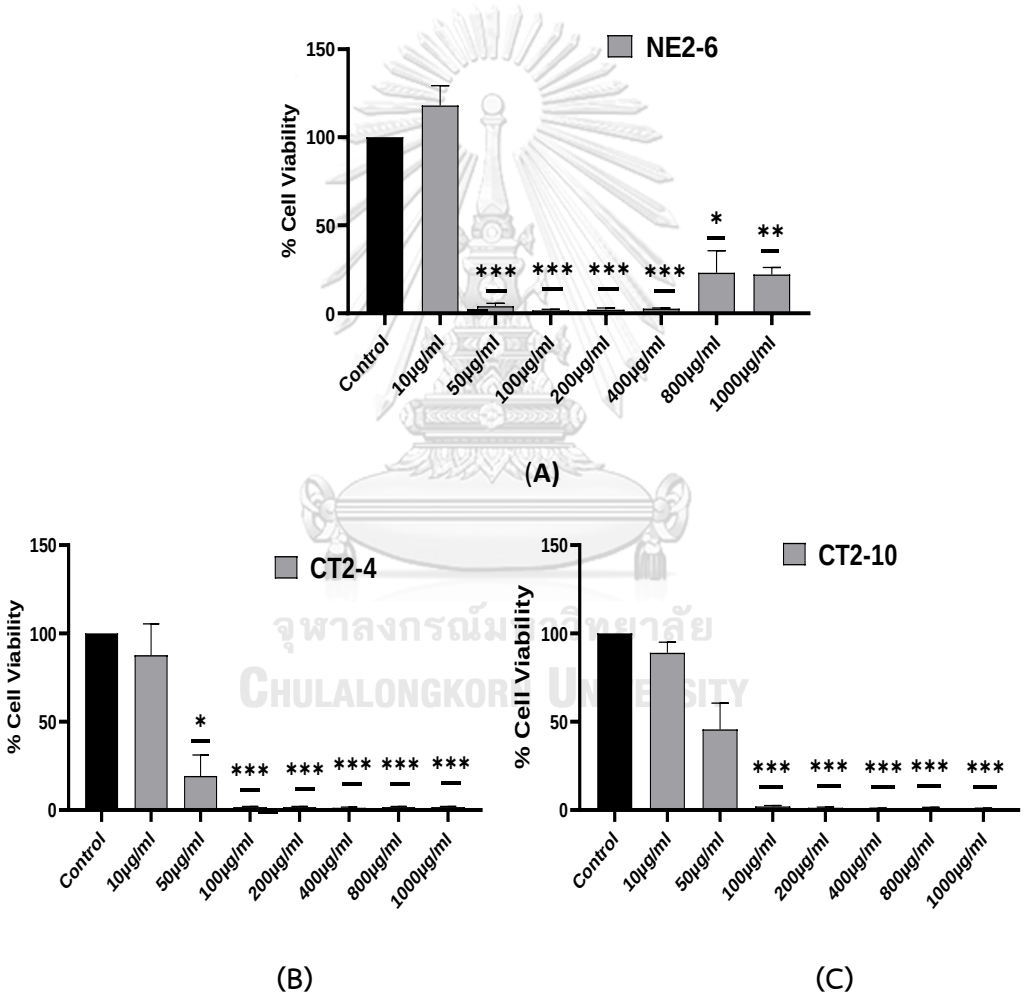



**Figure 24 (A-C). Effect of ethyl acetate crude extracts on cell viability on RAW 264.7 macrophage cells, *p<0.05, ***p<0.0001 compared to the control.**

**4.6.1 Determination of cytotoxicity with induction of hydrogen peroxide ($H_2O_2$)**

The selected non-toxic concentrations (up to 10μg/mL for CT2-4, CT2-10 and NE2-6 and up to 200μg/mL for JA03, MKP33, ET3-23, CT1-17 and NE1-12) were evaluated for cytotoxic assay in the presence or absence of $H_2O_2$ (100μM) for 30 mins.

The non-toxic concentration (10-200μg/mL) of CT1-17, ET3-23, NE1-12, JA03 and MKP33 were evaluated for cytotoxic assay in the presence or absence of $H_2O_2$ (100μM) for 30 mins followed by 3hrs incubation of MTT reagent and then addition of 100μl DMSO for solubility of formazan crystals. From the result of this study, NE1-12 exhibited no effect on cytotoxicity at 200μg/ml even though CT1-17, ET3-23, JA03 and MKP33 observed nearly 69±9.37%, 26±12.64%, 90±8.31% and 79±4.89% cell viability at 200μg/ml where decreasing cell viability with increasing the concentration of crude extracts shown in Figure 25. These findings suggested that NE1-12 might exert some enhancement playing a role in cell proliferation and no cytotoxic effect on RAW264.7 macrophage cells. Therefore, different concentration (10-200μg/mL) of these crude extracts were selected for further evaluation of intracellular reactive oxygen species generation.

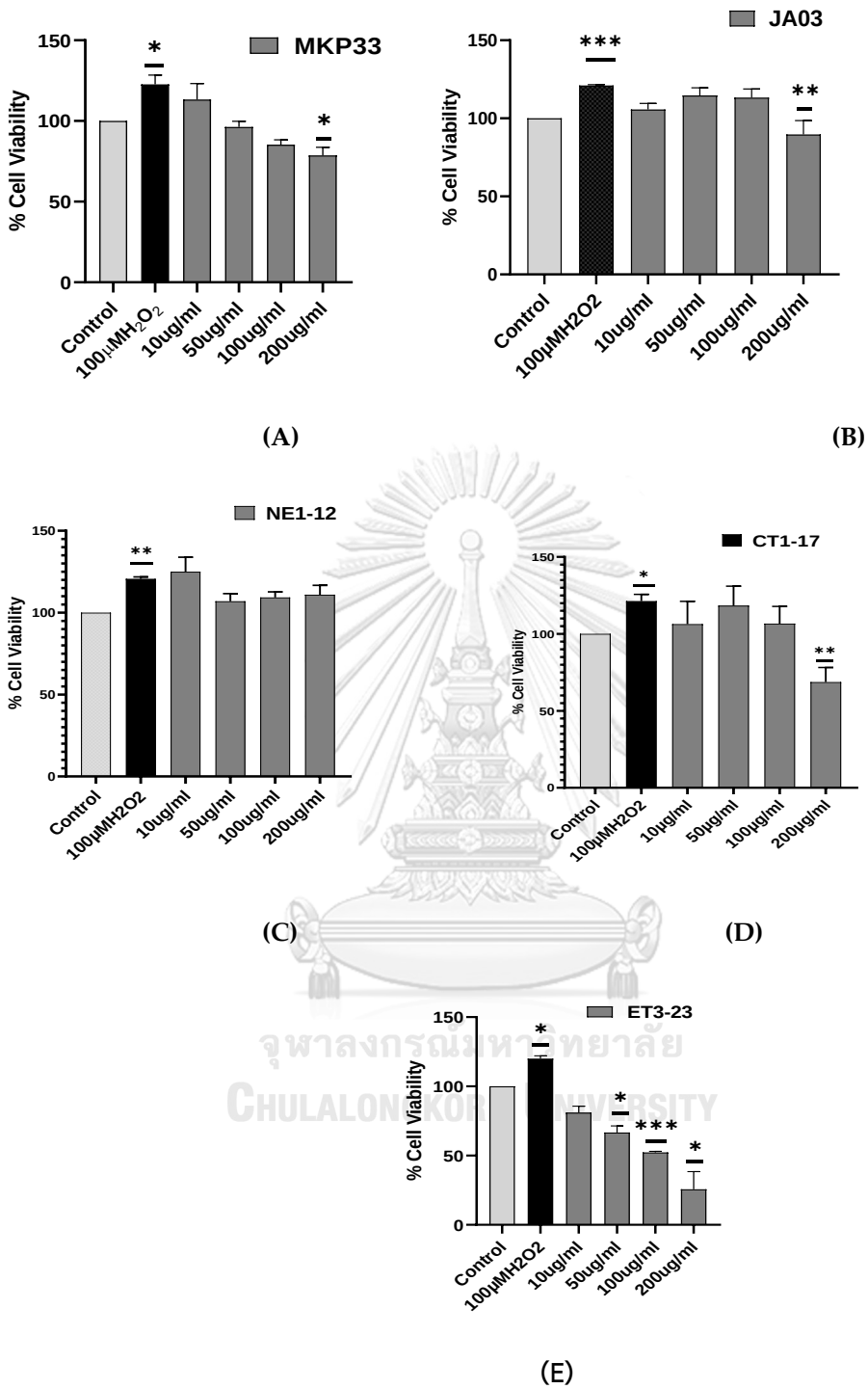
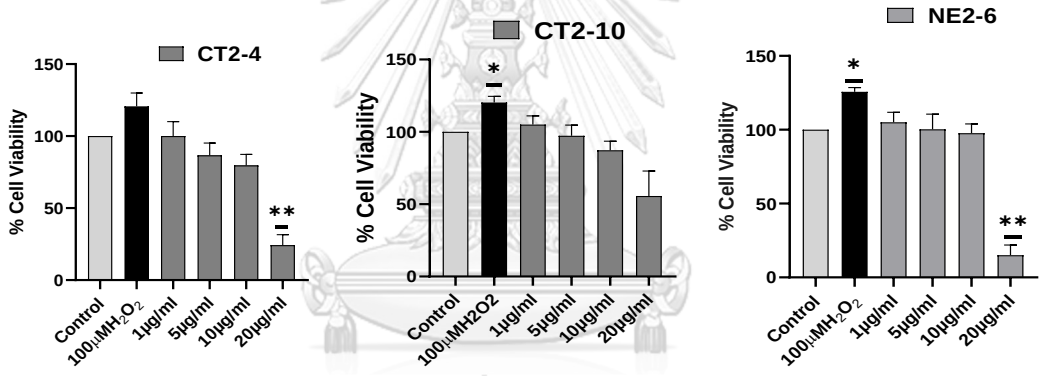



Figure 25 (A-E). Cytotoxic effect of ethyl acetate crude extracts on cell viability of macrophage induced by $H_2O_2$, *p<0.05, **p<0.005, ***p<0.0001 compared to the control.

The selected non-toxic concentration up to 20μg/mL for CT2-4, CT2-10 and NE2-6 were evaluated in the presence of 100μM $H_2O_2$ for 30 mins. After 100μM $H_2O_2$ incubation for 30 mins followed by 3hrs incubation of MTT reagent, 100μl 100%DMSO was added for solubility of formazan crystals. CT2-4, CT2-10 and NE2-6 observed nearly 80-90% cell viability at 10μg/ml where decreasing cell viability with increasing the concentration of crude extracts to 20μg/ml shown in Figure 26. These results clearly depicted that CT2-4, CT2-10 and NE2-6 at 20μg/ml showed cytotoxic effect of 25±7.21%, 55±10.29% and 15±6.72%.




(A) (B) (C)



**Figure 26 (A-C). Cytotoxic effect of ethyl acetate crude extracts on cell viability of macrophage induced by $H_2O_2$, *p<0.05, **p<0.005 compared to the control.**

**4.7 Determination of intracellular ROS generation**

DCFH-DA assay was performed for measuring ROS production in RAW264.7 macrophage cells. Non-fluorescent DCFH-DA hydrolyzed to DCFH inside the cells, produces fluorescent DCF in the presence of intracellular ROS. Induction with $H_2O_2$ on RAW264.7 cells pointedly encouraged intracellular ROS production determined by DCF fluorescence. In this present study, the cells were simultaneously pretreated with non-toxic concentrations (0.1-200μg/mL) of ethyl acetate crude extracts for 24 h and incubated with 10μM DCFH-DA for 30 mins followed by 100μM $H_2O_2$ for 30 mins.

Ethyl acetate crude extracts and ascorbic acid as the standard inhibited intracellular ROS production induced by $H_2O_2$ shown in Figure 27. Vitamin C showed intracellular ROS generation (65.29±1.98μM to 23.44±4.84μM) in the range of concentration (0.1-200μg/mL). Strain MKP33 inhibited intracellular ROS generation (33±6.56%) in $H_2O_2$ induced RAW 264.7 cells, followed by NE1-12 (38±4.31%), JA03 (56.69±3.39%) and CT1-17 (49.8±4.28%) at 200μg/mL.

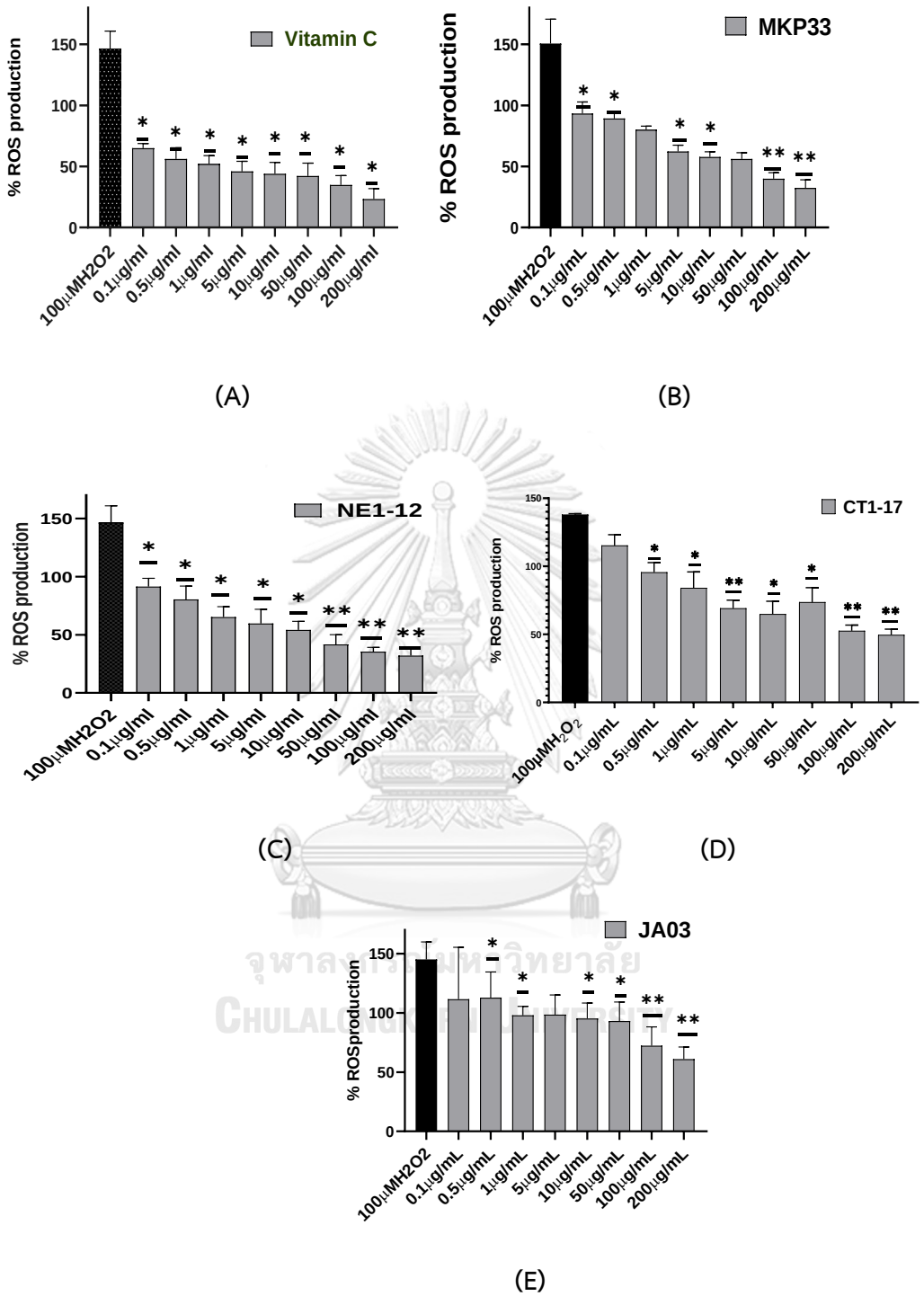



Figure 27 (A-E). Effect of ethyl acetate crude extracts on ROS production in $H_2O_2$ induced RAW264.7 macrophages cells, *p<0.05, **p<0.005 compared to the hydrogen peroxide.

ET3-23, NE2-6, CT2-4 and CT2-10 showed 44.6±9.44%, 56±4.26%, 86±9.85% and 88±6.86% intracellular ROS production induced by $H_2O_2$ at 10μg/mL compared with the control group shown in Figure 28. The low cytotoxicity of ET3-23 and NE2-6 give a possibility therapeutic agent for the diseases caused by free oxygen radicals.

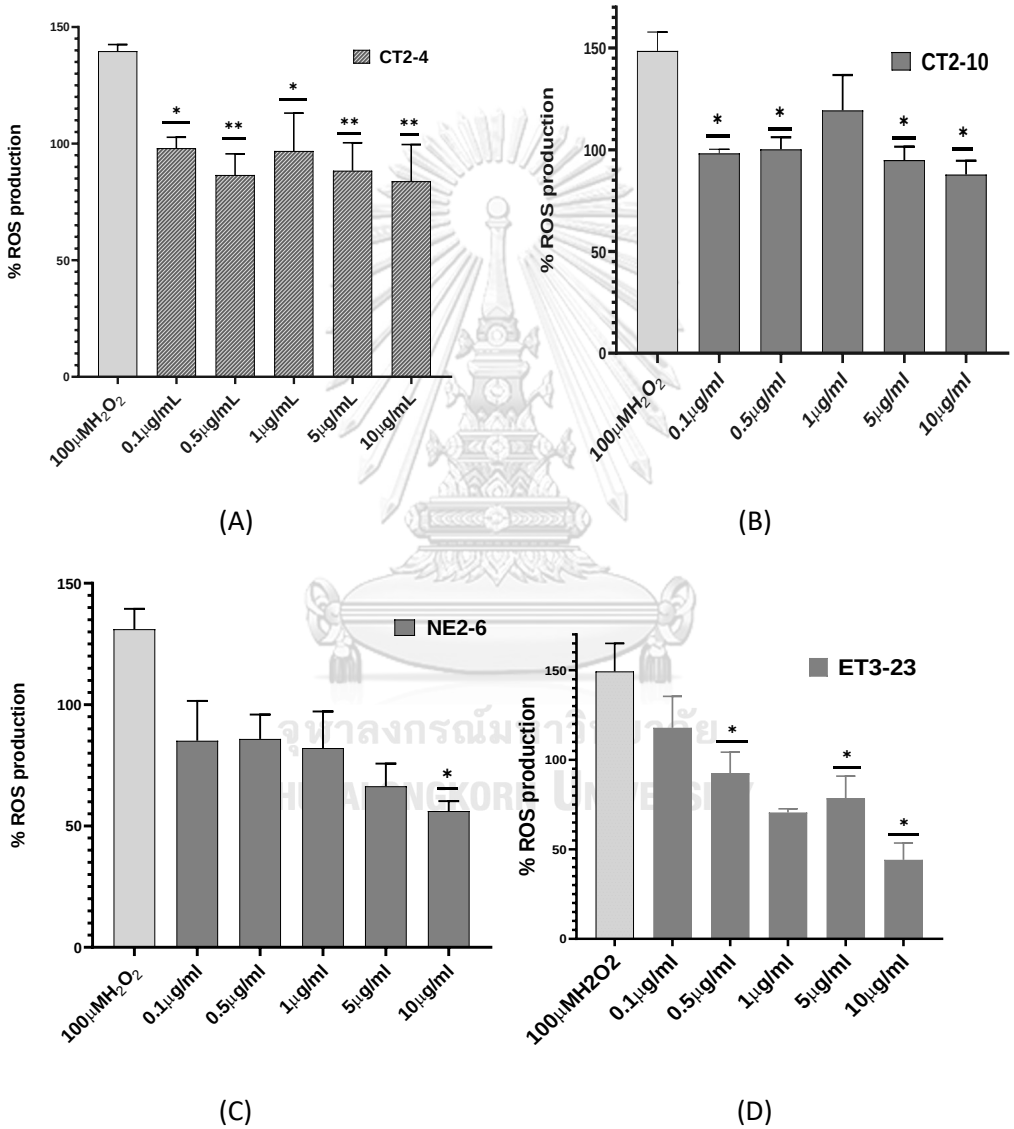



**Figure 28 (A-D). Effect of ethyl acetate crude extracts on ROS production in $H_2O_2$ induced RAW264.7 macrophages cells, *p<0.05, **p<0.005, compared to the hydrogen peroxide.**

**4.8 Determination of NO generation**

In murine RAW264.7 macrophage cells, induction of LPS proved an increase NO production. The Griess reaction, a spectrophotometric determination for nitrite, was approved to quantity the nitrite levels in the conditioned medium of RAW264.7 cells incubated with LPS. Ethyl acetate crude extracts were evaluated for NO inhibition activity with LPS (100ng/ml) induced RAW264.7 macrophages cells using Griess assay. The cells were incubated with or without LPS in the presence or absence of the tested crude extracts, ranging from 0.05 μg/ml to 200 μg/ml. Different concentration (1.5625-100μM) of sodium nitrite was prepared as a standard solution to calculate NO concentration. As expected, RAW 264.7 cells induced with LPS produced large amounts of NO compared with untreated cells, whereas cells treated with crude extracts produced significantly lower levels of NO.

The results demonstrated the ethyl acetate crude extracts significantly decreased the nitrite accumulation in LPS induced RAW 264.7 cells. The nitrite production measured as nitrite increased significantly 30-40μM when RAW 264.7 macrophages cells were induced with LPS (100ng/mL). Strains MKP33, JA03, NE1-12 and CT1-17 showed inhibitory effect on NO production by decreasing the nitrite accumulation in LPS stimulated RAW 264.7 cells shown in Figure 28. MKP33 and JA03 significantly showed reduction in NO production induced by LPS in RAW 264.7 cells (23.4±2.82μM to 2.9±1.02μM) and (16.50± 2.34μM to 3.92±1.12 μM), starting at the concentration of 0.05 μg/ml to 200 μg/ml. Moreover, strains CT1-17 and NE1-12

decreased 11.34 ±0.43μM and 11.73± 0.75μM NO production induced by LPS at 200 μg/ml.

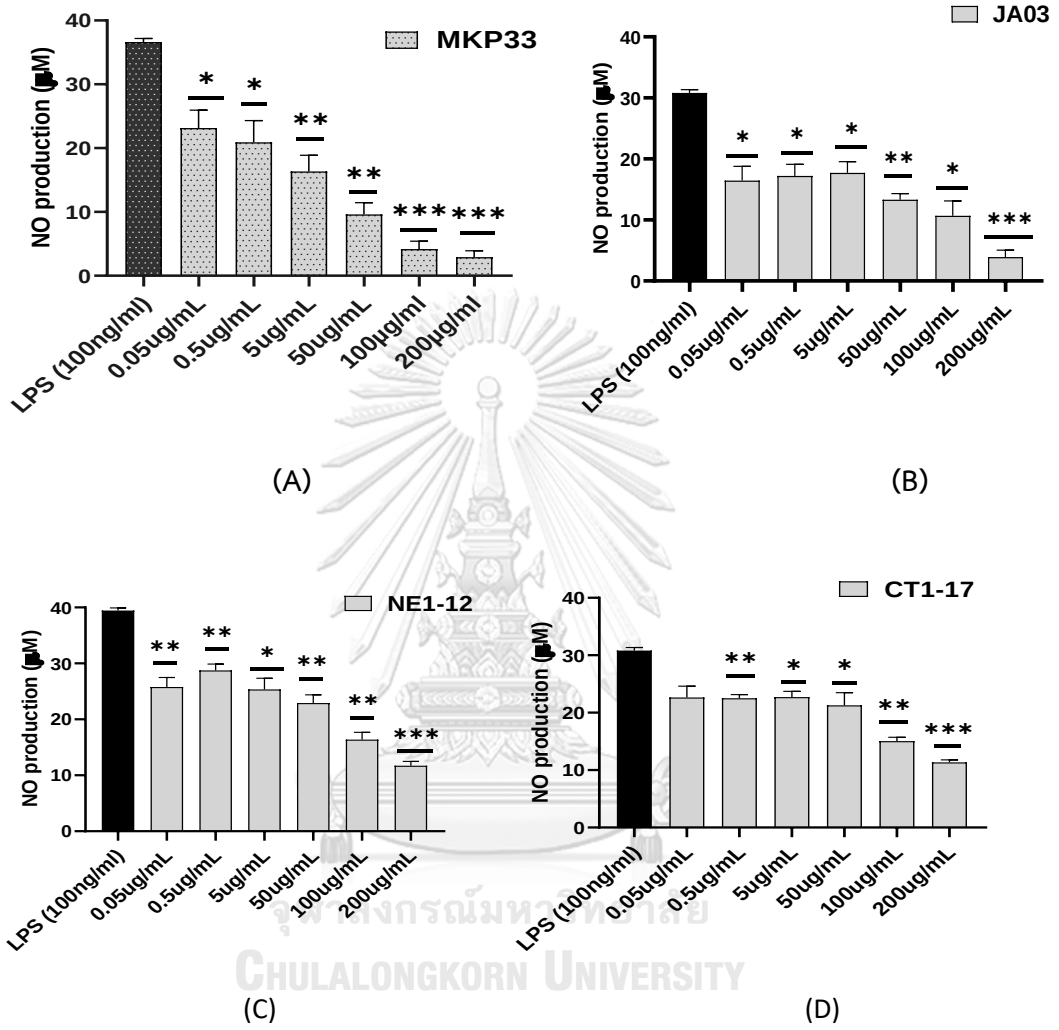



**Figure 29 (A-D). Effect of ethyl acetate crude extracts on NO production in LPS induced RAW264.7 macrophages cells, *p<0.05, **p<0.005, ***p<0.0001 compared to lipopolysaccharide.**

Concentration of nitrite, an indicator of NO production, were measured 24 h incubation with LPS (100ng/ml) and various concentrations of ethyl acetate crude extracts. CT2-4 and CT2-10 significantly decreased 15.7±0.65 μM and 13.87± 1.78μM

NO production induced by LPS (100ng/ml) in RAW264.7 macrophage cells at 10μg/mL when compared with the control group shown in Figure 30. Strains CT2-4 and CT2-10 in low concentration give a possibility usage for the treatment of diseases caused by free oxygen radicals.

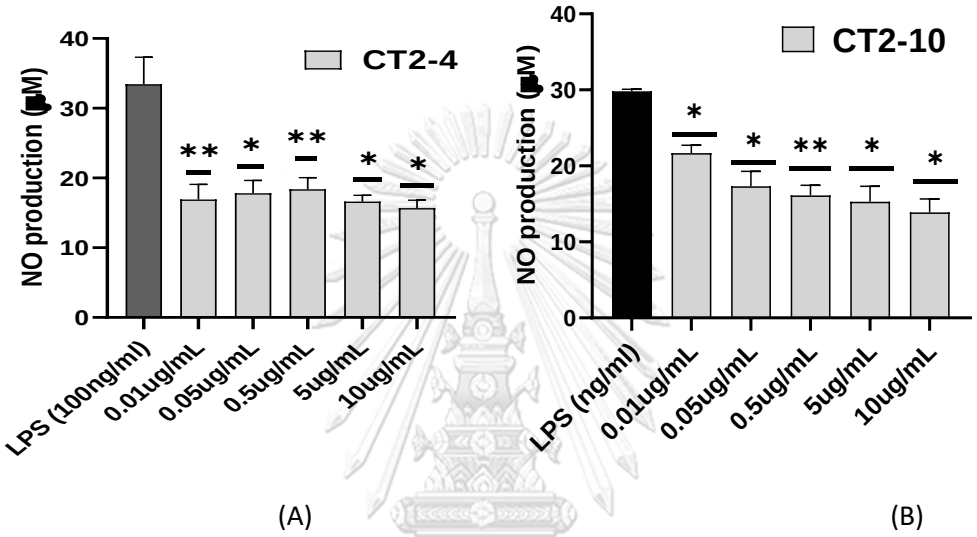



**Figure 30 (A-B). Effect of ethyl acetate crude extracts on NO production in LPS induced RAW264.7 macrophages cells.**

**4.9 Isolation and chemical profile analysis of secondary metabolites of *Streptomyces* strains CT2-10, ET3-23 and NE1-12**

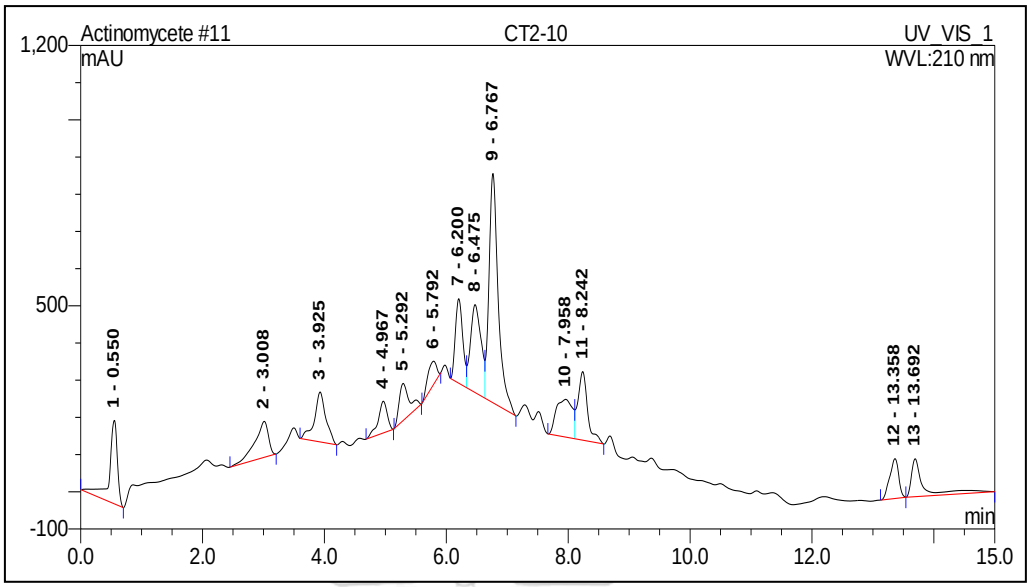
*Streptomyces* isolates CT2-10, ET3-23 and NE1-12 were cultured in ISP2 broth at 180 rpm, 30˚C for 14 days. These fermentation broths were extracted with ethyl acetate solvent. Ethyl acetate and the culture broth (2:1) were shaken vigorously in a separatory funnel. These crude extracts were evaporated on a rotary evaporator at 45˚C to get the dry extract. The chemical profiles were shown in Figures 31,32 and 33.

**Table 8. Retention time (min) and UV spectrum (nm) of *Streptomyces* strains CT2-10, ET3-23 and NE1-12.**

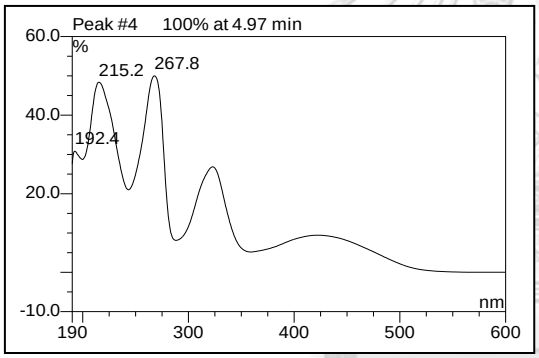
| Sample no | $^{t}R_{uv254}$ (min) | UV spectrum (λmax, nm) | Sample no | $^{t}R_{uv254}$ (min) | UV spectrum (λmax, nm) | Sample no | $^{t}R_{uv254}$ (min) | UV spectrum (λmax, nm) |
|---|---|---|---|---|---|---|---|---|
| CT2-10 | 0.55 | 195 | ET3-23 | 0.55 | 209, 299, 362 | NE1-12 | 0.55 | 194, 292 |
| | 3.01 | 191 | | 4.75 | 204, 231, 303 | | 4.57 | 202, 238, 290 |
| | 3.92 | 206, 249, 372 | | 5.01 | 203, 233, 290 | | 5.09 | 203, 291 |
| | 4.97 | 192, 215, 267, 325 | | 5.65 | 202, 232, 313 | | 5.97 | 194. 340 |
| | 5.29 | 192, 215, 267, 325 | | 5.97 | 195, 233, 343 | | 6.21 | 196, 236, 337 |
| | 6.20 | 192, 224, 265 | | 6.21 | 194, 237, 337 | | 6.66 | 193, 305 |
| | 6.47 | 203, 239 | | 13.32 | 222 | | 7.99 | 196 |
| | 6.77 | 192, 215, 267, 325 | | 13.59 | 210 | | 13.37 | 221 |
| | 7.96 | 219, 238, 306 | | | | | | |

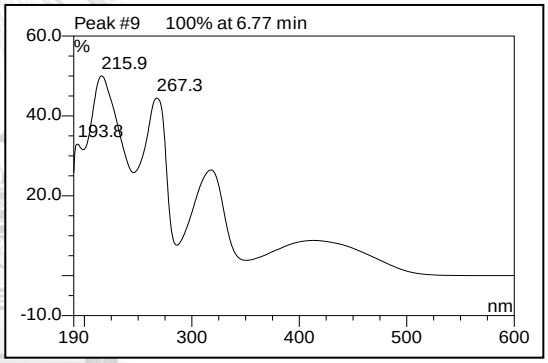


The main absorption of the phenolic groups is given by an intense band at 280 nm. The anthocyanin shows an absorption band around 320 nm. The flavanol group shows UV-visible absorption with an additional absorption band around 360 nm. This absorption band together with the 280 nm absorption features the UV-visible spectra of the flavanol group. These ethyl acetate crude extracts can be good metabolites for antioxidant compounds.




(A)




(B) (C)



Figure 31. HPLC chromatogram of ethyl acetate crude extract of *Streptomyces* sp. CT2-10 (A), UV profiles of peak at $^{t}R$ 4.97 mins (B) $^{t}R$ 6.77 mins (C) respectively. HPLC condition (Column: Purospher column RP-18 from Merck), (Mobile phase: 0-100% Acetonitrile in water) and (Flow rate: 0.5ml/min)

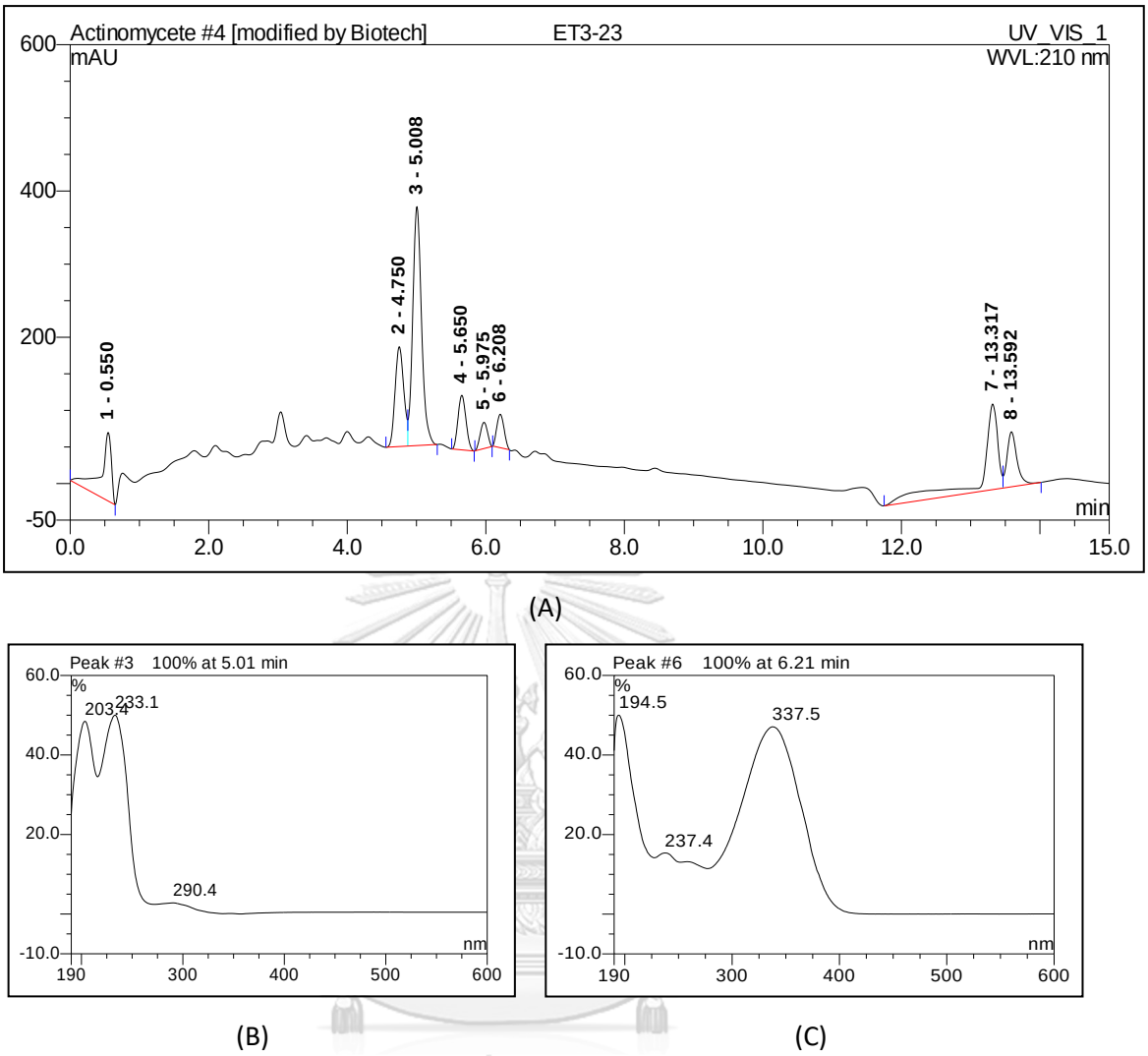
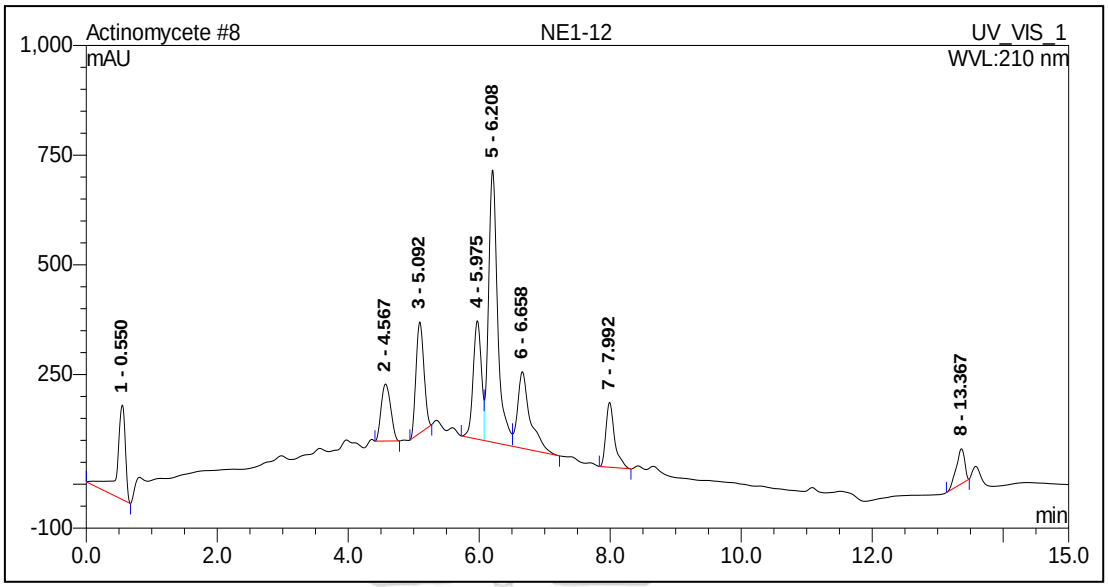
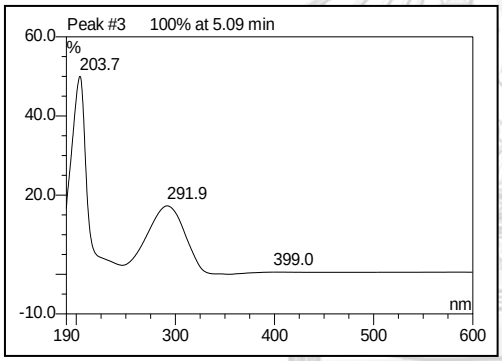


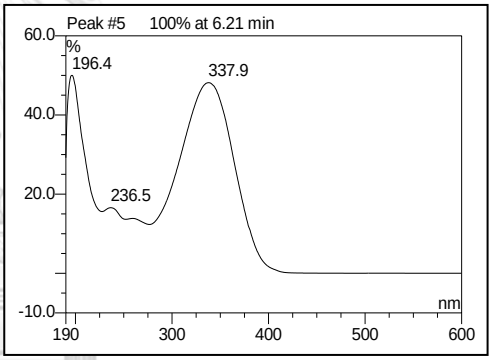



Figure 32. HPLC chromatogram of ethyl acetate crude extract of *Streptomyces* sp. ET3-23 (A), UV profiles of peak at $^{t}R$ 5.01 mins (B) $^{t}R$ 6.21 mins (C) respectively. HPLC condition (Column: Purospher column RP-18 from Merck), (Mobile phase: 0-100% Acetonitrile in water) and (Flow rate: 0.5ml/min)




(A)




(B) (C)



Figure 33. HPLC chromatogram of ethyl acetate crude extract of *Streptomyces* sp. NE1-12 (A), UV profiles of peak at $^{t}R$ 5.09 mins (B) $^{t}R$ 6.21 mins (C) respectively. HPLC condition (Column: Purospher column RP-18 from Merck), (Mobile phase: 0-100% Acetonitrile in water) and (Flow rate: 0.5ml/min).

# CHAPTER V

# DISCUSSION

Actinomycetes are widely distributed microorganisms in nature which primarily inhabit the soil and are one of the major groups of soil population. Kawahara et al., (2012) reported that actinomycetes efficiently produce bioactive molecules in a screening database for searching antibiotics and antioxidants from microbial sources.

Based on phenotypic characteristics including cultural, physiological and biochemical characteristics, phylogenetic analyses on the 16S rRNA gene sequences and the presence of LL-DAP and *meso*-DAP in the cell wall peptidoglycan, these strains are nearby to the genera *Streptomyces* and *Micromonospora*.

Based on the analysis of the biosynthesis secondary metabolites gene clusters of strains CT1-17, ET3-23 and JA03 using antiSMASH, divergolide isolated from *Strepromyces* sp. HKI0595 showed the broadest antibacterial activity of all members of the family (64). Coelichelin, a ferric-iron chelating peptide, is one such siderophore produced by the bacterium *Streptomyces coelicolor* (65). Germicidin A (1) and B (2) isolated from *Streptomyces coelicolor* A3, *Streptomyces viridochromogenes* NRRL B-1551 and *Streptomyces* sp. SCS525 reported as strong antioxidant compounds and was confirmed using DPPH and ABTS assays. Bacteriocins, antimicrobial peptides, treat pathogen disease and cancer therapy, and maintain human health (66). Lobophorin exhibited cytotoxic activity on the human pancreatic carcinoma (MiaPaca-2) and the

breast adenocarcinoma (MCF-7) human cells and moderate and selective antimicrobial activity against *Staphylococcus aureus* (67).

Huang et al., 2015 reported that neocarazostatin A 1 isolated from the soil bacterium *Streptomyces* sp. MA37 is a potent free scavenging agent to protect cell damage caused by the free radicals (68). Kato et al. 1991 reported the neocarazostatins A, B and C, the novel free radical scavenging substances, from *Streptomyces* strain GP 38 exhibited a strong inhibitory effect on the free radical induced lipid peroxidation in rat brain homogenate. (69). Neocarazostatins A–C, carbazole-3,4-quinone alkaloids, protect neuronal cells against oxidative damage caused by free radicals. (70). Berninamycins A-D isolated from *Streptomyces bernensis* is potent inhibition of protein biosynthesis in broad range of gram-positive bacteria by binding to their ribosomal subunits (71). Pentamycin is active against *Candida albicans*, *Trichomonas vaginalis*, and several pathogenic bacteria for the treatment of vaginal candidiasis, trichomoniasis, and mixed infections and has also been shown to increase the efficacy of the anticancer drug bleomycin (72). Cinnamycin binds to lipid molecules with a phosphatidylethanolamine having hemolytic and antimicrobial properties and inhibits angiotensin-I converting enzyme in the treatment of blood pressure regulation, inflammation, and viral infection. (73). Blasticidin S produced by some *Streptomyces* species, is an antibiotic that inhibits cytosolic protein synthesis by blocking ribosomal translation termination (74). Althiomycin from the actinobacteria *Streptomyces*

*althioticus,* displays wide spectrum antibiotic activity against Gram-positive and Gram-negative bacteria by inhibiting protein synthesis (75).

Albaflavenone exhibited antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Candida parapsilosis* ATCC 22019 and *Candida albicans* ATCC 10231 using the micro broth dilution method (76). In medicine, melanins are effective in radical scavenging capacity and protect patients against the harmful effects of gamma rays used in cancer therapy and could capture electrons and scavenge ROS produced through organism's metabolism or due to UV radiations and protect from oxidative stress. The melanin pigment extracted from *Pseudomonas balearica* possesses the efficient DPPH radical scavenging activity and its antimicrobial activity against some Gram-positive and *E. coli* (77). Aurantimycin isolated from *Streptomyces aurantiacus* JA 4570 exhibited antimicrobial activity against *Bacillus subtilis* ATCC 6633 (MIC 0.013 µg/mL) and *Staphylococcus aureus* 285 (MIC 0.013 µg/mL) and showed cytotoxic effects against L-929 mouse fibroblast cells from nontoxic to lethal concentrations (3-12 ng/mL) (78).



Even though antimicrobial activity of biosynthetic secondary metabolites gene clusters, *Streptomyces* strains CT1-17, ET3-23 and JA03 have antimicrobial resistance genes based on AMR genes detection method and showed no antimicrobial activity.

During the cultivation for 14-days, fermentation process was accomplished to enhance cell growth and secondary metabolites production extracted with ethyl acetate. The antimicrobial and antioxidant activities, cytotoxicity, intracellular ROS generation and NO production on RAW264.7 macrophage cells induced by hydrogen peroxide and lipopolysaccharides of crude extracts were evaluated.

Filamentous soil bacteria belonging to the genus *Streptomyces* are rich sources of a high number of bioactive natural products with biological activity; and these bacteria produce about 75% of commercially and medically useful antibiotics and are useful in the production of antimicrobials against bacteria and fungi. McCann P.A and Pogell B.M., (1979) reported that pamamycin is a new antibiotic isolated from *Streptomyces alboniger* ATCC 12461. *In vitro* antimicrobial spectrum of pamamycin was highly active against Gram-positive bacteria, *Neurospora crassa,* and *Mycobacteria* smegmatis, but inactive against Gram-negative bacteria (79). Yan X et al., (2018) reported that three cryptic nucleosides products, including puromycin A, B and C isolated from *Streptomyces alboniger* NRRL B-1832, aminonucleoside antibiotic were the broad spectrum secondary metabolites and effective against Gram-positive bacteria, protozoans, and mammalian cells, including tumor cells by inhibiting both 70s and 80s ribosomes(80). 17 actinomycetes were isolated from soil collected from various sources. The obtained result is that all of the culture broth cultivated in 4 production media did not inhibit growth of *P. aeruginosa* and *E. coli* but CT2-4, ET1-12, ET2-2, NE1-3, NE2-4 and NE2-6 inhibit Gram-positive bacteria and fungi; *S. aureus*,

*B. subtilis, K.rhizophila* and *C.albicans*. The antimicrobial profile for each strain depended upon the culture medium. Among the extracts from four media, NE2-6 gave the highest inhibition zone against *K.rhizophila* followed by ET1-12 and ET2-2. However, NE2-6 gave only little activity against *S. aureus* with the inhibition zone around 14.1 mm. Moreover, ET1-12 gave less inhibition zone against *B.subtilis* when compared with *K.rhizophila* and *S. aureus*. ET2-2 inhibit on *B.subtilis* and CT2-4 showed moderate inhibition zone against *S. aureus*. These results demonstrated that NE2-6 showed higher bacterial inhibitory activity when compared to other extracts. Furthermore, NE2-6 nearest to the type strain, *Streptomyces alboniger* NRRL B-1832$^{T}$, contains puromycin A, B and C, aminonucleoside antibiotic, which are effective on Gram positive bacteria. Further studies should be investigated to produce pure compounds from natural microbial sources and novel sources of natural bioactive molecules.



Hong et al., (2009) revealed that potent antioxidants from microbial sources own high antioxidant activity and these microbes have attained the ability to synthesize specific antioxidants (81). Luis Aleixandre et al., (2019) reported that phenolic compounds are the effective antioxidants and can prevent the inflammation, neurodegenerative, cardiovascular diseases or even against cancer. To quantify phenolic compounds, UV-visible spectroscopy has proven. The absorbance at 280 nm reported the best indicator of the phenolic content possessing the phenolic ingredients, and the phenolic ring, to absorb UV light. Depending on the spectral

characteristics, phenolic composites can quantify their absorption maxima, i.e. sub-families of phenolics at 280 nm for flavanol monomers and polymers and 320 nm for hydroxicinnamic acids, 360 nm for flavonols and finally 520 nm for anthocyanins (82).

Free radicals are the major cause of chronic and degenerative diseases causing aging, cardiovascular disease, inflammation, diabetes mellitus and cancer. Therefore, natural antioxidants are more desirable, and many current studies have exposed the source of antioxidants, microorganisms become interested in searching for the manufacture of natural antioxidants. Actinomycetes have been evidenced an important microbial source of secondary metabolites with extensive applications. Phenolic compounds have been known to act as antioxidants due to their capability to donate electrons as well as the effectiveness of stabilizing radical intermediates in the prevention of oxidation at cellular and physiological level (83). Lee et al., (2014) revealed that total phenolic content of ethyl acetate extract of *Streptomyces* sp. strain MJM 10778 isolated from mountain forest soil was expressed as gallic acid equivalence (GAE) in μg and was found to be 8.8±0.2 μg GAE/g dry weight (84). Phenolic compounds are involved in antioxidant activity. Under the basic reaction conditions, a phenol loses an $H^+$ ion to produce a phenolate ion, which reduce the Folic-Ciocalteu reagent. Furthermore, the higher antioxidant activity was found in MKP33 and JA03 with the higher total phenolic content in the presence of phenolic hydroxyl groups. By contrast, the lower antioxidant activity was determined in NE2-4 and ET1-12 with the lower total phenolic content. Many researchers reported that there is correlation between

antioxidant activity and the content of phenolic compounds. Radical scavenging activities by the tested samples might be due to the presence of the hydroxyl groups and electron donating ability. These results suggested that MKP33 was able to reduce the stable radical DPPH to the yellow colored diphenylpicrylhydrazine and has potential antioxidant activity. Additionally, phenolic compounds are correlated with antioxidant activity and anticancer effect and may prevent the development of aging and disease.

Prior et al., (2005) showed that FRAP assay is measured the reducing capability of antioxidants by increasing the absorbance based on the release of the blue colored ferrous ions released at pH 3.6 at 593 nm (85). Abdelmohsen U.R et.al., (2012) exhibited that diazepinomicin, a dibenzodiazepine alkaloid among the microbial metabolites, was isolated from the marine sponge-associated strain *Micromonospora* sp. RV115. A strong antioxidant potential of diazepinomicin was demonstrated by the ferric reducing antioxidant power (FRAP) assay. This result demonstrated that diazepinomicin exhibited significant antioxidant activity, with 30 nM of diazepinomicin with an equal potential of 50 μM tempol as positive control, indicating the strong antioxidant potential of diazepinomicin (86). The antioxidant activity was measured by FRAP assay based on the redox capacities of the compounds characterized by the complex molecules. From the present results of the reducing ability of polyphenols as determined by the FRAP assay, the reducing capacity of ET3-23 might assist as a significant indicator of its potential antioxidant capacity with reducing capacity and the

proton-donating ability and followed by JA03 and CT1-17. The reducing capacity of MKP33 might assist as a significant indicator of its potential antioxidant capacity. MKP33 nearest to the type strain, *Micromonospora yasonensis* DS 3186$^{T}$ had potent total phenolic compounds and FRAP values compared to others. Therefore, ethyl acetate extract of MKP33 exhibited maximum reducing capacity and could serve as free radical inhibitors or scavengers and primary antioxidants.

DPPH is a stable free radical dissolved in ethanol, and its colors show characteristic absorptions at 515nm. When an antioxidant scavenges the free radicals by hydrogen donation, the colors of DPPH solutions become yellow colour (87). Taechowisan et al. (2017) studied that antioxidative phenolic compounds identified as JBIR-94 and JBIR-125 from *Streptomyces* sp. R56-07 exhibited DPPH radical scavenging activity with an $IC_{50}$ 11.4 μM and 35.1 μM, respectively (88). Tan et al (2015) reported that pyrrolopyrazine compounds isolated from *Streptomyces* sp. had been identified to possess potent antioxidant activity (89). Sowndhararajan and Kang (2013) reported that ethyl acetate extract of *Streptomyces* sp. AM-S1 culture filtrate registered very strong DPPH ($IC_{50}$ of 68.4 lg/ml) radical scavenging activity which indicated that the mechanism of antioxidant action was as a hydrogen donor that terminates the oxidation process by converting free radicals into the stable forms (90). Lee et al., (2014) exhibited the soil-borne actinobacteria, *Streptomyces* sp. MJM 10778, possessing DPPH free radical scavenging (81.6%) at 500 μg/mL and nitric oxide free radical scavenging (95.4%) at 1000 μg/mL (84).

Antioxidants can reduce the purple-colored DPPH radical to a yellow-colored non-DPPH radical, and the extent of reaction was related to the ability of hydrogen donation. Sanjivkumar M, et al., (2016) presented that in DPPH scavenging assay, ethyl acetate crude extracts isolated from *Streptomyces olivaceus* (MSU3) showed increasing percentage of inhibition with increasing concentration of extract. The DPPH scavenging activity of the crude extract exhibited as 38.5, 44.52, 57.36% and 62.06% against 12.5, 25, 50 and 100 mg/ml concentrations respectively with the $IC_{50}$ of 75.21 mg/ml (91). Sanjivkumar M, et al., (2019) reported that in *in-vitro* antioxidant activity, silver nanoparticles (AgNPs) from *Streptomyces olivaceus* (MSU3), expressed highest (60.38%) inhibition at 100 µg/ml, while lowest (28.0%) inhibition was observed at 12.5 µg/ml. In DPPH radical scavenging activity, the AgNPs exhibited increased % inhibition (36.12%, 41.87%, 47.00% and 58.73%) with the increasing concentrations (12.5, 25, 50 and 100µg/ml) of AgNPs with the $IC_{50}$ value of 52.31 µg/ml (92). As the outcome of this present study, a decrease in the absorbance of DPPH solution at 515 nm observed that strain CT1-17 closely related to the type strain, *Streptomyces olivaceus NRRBL 3009* $^{T}$, showed potent DPPH radical scavenging of extract with concentration dependent manner and $IC_{50}$ value 218.645µg/mL. The lower $IC_{50}$ value in JA03 and ET3-23 indicated that it could be hydroxyl radical scavenger. This proofs that these extracts contain some active constituents that can donate hydrogen to a free radical for removal of odd electron responsible for radical's reactivity and also has greater antioxidant activity than the others. Depending on the results of whole genome

analysis, secondary metabolites of *Streptomyces* strains reported that germicidin from strain CT1-17 has strong antioxidant compounds with their antioxidant activity using DPPH and ABTS assays, melanins from strain JA03 have a very effective radical scavenging capacity and could capture electrons and scavenge Reactive Oxygen Species (ROS) produced through organisms metabolism or due to UV radiations and protect from oxidative stress and neocarazostatins from strain ET3-23 possess a potent free scavenging agent for protecting cell damage caused by free radicals.

Doshi et al. (2006) and Orhan et al. (2009) exhibited a positive correlation between the phenolic contents and antioxidant potential and crude extract comprises some active ingredients that can donate hydrogen atom to a free radical to remove odd electron accountable for radical's reactivity. Abdelmohsen U.R et.al., (2012) exhibited that diazepinomicin, a dibenzodiazepine alkaloid was isolated from the marine sponge-associated strain *Micromonospora* sp. RV115. Based on the ferric reducing antioxidant power (FRAP) assay, this result demonstrated that diazepinomicin exhibited strong antioxidant potential with 30 nM of diazepinomicin with an equal potential of 50 μM tempol as positive control, indicating the strong antioxidant potential of diazepinomicin (86). Strain MKP33 nearest to the type strain, *Micromonospora yasonensis* DS 3186$^{T}$ exhibited higher DPPH radical scavenging activity and indicated the presence of a higher content of phenolic and FRAP value. These results indicated that these extracts reduce the DPPH radical and has good antioxidant activity. MKP33 with low $IC_{50}$ on DPPH radical scavenging might assist as a significant

indicator of its potential antioxidant capacity even though JA03 and ET3-23 with lower $IC_{50}$ showed potent DPPH radical scavenging activity. Its higher radical scavenging activity showed that the mechanism of action of antioxidant was based on a hydrogen donor and could eliminate the oxidation procedure by altering free radicals into the stable forms. The percentage of DPPH radical scavenging activity of MKP33 showed concentration dependent manner even though in decreasing in the range of concentrations (1-200µg/ml).

Lee et al., (2014) showed *Streptomyces* strain MJM 10778 extract having NO scavenging activities with increasing concentration of extract as follows: (89.6±0.2)% at 31.25 µg/mL, (90.8±0.2)% at 62.5 µg/mL, (91±0.2)% at 125 µg/mL, (92.4±0.2)% at 250 µg/mL, (92.6±0.6)% at 500 g/mL, and (95.4±0.1)% at 1 000 µg/mL and $IC_{50}$ value (0.02 µg/mL) (84). Incubation with sodium nitroprusside in phosphate buffer solution for 2hrs resulted in linear time-dependent nitrite production, which is reduced by ethyl acetate extracts. This may be due to the antioxidant potency in these extracts, thereby decreasing in nitrite generation. Both of NE2-6 and MKP33 showed NO scavenging activity at 500 µg/mL and 100µg/mL and then exhibited decrement in NO scavenging activity at higher concentration 1000µg/mL and 200µg/mL and JA03, NE1-12 and ET3-23 showed scavengers of nitric oxide with 44-%-48% scavenging. Abdelmohsen U.R et.al., (2012) exhibited that diazepinomicin isolated from the marine sponge-associated strain *Micromonospora* sp. RV115 had the strong antioxidant potential by the ferric reducing antioxidant power (FRAP) assay (86). Moreover, MKP33 showed scavenger of

nitric oxide and inhibited nitrite formation by competing with oxygen to react with nitric oxide thereby inhibiting nitrite generation. Diazepinomicin with antioxidant potential was isolated from *Micromonospora sp. RV115.* MKP33 nearest to the type strain, *Micromonospora yasonensis* DS 3186$^{T}$ can scavenge nitric oxide radicals and the potent antioxidant potential of FRAP values. Strain MKP33 contains more polyphenols which are one of the major antioxidant components and higher FRAP values and higher amount in total phenolic content compared to other extracts and are effective in nitric oxide scavenging.

Mantani, 2001 exhibited that in MTT assay, (3-(4, 5 - dimethyl thiazol-2yl)-2, 5-diphenyl tetrazolium bromide (MTT) cleaved by mitochondrial succinate dehydrogenase and reductase of viable cells, yields a quantifiable purple formazan crystal which is directly proportional to the number of cells viability and in reverse proportional to the degree of cytotoxicity. Lee et al., (2014) exhibited the cytotoxicity of ethyl acetate extract of the strain MJM 10778 on RAW 264.7 cells was no cytotoxicity (81.50% of cell viability at 100 µg/mL) (84). Taechowisan et al., (2019) showed that cell viability of biphenyl compounds (compound 1 and 2) isolated from the culture of *Streptomyces* sp. BO07, an endophyte in *Boesenbergia rotunda* (L.) were observed to be over 90% at non-toxic concentrations of up to 20 µg mL by the MTT assay (93). The number of activated viable macrophages were not altered by ethyl acetate extracts as determined by the MTT assay and this was indicated that intracellular radical inhibition was not owing to cytotoxicity of ethyl acetate extracts. The cytotoxic effects of

*Streptomyces* sp. were evaluated by MTT assay on RAW 264.7 macrophage cells. Based on cytotoxic assay, ethyl acetate extracts have no cytotoxicity in $H_2O_2$ induced RAW 264.7 macrophages in tested concentration and non-toxic concentration of ethyl acetate extracts were selected for intracellular ROS production and also had an antioxidant potential by suppressing ROS production in $H_2O_2$ induced RAW 264.7 macrophages.

As proved in DCFH-DA assay, intracellular radical release in induction of H2O2 led to an increase in DCF fluorescence intensity related to the amount of ROS generation. Leiros et al., (2013) proved that undecylprodigiosin (0.01 μM) from *Streptomyces* sp. was also effective against $H_2O_2$ -mediated induction of ROS (94). Intracellular ROS generation by $H_2O_2$ induction were monitored by DCFH-DA on RAW 264.7 macrophages. As proved in DCFH-DA assay, intracellular radical released in induction of $H_2O_2$ led to an increase in DCF fluorescence intensity related to the amount of ROS generation. However, pretreatment with ethyl acetate extracts CT1-17, CT2-4, CT2-10, NE1-12, NE2-6, JA03, MKP33 and ET3-23 efficiently suppressed $H_2O_2$ induced ROS production, as significantly evidenced by the lower DCF fluorescence intensity having an antioxidant potential by suppressing the radical release in $H_2O_2$ induced RAW 264.7 macrophages. It was noted that NE1-12 and MKP33 have decrement in ROS production and antioxidant potential by defeating the radical release.

Macrophages produce nitric oxide (NO) and pro-inflammatory cytokines in response to lipopolysaccharide (LPS). NO, a type of ROS, causes the destruction of the cells and tissue injury causes by induction of inflammatory reaction. Taechowisan et al., (2009) observed that the lansai A-D produced by *Streptomyces* sp. SUC1 isolated from the aerial roots of *Ficus benjamina* prevented NO production in macrophages stimulated with lipopolysaccharide (LPS) in vitro (95). Taechowisan et al., (2019) reported that the production of NO in LPS-induced RAW 264.7 incubated with biphenyl compounds (compound 1 and 2) isolated from the culture of *Streptomyces* sp. BO07, an endophyte in *Boesenbergia rotunda* (L.) at 5, 10 and 20 µg/mL for 24 h and prevented NO production in dose-dependent fashion with 43.67±6.11, 31.86±5.46 and 22.13±6.76 µM, respectively, 36.74±5.05, 26.67±6.81 and 20.87±4.38 µM, respectively (93). Taechowisan et al., (2019) showed that the production of NO in LPS-induced RAW 264.7 incubated with geldanamycin isolated from *Streptomyces* sp W14 from the rhizome tissue of *Zingiber zerumbet* (L.) Smith at 1, 2.5 and 5 µg/ml for 24 h were 48.72 ± 7.43, 36.51 ± 5.84 and 20.28 ± 4.66 µM, respectively (96). Park et al., (2012) observed that dechlorothienodolin and thienodolin isolated from *Streptomyces* sp. (strain CNY325) inhibited NO production in LPS stimulated RAW 264.7 cells, and therefore may represent promising candidates for anti-inflammatory and cancer chemoprevention (97). The result of the present study demonstrated that strains MKP33 and JA03 significantly decreased the nitrite accumulation in LPS stimulated RAW264.7 macrophage cells. The inhibition of NO production in cells indicates the

presence of antioxidant molecules responsible for the inhibitory action. Diazepinomicin with antioxidant potential was isolated from *Micromonospora sp.* These results indicate that MKP33 nearest to the type of the strain, *Micromonospora yasonensis* DS 3186 $^{T}$ and strain JA03 can prevent LPS induced oxidative stress in RAW 264.7 cells.

# CHAPTER VI

# CONCLUSION

Based on laboratory analysis of phenotypic characteristics of the strains, these strains are closely related to the genera of *Streptomyces and Micromonospora.* On the results of phenotypic and genotypic characteristics, strain JA03 showed 98.95% similarity of 16S rRNA gene sequence to *Streptomyces puniciscabiei* DSM 41929$^{T}$, this might be the novel species of *Streptomyces*. On the results of antimicrobial activity, strain NE2-6 exhibited the good antimicrobial activity against *Kocuria rhizophila* ATCC 9341, while strain ET1-12 showed the good anti-fungal activity against *Candida albicans* ATCC 10231. Based on the antioxidant activity, strain JA03 with high phenolic contents and low $IC_{50}$ value indicated the potent DPPH radical scavenging activity and MKP33 with high FRAP values presented moderate NO scavenging activity. In addition, strain ET3-23 showed the inhibition of intracellular ROS generation in hydrogen peroxide induced RAW264.7 macrophage cells. Moreover, strain CT2-10 showed the reduction in NO production in LPS induced RAW264.7 macrophage cells. These findings supported that *Streptomyces* strains might be a good bioactive metabolite sources for drug discovery, especially in the progress of the effective antibiotics and antioxidant agents. The further study for the purification and activities of bioactive compounds from candidate actinomycetes from soil should be investigated.

# REFERENCES

1. Subhan Sa, Wahab A, Mujahid T, Abbas T, Khan I, Idrees S. Screening of actinomycetes from indigenous soil for production of extracellular metabolites. *International Journal of Current Research*. 2015;7:12078-83.

2. Sharma M, Dangi P, Choudhary M. Actinomycetes: Source, identification, and their applications. *International Journal of Current Microbiology and Applied Sciences* 2014:801-32.

3. Bhatti AA, Haq S, Bhat RA. Actinomycetes benefaction role in soil and plant health. *Microbial Pathogenesis*. 2017;111:458-67.

4. Alam MN, Bristi NJ, Rafiquzzaman M. Review on in vivo and in vitro methods evaluation of antioxidant activity. *Saudi Pharmaceutical Journal.* 2013;21(2):143-52.

5. Jung H, Kwak H-K, Hwang KT. Antioxidant and antiinflammatory activities of cyanidin-3-glucoside and cyanidin-3-rutinoside in hydrogen peroxide and lipopolysaccharide-treated RAW264.7 cells. *Food Science and Biotechnology.* 2014;23(6):2053-62.



6. Kim M-C, Lee J, Kim D-H, Son H-J, Heo M-S. Isolation and identification of antioxidant producing marine-source actinomycetes and optimal medium conditions. *Food Science and Biotechnology*. 2014;23(5):1629-35.

7. Pham-Huy LA, He H, Pham-Huy C. Free radicals, antioxidants in disease and health. *International Journal of Biomedical Science.* 2008;4(2):89-96.

8. El-Sousy S, Easa S, Hassan A, Ismail A-M. Production of a bacterial extracellular L-glutaminase possessing high antioxidant capability. *Egyptian Pharmaceutical Journal.* 2021;20:59.

9. Karthik L, Kumar G, Rao KVB. Antioxidant activity of newly discovered lineage of marine actinobacteria. *Asian Pacific Journal of Tropical Medicine.* 2013;6(4):325-32.

10. Wang X, Fang H, Huang Z, Shang W, Hou T, Cheng A, et al. Imaging ROS signaling in cells and animals. *Journal of Molecular Medicine*. 2013;91(8):917-27.

11. Sarma A, Rahaman Mallick A, Ghosh A. Free radicals and their role in different clinical conditions: An overview. *International Journal of Pharma Sciences and Research*. 2010;1.

12. Zhu H, Zhang L, Amin AR, Li Y. Coordinated upregulation of a series of endogenous antioxidants and phase 2 enzymes as a novel strategy for protecting renal tubular cells from oxidative and electrophilic stress. *Experimental Biology and Medicine*. 2008;233(6):753-65.



13. Annappa G, Onkarappa R. In Vitro Antioxidant activity of Metabolite from *Streptomyces fradiae* strain GOS1. *International Journal of Drug Development and Research.* 2013;5:235-44.

14. Naik DV, Prakash DS, Thomas DLA, editors. Role of free radicals and effectiveness of antioxidant in patients with OSMF and OLP. a systemic review. *International Journal of Life Sciences and Pharma Research.* 2018;1:58-69

15. Gunawardena D, Raju R, Münch G. Hydrogen peroxide mediates pro-inflammatory cell-to-cell signaling: a new therapeutic target for inflammation? *Neural Regeneration Research*. 2019;14(8):1430.

16. Genilloud O, González I, Salazar O, Martín J, Tormo JR, Vicente F. Current approaches to exploit actinomycetes as a source of novel natural products. *Journal of Industrial Microbiology & Biotechnology.* 2011;38(3):375-89.

17. Zhou X, Li Y, Basic Biology of Oral Microbes. *Atlas of Oral Microbiology.* Oxford: Academic Press; 2015;1-14.

18. Li Q, Chen X, Jiang Y, Jiang C. Morphological identification of actinobacteria. *Actinobacteria - Basics and Biotechnological Applications*. 2016.

19. De Simeis D, Serra S. A never-ending source of bioactive compounds—An overview on antibiotics production. *Antibiotics*. 2021;10(5):483.

20. Hazarika SN, Thakur D. Chapter 21 - Actinobacteria. In: Amaresan N, Senthil Kumar M, Annapurna K, Kumar K, Sankaranarayanan A, editors. *Beneficial Microbes in Agro-Ecology: Academic Press;* 2020. p. 443-76.



21. Barka E, Vatsa P, Sanchez L, Vaillant-Gaveau N, Jacquard C, Klenk H-P, et al. Taxonomy, physiology, and natural products of actinobacteria. *Microbiology and Molecular Biology Reviews*. MMBR. 2015;80:1-43.

22. Loganayaki N, Siddhuraju P, Manian S. Antioxidant activity and free radical scavenging capacity of phenolic extracts from *Helicteres isora* L. and *Ceiba pentandra* L. *Journal of Food Science and Technology.* 2013;50(4):687-95.

23. Chandra P, Sharma RK, Arora DS. Antioxidant compounds from microbial sources: A review. *Food Research International*. 2020;129:108849.

24. Fadzai Boora EC, and Stanley Mukanganyama. Evaluation of nitrite radical scavenging properties of selected Zimbabwean plant extracts and their phytoconstituents. *Journal of Food Processing*. 2014:1-7.

25. Kato S, Kawasaki T, Urata T, Mochizuki J. In vitro and ex vivo free radical scavenging activities of carazostatin, carbazomycin B and their derivatives. *Journal of Antibiotics.* 1993;46(12):1859-65.

26. Komiyama K, Funayama S, Anraku Y, Mita A, Takahashi Y, Omura S, et al. Isolation of isoflavonoids possessing antioxidant activity from the fermentation broth of *Streptomyces* sp. *Journal of Antibiotics*.. 1989;42(9):1344-9.

27. Shin-ya K, Furihata K, Teshima Y, Hayakawa Y, Seto H. Benthocyanins B and C, new free radical scavengers from *Streptomyces prunicolor*. *Journal of Organic Chemistry.* 1993;58:4170-2.

28. Kn R, Junaid S, N D, Kekuda PTR. Antibacterial and antioxidant activities of *Streptomyces* species SRDP-H03 isolated from soil of Hosudi, Karnataka, India. *Journal of Drug Delivery and Therapeutics*. 2013;3(4).

29. Lertcanawanichakul M, Pondet K, Kwantep J. In vitro antimicrobial and antioxidant activities of bioactive compounds (secondary metabolites) extracted from *Streptomyces lydicus* A2. *Journal of Applied Pharmaceutical Science.* 2015;5:017-21.

30. Lee D-R, Lee S-K, Choi B-K, Cheng J, Lee Y-S, Yang SH, et al. Antioxidant activity and free radical scavenging activities of *Streptomyces* sp. strain MJM 10778. *Asian Pacific Journal of Tropical Medicine*. 2014;7(12):962-7.

31. Shindo K, Takenaka A, Noguchi T, Hayakawa Y, Seto H. Thiazostatin A and thiazostatin B, new antioxidants produced by *Streptomyces tolurosus*. *Journal of Antibiotics.* 1989;42(10):1526-9.

32. Takagi H, Motohashi K, Miyamoto T, Shin-ya K, Furihata K, Seto H. Studies on terpenoids produced by actinomycetes. *Journal of Antibiotics*.. 2005;58(4):275-8.

33. Arai T, Tamotsu F, Masa H, Akihiro M, Yuzuru M, Akio S. Culture media for actinomycetes. *Tokyo: The Society for Actinomycetes Japan.* 1975:1-20.

34. Teng Hern T, Ser HL, Yin W-F, Chan K-G, Lee LH, Goh BH. Investigation of antioxidative and anticancer potentials of *Streptomyces* sp. MUM256 Isolated from Malaysia mangrove soil. *Frontiers in Microbiology*. 2015;6.

35. Narendhran S, Rajiv P, Vanathi P, Sivaraj R. Spectroscopic analysis of bioactive compounds from *Streptomyces cavouresis* KUV39: Evaluation of antioxidant and cytotoxicity activity. *International Journal of Pharmacy and Pharmaceutical Sciences* 2014;6(7):319-22.

36. Rahbar Saadat Y, Yari Khosroushahi A, Pourghassem Gargari B. A comprehensive review of anticancer, immunomodulatory and health beneficial effects of the lactic acid bacteria exopolysaccharides. *Carbohydrate Polymers*. 2019;217:79-89.

37. Jain S, Yadav H, Sinha PR. Antioxidant and cholesterol assimilation activities of selected lactobacilli and lactococci cultures. *Journal of Dairy Research*. 2009;76(4):385-91.

38. Virtanen T, Pihlanto A, Akkanen S, Korhonen H. Development of antioxidant activity in milk whey during fermentation with lactic acid bacteria. *Journal of Applied Microbiology*. 2007;102(1):106-15.

39. Liu J, Steiner M, Dunlop JA, Keupp H, Shu D, Ou Q, et al. Antiproliferative and anticytotoxic effects of cell fractions and exopolysaccharides from *Lactobacillus casei* 01. *Mutation Research.* 2011;721(2):157-62.

40. Kato S, Shindo K, Kawai H, Odagawa A, Matsuoka M, Mochizuki J. Pyrrolostatin, a novel lipid peroxidation inhibitor from *Streptomyces chrestomyceticus* taxonomy, fermentation, isolation, structure elucidation and biological properties. *Journal of Antibiotics.* 1993;46(6):892-9.

41. Martínez-Horta S, Riba J, de Bobadilla RF, Pagonabarraga J, Pascual-Sedano B, Antonijoan RM, et al. Apathy in Parkinson's disease: neurophysiological evidence of impaired incentive processing. *Journal of Neuroscience : The Official Journal of The Society for Neuroscience*.. 2014;34(17):5918-26.

42. Chen B, Liu Y, Song WM, Hayashi Y, Ding XC, Li WH. In vitro evaluation of cytotoxicity and oxidative stress induced by multiwalled carbon nanotubes in murine RAW 264.7 macrophages and human A549 lung cells. *Biomedical and Environmental Sciences* : BES. 2011;24(6):593-601.

43. Tan H-Y, Wang N, Li S, Hong M, Wang X, Feng Y. The reactive oxygen species in macrophage polarization: Reflecting its dual role in progression and treatment of human diseases. *Oxidative Medicine and Cellular Longevity.* 2016;2016:2795090.

44. Yang EJ, Yim EY, Song G, Kim GO, Hyun CG. Inhibition of nitric oxide production in lipopolysaccharide-activated RAW 264.7 macrophages by Jeju plant extracts. *Interdisciplinary Toxicology.* 2009;2(4):245-9.

45. Hadizadeh S, Forootanfar H, Shahidi Bonjar GH, Falahati Nejad M, Karamy Robati A, Ayatollahi Mousavi SA, et al. Antifungal activity of terrestrial *Streptomyces rochei* strain HF391 against clinical azole -resistant *Aspergillus fumigatus*. *Current Medical Mycology*. 2015;1(2):19-24.

46. Ranjan R, Jadeja V. Isolation of actinomycetes: A complete approach. *International Journal of Current Microbiology and Applied Sciences*. 2016;5:606-18.

47. Shirling EB, Gottlieb D. Methods for characterization of *Streptomyces* species. *International Journal of Systematic and Evolutionary Microbiology*. 1966;16:313-40.

48. Kelly KL, Judd DB, Inter-Society Color C, United S, National Bureau of S. ISCC-NBS color-name charts illustrated with centroid colors. [Washington, DC]: *[U.S. National Bureau of Standards];* 1965.

49. Gordon RE, Barnett DA, Handerhan JE, Pang CH-N. *Nocardia coeliaca, Nocardia autotrophica,* and the Nocardin Strain. *International Journal of Systematic and Evolutionary Microbiology.* 1974;24(1):54-63.

50. Seino A. Actinomycete culture collection: A brief outline. *Actinomycetologica.* 1991;5(2):72-7.

51. Dietz A, Mathews J. Classification of *Streptomyces* spore surfaces into five groups. *Applied Microbiology.* 1971;21(3):527-33.

52. Wattam AR, Abraham D, Dalay O, Disz TL, Driscoll T, Gabbard JL, et al. PATRIC, the bacterial bioinformatics database and analysis resource. *Nucleic Acids Research.* 2014;42:D581-91.

53. Blin K, Shaw S, Steinke K, Villebro R, Ziemert N, Lee SY, et al. antiSMASH 5.0: updates to the secondary metabolite genome mining pipeline. *Nucleic Acids Research.* 2019;47(W1):W81-w7.

54. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution.* 1987;4(4):406-25.

55. Klevay M, Ebinger A, Diekema D, Messer S, Hollis R, Pfaller M. Disk diffusion testing using *Candida* sp. Colonies taken directly from CHROM agar medium. *Journal of Clinical Microbiology.* 2005;43(7):3497.

56. Ford L, Theodoridou K, Sheldrake G, Walsh P. A critical review of analytical methods used for the chemical characterisation and quantification of phlorotannin compounds in brown seaweeds. *Phytochemical Analysis*. 2019;30:1-13.

57. Slinkard K, Singleton VL. Total Phenol Analysis: Automation and comparison with manual methods. *American Journal of Enology and Viticulture.* 1977;28(1):49-55.

58. Thawai C, Tanasupawat S, Itoh T, Suwanborirux K, Suzuki K-i, Kudo T. *Micromonospora eburnea* sp. nov., isolated from a Thai peat swamp forest. *International Journal of Systematic and Evolutionary Microbiology* 2005;55(1):417-22.

59. Merly L, Smith SL. Murine RAW 264.7 cell line as an immune target: are we missing something? *Immunopharmacology and Immunotoxicology*. 2017;39(2):55-8.

60. Kuete V, Karaosmanoğlu O, Sivas H. Chapter 10 - Anticancer activities of African medicinal spices and vegetables. In: Kuete V, editor. *Medicinal Spices and Vegetables from Africa: Academic Press*. 2017. p. 271-97.

61. Bao D, Wang J, Pang X, Liu H. Protective effect of quercetin against oxidative stress-induced cytotoxicity in rat pheochromocytoma (PC-12) cells. *Molecules*. 2017;22(7).

62. Kalyanaraman B, Darley-Usmar V, Davies KJA, Dennery PA, Forman HJ, Grisham MB, et al. Measuring reactive oxygen and nitrogen species with fluorescent probes: challenges and limitations. *Free Radical Biololgy Medicine*. 2012;52(1):1-6.

63. Shaaban S. Synthesis and Biological Activity of Multifunctional Sensor/Effector Catalysts. 2011.

64. Terwilliger DW, Trauner D. Selective synthesis of divergolide I. *Journal of the American Chemical Society.* 2018;140(8):2748-51.

65. Williams JC, Sheldon JR, Imlay HD, Dutter BF, Draelos MM, Skaar EP, et al. Synthesis of the siderophore coelichelin and its utility as a probe in the study of bacterial metal sensing and response. *Organic Letters*.. 2019;21(3):679-82.

66. Yang S-C, Lin C-H, Sung CT, Fang J-Y. Antibacterial activities of bacteriocins: application in foods and pharmaceuticals. *Frontiers in Microbiology*. 2014;5(241).

67. Braña AF, Sarmiento-Vizcaíno A, Osset M, Pérez-Victoria I, Martín J, de Pedro N, et al. Lobophorin K, A new natural product with cytotoxic activity produced by *Streptomyces* sp. M-207 associated with the deep-sea coral *Lophelia pertusa*. *Marine Drugs.* 2017;15(5):144.

68. Huang S, Tabudravu J, Elsayed SS, Travert J, Peace D, Tong MH, et al. Discovery of a single monooxygenase that catalyzes carbamate formation and ring contraction in the biosynthesis of the legonmycins. *Angewandte Chemie*. 2015;54(43):12697-701.

69. Czerwonka R, Reddy KR, Baum E, Knölker H-J. First enantioselective total synthesis of neocarazostatin B, determination of its absolute configuration and transformation into carquinostatin A. *Chemical Communications.* 2006(7):711-3.

70. Kobayashi M, Kuzuyama T. Recent advances in the biosynthesis of carbazoles produced by actinomycetes. *Biomolecules*.2020;10(8).1-14.

71. Kodani S, Ninomiya A. Isolation of new thiopeptide berninamycin E from *Streptomyces atroolivaceus. Asian Journal of Chemistry.* 2013;25:490-2.

72. Zhou S, Song L, Masschelein J, Sumang FAM, Papa IA, Zulaybar TO, et al. Pentamycin biosynthesis in Philippine *Streptomyces* sp. S816: cytochrome p450-catalyzed installation of the C-14 hydroxyl group. *ACS Chemical Biology*. 2019;14(6):1305-9.

73. Vestergaard M, Berglund NA, Hsu P-C, Song C, Koldsø H, Schiøtt B, et al. Structure and dynamics of cinnamycin–lipid complexes: Mechanisms of selectivity for phosphatidylethanolamine lipids. *ACS Omega.* 2019;4(20):18889-99.

74. Svidritskiy E, Ling C, Ermolenko DN, Korostelev AA. Blasticidin S inhibits translation by trapping deformed tRNA on the ribosome. *Proceedings of the National Academy of Sciences of the United States of America.* 2013;110(30):12283-8.

75. Kunze B, Reichenbach H, Augustiniak H, Höfle G. Isolation and identification of althiomycin from *Cystobacter fuscus* (myxobacterales). *Journal of Antibiotics* 1982;35(5):635-6.

76. Zheng D, Ding N, Jiang Y, Zhang J, Ma J, Chen X, et al. Albaflavenoid, a new tricyclic sesquiterpenoid from *Streptomyces violascens*. *Journal of Antibiotics*. 2016;69(10):773-5.

77. Zerrad A, Anissi J, Ghanam J, Sendide K, Mohammed EH. Article: Antioxidant and antimicrobial activities of melanin produced by a *Pseudomonas balearica* strain. *Journal of Biotechnology Letters.* 2014;5.87-94.

78. Zhao H, Wang L, Wan D, Qi J, Gong R, Deng Z, et al. Characterization of the aurantimycin biosynthetic gene cluster and enhancing its production by manipulating two pathway-specific activators in *Streptomyces aurantiacus* JA 4570. *Microbial Cell Factories.* 2016;15(1):160.

79. McCann PA, Pogell BM. Pamamycin: a new antibiotic and stimulator of aerial mycelia formation. *Journal of Antibiotics*. 1979;32(7):673-8.

80. Yan X, Zhang B, Tian W, Dai Q, Zheng X, Hu K, et al. Puromycin A, B and C, cryptic nucleosides identified from *Streptomyces alboniger* NRRL B-1832 by PPtase-based activation. *Synthetic and Systems Biotechnology*. 2018;3:76 - 80.

81. Hong K, Gao AH, Xie QY, Gao H, Zhuang L, Lin HP, et al. Actinomycetes for marine drug discovery isolated from mangrove soils and plants in China. *Marine Drugs.* 2009;7(1):24-44.

82. Luis Aleixandre-Tudo J, du Toit W. The role of UV-visible spectroscopy for phenolic compounds quantification in winemaking. *Frontiers and New Trends in the Science of Fermented Food and Beverage.* 2019.

83. Sánchez-Rangel JC, Benavides J, Heredia JB, Cisneros-Zevallos L, Jacobo-Velázquez DA. The Folin–Ciocalteu assay revisited: improvement of its specificity for total phenolic content determination. *Analytical Methods*. 2013;5(21):5990-9.

84. Lee DR, Lee SK, Choi BK, Cheng J, Lee YS, Yang SH, et al. Antioxidant activity and free radical scavenging activities of *Streptomyces* sp. strain MJM 10778. *Asian Pacific Journal of Tropical Medicine*. 2014;7(12):962-7.

85. Prior RL, Wu X, Schaich K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal of Agricultural and Food Chemistry*. 2005;53(10):4290-302.

86. Abdelmohsen UR, Szesny M, Othman EM, Schirmeister T, Grond S, Stopper H, et al. Antioxidant and anti-protease activities of diazepinomicin from the sponge-associated *Micromonospora* strain RV115. *Marine Drugs*. 2012;10(10):2208-21.

87. Li X, Wu X, Huang L. Correlation between antioxidant activities and phenolic contents of radix *Angelicae sinensis* (Danggui). *Molecules*. 2009;14(12):5349-61.

88. Taechowisan T, Chaisaeng S, Phutdhawong WS. Antibacterial, antioxidant and anticancer activities of biphenyls from *Streptomyces* sp. BO-07: an endophyte in *Boesenbergia rotunda* (L.) Mansf A. *Food and Agricultural Immunology.* 2017;28(6):1330-46.

89. Tan LT, Ser HL, Yin WF, Chan KG, Lee LH, Goh BH. Investigation of antioxidative and anticancer potentials of *Streptomyces* sp. MUM256 isolated from Malaysia mangrove soil. *Frontier Microbiology*. 2015;6:1316.

90. Sowndhararajan K, Kang SC. Evaluation of in vitro free radical scavenging potential of *Streptomyces* sp. AM-S1 culture filtrate. *Saudi Journal of Biological Sciences.* 2013;20(3):227-33.

91. Sanjivkumar M, Babu DR, Suganya AM, Silambarasan T, Balagurunathan R, Immanuel G. Investigation on pharmacological activities of secondary metabolite extracted from a mangrove associated actinobacterium *Streptomyces olivaceus* (MSU3). *Biocatalysis and Agricultural Biotechnology.* 2016;6:82-90.

92. Sanjivkumar M, Vaishnavi R, Neelakannan M, Kannan D, Silambarasan T, Immanuel G. Investigation on characterization and biomedical properties of silver nanoparticles synthesized by an actinobacterium *Streptomyces olivaceus* (MSU3). *Biocatalysis and Agricultural Biotechnology.* 2019;17:151-9.

93. Taechowisa T, Chaiseang S, Puckdee W, Phutdhawon W. Anti-inflammatory Activity of biphenyls from *Streptomyces* sp. BO07 in LPS-induced RAW 264.7 Cells. *Asian Journal of Biological Sciences.* 2019;12:148-55.

94. Leirós M, Alonso E, Sanchez JA, Rateb ME, Ebel R, Houssen WE, et al. Mitigation of ROS insults by *Streptomyces* secondary metabolites in primary cortical neurons. *ACS Chemical Neuroscience.* 2014;5(1):71-80.

95. Taechowisan T, Wanbanjob A, Tuntiwachwuttikul P, Liu J. Anti-inflammatory activity of lansais from endophytic *Streptomyces* sp. SUC1 in LPS-induced RAW 264.7 cells. *Food and Agricultural Immunology*. 2009;20(1):67-77.

96. Taechowisan T, Puckdee W, Phutdhawong W. *Streptomyces zerumbet* , a novel species from *Zingiber zerumbet* (L.) Smith and isolation of its bioactive compounds. *Advances in Microbiology*. 2019;09:194-219.

97. Park EJ, Pezzuto JM, Jang KH, Nam SJ, Bucarey SA, Fenical W. Suppression of nitric oxide synthase by thienodolin in lipopolysaccharide-stimulated RAW 264.7 murine macrophage cells. *Natural Product Communications*. 2012;7(6):789-94.

## APPENDIX A

All media were sterilized in autoclave at 121°C for 15 min but skim milk and media for carbon utilization test were autoclaved at 110 °C for 10 min.

### 1. Humic acid vitamin agar (HV)

| | | |
|---|---|---|
| Humic acid (dissolved in 1 mL of 0.2 N NaOH) | 0.1 | g |
| $Na_2HPO_4$ | 0.05 | g |
| KCl | 0.171 | g |
| $MgSO_4 \cdot 7H_2O$ | 0.005 | g |
| $FeSO_4 \cdot 7H_2O$ | 0.001 | g |
| $CaCO_3$ | 0.002 | g |
| Vitamin B solution | 1 | mL |
| Agar | 1.5 | g |

Vitamin B solution

| | | |
|---|---|---|
| Thiamine-HCl | 0.005 | g |
| Riboflavin | 0.005 | g |
| Nicotinate (Niacin) | 0.005 | g |
| Pyridoxine-HCl | 0.005 | g |
| Inositol | 0.005 | g |
| Ca-pantothenate | 0.005 | g |
| *p*-aminobenzoate | 0.005 | g |
| d-biotin | 0.0025 | g |

To add vitamin B solution, cycloheximide (50 mg/L) and nalidixic acid (25 mg/L) that was filter sterilized.

**2. Starch-casein nitrate agar (SCN)**

| | | |
|---|---|---|
| Soluble starch | 1 | g |
| Sodium caseinate | 0.3 | g |
| $KNO_3$ | 0.2 | g |
| Agar | 1.5 | g |

To add cycloheximide (50 mg/L) and nalidixic acid (25 mg/L) that was filter-sterilized.

**3. 301 Seed medium**

| | | |
|---|---|---|
| Soluble starch | 2.4 | g |
| Glucose | 0.1 | g |
| Peptone | 0.3 | g |
| Meat extract | 0.3 | g |
| Yeast extract | 0.5 | g |
| $CaCO_3$ | 0.4 | g |

**4. Production medium no. 54 (M60)**



| | | |
|---|---|---|
| Soluble starch | 2 | g |
| Glycerol | 0.5 | g |
| Defatted wheat germ (B) | 1 | g |
| Meat extract | 0.3 | g |
| Yeast extract | 0.3 | g |
| $CaCO_3$ | 0.3 | g |

**5. Yeast extract-glucose broth**

| | | |
|---|---|---|
| Yeast extract | 1 | g |
| Glucose | 1 | g |

**6. Production medium no. 30**

| | | |
|---|---|---|
| Soluble starch | 2.4 | g |
| Glucose | 0.1 | g |
| Peptone | 0.3 | g |
| Meat extract | 0.3 | g |
| Yeast extract | 0.5 | g |
| $CaCO_3$ | 0.4 | g |

Trace salts solution A

| | | |
|---|---|---|
| $FeSO_4 \cdot 7H_2O$ | 0.1 | g |
| $MnCl_2 \cdot 4H_2O$ | 0.1 | g |
| $ZnSO_4 \cdot 7H_2O$ | 0.1 | g |
| distilled water | 100 | mL |

**7. Production medium no. 57**

| | | |
|---|---|---|
| Glucose | 2 | g |
| Peptone | 0.5 | g |
| Dry yeast | 0.3 | g |
| Meat extract | 0.5 | g |
| NaCl | 0.5 | g |
| $CaCO_3$ | 0.3 | g |

**8. Yeast extract-malt extract agar (ISP no.2)**

| | | |
|---|---|---|
| Yeast extract | 0.4 | g |
| Malt extract | 1 | g |
| Glucose (Dextrose) | 0.4 | g |
| Agar | 1.5 | g |

**9. Carbon utilization medium**

| | | |
|---|---|---|
| Carbohydrate | 1 | g |
| $(NH_4)_2SO_4$ | 0.264 | g |
| $K_2HPO_4 \cdot 3H_2O$ | 0.565 | g |
| $KH_2PO_4$ anhydrous | 0.238 | g |
| $MgSO_4 \cdot 7H_2O$ | 0.1 | g |
| Trace salts solution B | 0.1 | mL |
| Agar | 1.5 | g |

Trace salts solution B (Pridham and Gottlieb trace salts)

| | | |
|---|---|---|
| $CuSO_4 \cdot 5H_2O$ | 0.64 | g |
| $FeSO_4 \cdot 7H_2O$ | 0.11 | g |
| $MnCl_2 \cdot 4H_2O$ | 0.79 | g |
| $ZnSO_4 \cdot 7H_2O$ | 0.15 | g |
| Distilled water | 100 | mL |



**10. 10% Skim milk**

| | | |
|---|---|---|
| Skim milk | 10 | g |

To sterilize at 110 °C for 10 min

**11. Mueller-Hinton agar (Difco)**

| | | |
|---|---|---|
| Beef infusion form | 3 | g |
| Casamino acid, technical | 1.75 | g |
| Starch | 0.15 | g |
| Agar | 1.5 | g |

**12. Nitrate reduction test reagent**

Sulphanilic acid solution

| | | |
|---|---|---|
| Sulphanilic acid | 0.8 | g |
| 5 N acetic acid | 100 | mL |

To dissolve by gentle heating in fume hood.

N,N-dimethyl-1-naphthylamine solution

| | | |
|---|---|---|
| N,N-dimethyl-1-naphthylamine | 0.5 | g |
| 5 N acetic acid | 100 | mL |

To dissolve by gentle heating in fume hood.

**13. Ninhydrin solution**

| | | |
|---|---|---|
| Ninhydrin | 0.3 | g |
| n-butanol | 100 | mL |
| Glacial acetic acid | 3 | mL |

## APPENDIX B

### 1. Total phenolic content assay

**Folin-Ciocalteu reagent preparation**

Folin-Ciocalteu reagent (1ml) was dissolved in DI water (9ml) (1:10 v/v) and sodium bicarbonate ($NaHCO_3$) (1g) (10% v/v) was dissolved in DI water (10ml).

### 2. Ferric reducing antioxidant potential assay

**FRAP reagent preparation**

**Reagent A: 300 mM sodium acetate buffer solution** (pH 3.6): Glacial acetic acid (1.6ml) was added to sodium acetate trihydrate (0.31g). This solution was made up to volume (100ml) with DI water.

**Reagent B: 10 mM TPTZ solution:** TPTZ (0.0312g) was added in 40mM HCl (10mL).

**Reagent C: 20mM $FeCL_3.6H_2O$ solution:** Ferric chloride (0.054g) was dissolved in DI water (10mL). FRAP reagent (pale yellow orange in color) was prepared by mixing 300mM sodium acetate buffer, 10mM TPTZ in 40mM HCl and 20mM $FeCl_3.6H_2O$ (10:1:1) at 37˚C.



### 3. DPPH assay

**DPPH reagent preparation**

For the stock solution, DPPH (1,1-diphenyl-2-picrylhydraxyl) (1mM) was dissolved in ethanol (V= mg/MW). DPPH solution was diluted with ethanol (50μM DPPH solution). (M.wt of DPPH=394.32g/mol)

4. **Nitric oxide (NO) scavenging assay**

**Griess reagent and sodium nitroprusside preparation**

Griess reagent (1g) was dissolved in ultrapure water (25mL) and 10 mM sodium nitroprusside dihydrate (0.29795g) was dissolved in 10mM phosphate buffer solution (PBS) (100mL). (M.wt of sodium nitroprusside=297.95g/mol)

**5. Cell viability assay**

**Dulbecco's Modified Eagle Medium (DMEM) preparation (pH7.2)**

| | |
|---|---|
| DMEM | 1 package |
| Fetal Bovine Serum | 10% |
| Penicillin and Streptomycin | 1% |
| Sodium bicarbonate | 3.7g |
| Autoclaved ultrapure water | 1000ml |

**Hydrogen peroxide ($H_2O_2$)**

30%V/V, M.wt = 34.01g/mol, Density=1.14g/mol

**DCFH-DA**

For 10 mM DCFH-DA stock solution, 4.8729 g of DCFH-DA was dissolved in 1ml DMSO (100%). (M.wt of DCFH-DA = 487.29g/mol)

**Lipopolysaccharide (LPS)**

For LPS stock solution, LPS (1.5mg) was dissolved in 1mL PBS.

## TABLES OF EXPERIMENTAL RESULTS

### 1. Total Phenolic Contents of ethyl acetate extracts of *Streptomyces* sp.

| Isolate no | Mean ± SEM |
|---|---|
| NE1-3 | 11.4 ± 0.40 |
| NE1-4 | 3.3 ± 0.56 |
| NE1-6 | 15.6 ± 1.42 |
| NE1-12 | 21.0 ± 1.137 |
| NE2-4 | 20.2 ± 0.44 |
| NE2-6 | 19.3 ± 0.79 |
| NE3-2 | 6.5 ± 0.91 |
| CT1-7 | 7.9 ± 0.98 |
| CT2-4 | 9.1 ± 0.53 |
| CT2-10 | 16.8 ± 0.66 |
| ET1-7 | 5.8 ± 0.55 |
| ET1-12 | 9.7 ± 0.58 |
| ET1-13 | 3.1 ± 1.07 |
| ET2-2 | 7.0 ± 0.26 |
| ET3-23 | 9.1 ± 0.54 |
| JA03 | 33.4 ± 0.66 |
| MKP33 | 31.2 ± 0.60 |

2. FRAP values of ethyl acetate extracts of *Streptomyces* sp.

| Isolate no | Mean ± SEM |
|---|---|
| NE1-3 | 32.9 ± 2.82 |
| NE1-4 | 27.6 ± 0.36 |
| NE1-6 | 31.3 ± 0.81 |
| NE1-12 | 33.9 ± 0.55 |
| NE2-4 | 17.0 ± 0.79 |
| NE2-6 | 31.0 ± 0.88 |
| NE3-2 | 38.4 ± 4.18 |
| CT1-17 | 70.8 ± 1.04 |
| CT2-4 | 30.9 ± 4.61 |
| CT2-10 | 31.4 ± 3.84 |
| ET1-7 | 23.7 ± 0.78 |
| ET1-12 | 13.8 ± 1.26 |
| ET1-13 | 18.3 ± 0.61 |
| ET2-2 | 45.9 ± 5.10 |
| ET3-23 | 95.0 ± 3.14 |
| JA03 | 70.8 ± 1.75 |
| MKP33 | 137.5 ± 5.66 |

**3. % Inhibition of *Streptomyces* sp. on DPPH radicals scavenging assay**

**1. Vitamin C**

| Concentration (µg/mL) | Mean ± SEM |
|---|---|
| 0.5 | 0.535 ± 1.15 |
| 1 | 4.708 ± 3.30 |
| 2 | 11.302 ± 0.29 |
| 4 | 29.002 ± 5.08 |
| 8 | 66.812 ± 7.49 |
| 16 | 86.492 ± 2.20 |
| 32 | 88.061 ± 1.60 |

**2. NE1-3**

| Concentration (µg/mL) | Mean ± SEM |
|---|---|
| 50 | 5.872 ± 0.72 |
| 100 | 11.699 ± 1.78 |
| 200 | 23.253 ± 1.97 |
| 300 | 30.678 ± 4.33 |
| 400 | 39.161 ± 3.91 |
| 500 | 43.984 ± 3.23 |
| 1000 | 61.327 ± 5.28 |

**3. NE1-6**

| Concentration (µg/mL) | Mean ± SEM |
|---|---|
| 50 | 11.734 ± 0.66 |
| 100 | 11.740 ± 0.94 |
| 200 | 25.534 ± 0.86 |
| 300 | 41.068 ± 0.21 |
| 400 | 36.273 ± 4.27 |
| 500 | 52.470 ± 4.29 |
| 1000 | 68.774 ± 1.31 |

**4. NE1-12**

| Concentration (µg/mL) | Mean ± SEM |
|---|---|
| 50 | 12.206 ± 0.77 |
| 100 | 12.82 ± 0.86 |
| 200 | 25.275 ± 0.64 |
| 300 | 40.691 ± 0.27 |
| 400 | 41.407 ± 1.25 |
| 500 | 57.832 ± 1.27 |
| 1000 | 69.799 ± 2.32 |

**5. NE2-4**

| Concentration (µg/mL) | Mean ± SEM |
|---|---|
| 50 | 4.209 ± 0.13 |
| 100 | 6.288 ± 0.51 |
| 200 | 11.432 ± 0.69 |
| 300 | 16.626 ± 0.68 |
| 400 | 20.565 ± 0.47 |
| 500 | 19.826 ± 1.05 |
| 1000 | 33.232 ± 1.00 |

**6. NE2-6**

| Concentration (µg/mL) | Mean ± SEM |
|---|---|
| 50 | 5.373 ± 0.58 |
| 100 | 9.850 ± 0.66 |
| 200 | 16.900 ± 1.85 |
| 300 | 25.907 ± 3.04 |
| 400 | 34.748 ± 2.18 |
| 500 | 45.712 ± 1.98 |
| 1000 | 67.389 ± 0.82 |

**7. NE3-2**

| Concentration (µg/mL) | Mean ± SEM |
|---|---|
| 50 | 7.156 ± 0.84 |
| 100 | 19.064 ± 1.39 |
| 200 | 31.033 ± 1.47 |
| 300 | 38.458 ± 2.84 |
| 400 | 42.858 ± 2.01 |
| 500 | 50.846 ± 4.30 |
| 1000 | 70.811 ± 3.13 |

**8. CT1-17**

| Concentration (µg/mL) | Mean ± SEM |
|---|---|
| 50 | 24.472 ± 0.34 |
| 100 | 31.869 ± 0.42 |
| 200 | 47.528 ± 0.50 |
| 300 | 64.517 ± 0.62 |
| 400 | 80.211 ± 0.04 |
| 500 | 72.088 ± 0.05 |
| 1000 | 58.434 ± 0.32 |

**9. CT2-4**

| Concentration (µg/mL) | Mean ± SEM |
|---|---|
| 50 | 15.460 ± 1.43 |
| 100 | 29.134 ± 1.53 |
| 200 | 42.924 ± 2.27 |
| 300 | 57.338 ± 3.16 |
| 400 | 60.940 ± 2.37 |
| 500 | 74.194 ± 1.93 |
| 1000 | 77.822 ± 1.59 |

**10. CT2-10**

| Concentration (µg/mL) | Mean ± SEM |
|---|---|
| 50 | 7.533 ± 0.54 |
| 100 | 12.708 ± 0.41 |
| 200 | 19.303 ± 2.42 |
| 300 | 30.935 ± 1.80 |
| 400 | 37.609 ± 1.24 |
| 500 | 47.312 ± 3.18 |
| 1000 | 74.281 ± 1.12 |

**10. ET1-7**

| Concentration (µg/mL) | Mean ± SEM |
|---|---|
| 50 | 2.721 ± 1.04 |
| 100 | 4.840 ± 0.71 |
| 200 | 9.637 ± 1.13 |
| 300 | 15.839 ± 0.48 |
| 400 | 17.830 ± 0.34 |
| 500 | 23.346 ± 1.39 |
| 1000 | 46.622 ± 2.06 |

**11. ET1-13**

| Concentration (µg/mL) | Mean ± SEM |
|---|---|
| 50 | 3.551 ± 0.36 |
| 100 | 7.167 ± 1.54 |
| 200 | 18.322 ± 0.92 |
| 300 | 22.373 ± 0.62 |
| 400 | 26.387 ± 2.13 |
| 500 | 26.844 ± 0.80 |
| 1000 | 36.786 ± 1.70 |

**12. ET1-12**

| Concentration (µg/mL) | Mean ± SEM |
|---|---|
| 50 | 5.472 ± 0.94 |
| 100 | 5.220 ± 1.14 |
| 200 | 9.776 ± 2.91 |
| 300 | 15.128 ± 2.44 |
| 400 | 18.143 ± 1.67 |
| 500 | 20.680 ± 0.80 |
| 1000 | 27.288 ± 1.27 |

**13. ET2-2**

| Concentration (µg/mL) | Mean ± SEM |
|---|---|
| 50 | 17.968 ± 2.11 |
| 100 | 28.336 ± 2.90 |
| 200 | 45.243 ± 4.24 |
| 300 | 56.778 ± 3.54 |
| 400 | 62.669 ± 3.05 |
| 500 | 66.823 ± 2.44 |
| 1000 | 67.973 ± 2.09 |

**14. ET3-23**

| Concentration (µg/mL) | Mean ± SEM |
|---|---|
| 50 | 41.936 ± 4.34 |
| 100 | 59.822 ± 2.04 |
| 200 | 77.759 ± 0.98 |
| 300 | 74.799 ± 2.36 |
| 400 | 70.386 ± 4.62 |
| 500 | 65.316 ± 4.79 |
| 1000 | 47.966 ± 10.06 |

**15. JA03**

| Concentration (µg/mL) | Mean ± SEM |
|---|---|
| 50 | 40.614 ± 3.23 |
| 100 | 53.688 ± 1.57 |
| 200 | 64.985 ± 2.06 |
| 300 | 67.304 ± 1.18 |
| 400 | 69.310 ± 2.13 |
| 500 | 68.997 ± 3.25 |
| 1000 | 59.114 ± 2.00 |

**16. NE1-4**

| Concentration (µg/mL) | Mean ± SEM |
|---|---|
| 1 | -7.727 ± 0.74 |
| 10 | -2.595 ± 0.50 |
| 25 | 4.789 ± 1.93 |
| 50 | 19.740 ± 2.53 |
| 100 | 27.462 ± 0.55 |
| 200 | 46.416 ± 1.16 |

**17. MKP33**

| Concentration (µg/mL) | Mean ± SEM |
|---|---|
| 1 | 2.047 ± 1.476 |
| 10 | 4.841 ± 0.52 |
| 25 | 12.204 ± 0.99 |
| 50 | 22.122 ± 1.59 |
| 100 | 39.816 ± 1.91 |
| 200 | 64.514 ± 1.36 |

**4. Nitric oxide (NO) scavenging activity of *Streptomyces* sp.**

**1. Vitamin C**

| Concentration (µg/mL) | Mean ± SEM |
|---|---|
| 50 | 19.743 ± 5.60 |
| 100 | 30.456 ± 3.87 |
| 200 | 44.320 ± 2.78 |
| 300 | 68.238 ± 1.51 |
| 400 | 81.007 ± 2.48 |
| 500 | 87.011 ± 0.48 |
| 1000 | 87.831 ± 0.40 |

**2. ET3-23**

| Concentration (µg/mL) | Mean ± SEM |
|---|---|
| 50 | -3.917 ± 1.74 |
| 100 | 7.143 ± 1.94 |
| 200 | 42.732 ± 2.58 |
| 300 | 37.923 ± 1.63 |
| 400 | 45.02 ± 0.55 |
| 500 | 39.908 ± 6.85 |
| 1000 | 20.569 ± 5.89 |

**3. NE2-6**

| Concentration (µg/mL) | Mean ± SEM |
|---|---|
| 50 | 13.863 ± 1.11 |
| 100 | 21.866 ± 0.75 |
| 200 | 30.830 ± 1.72 |
| 300 | 42.089 ± 1.85 |
| 400 | 47.061 ± 1.14 |
| 500 | 49.681 ± 1.34 |
| 1000 | 42.992 ± 10.48 |

**4. JA03**

| Concentration (µg/mL) | Mean ± SEM |
|---|---|
| 50 | 4.393 ± 2.93 |
| 100 | 14.180 ± 2.35 |
| 200 | 31.177 ± 1.40 |
| 300 | 40.628 ± 4.79 |
| 400 | 44.709 ± 2.53 |
| 500 | 48.133 ± 4.13 |
| 1000 | 38.840 ± 8.87 |

**5. NE1-12**

| Concentration (µg/mL) | Mean ± SEM |
|---|---|
| 50 | 17.385 ± 2.19 |
| 100 | 23.918 ± 1.12 |
| 200 | 40.862 ± 1.06 |
| 300 | 43.948 ± 1.56 |
| 400 | 44.123 ± 3.68 |
| 500 | 27.910 ± 2.10 |
| 1000 | 23.432 ± 7.19 |

**6. MKP33**

| Concentration (µg/mL) | Mean ± SEM |
|---|---|
| 1 | -6.886 ± 2.18 |
| 10 | -3.525 ± 1.33 |
| 25 | 5.258 ± 3.60 |
| 50 | 25.951 ± 2.04 |
| 100 | 49.363 ± 1.96 |
| 200 | **33.886** ± 8.79 |

**5. % Cell viability of crude extracts of *Streptomyces* strains on RAW 264.7 macrophage cells**

**1. JA03**

| Conc: µg/mL | Mean ± SEM |
|---|---|
| Control (0.5%DMSO) | 100 ± 0.01 |
| 10 | 103 ± 5.46 |
| 50 | 107 ± 7.63 |
| 100 | 103 ± 8.27 |
| 200 | 92 ± 6.52 |
| 400 | 73 ± 5.99 |
| 800 | 31 ± 13.42 |
| 1000 | 9 ± 2.41 |

**2. MKP33**



| Conc: µg/mL | Mean ± SEM |
|---|---|
| Control (0.5%DMSO) | 100 ± 0.01 |
| 10 | 126 ± 6.25 |
| 50 | 134 ± 10.10 |
| 100 | 96 ± 2.88 |
| 200 | 86 ± 4.47 |
| 400 | 71 ± 6.37 |
| 800 | 57 ± 9.17 |
| 1000 | 44 ± 2.68 |

**3. CT1-17**

| Conc: µg/mL | Mean ± SEM |
|---|---|
| Control (0.5%DMSO) | 100 ± 0.01 |
| 10 | 102 ± 3.25 |
| 50 | 108 ± 8.45 |
| 100 | 86 ± 3.16 |
| 200 | 82 ± 3.78 |
| 400 | 61 ± 4.81 |
| 800 | 17 ± 9.74 |
| 1000 | 9 ± 6.53 |

**4. NE1-12**

| Conc: µg/mL | Mean ± SEM |
|---|---|
| Control (0.5%DMSO) | 100 ± 0.01 |
| 10 | 124 ± 5.33 |
| 50 | 136 ± 8.89 |
| 100 | 126 ± 7.98 |
| 200 | 133 ± 0.82 |
| 400 | 129 ± 5.99 |
| 800 | 67 ± 7.98 |
| 1000 | 55 ± 5.41 |

5. **ET3-23**

| Conc: µg/mL | Mean ± SEM |
|---|---|
| Control (0.5%DMSO) | 100 ± 0.03 |
| 10 | 108 ± 9.35 |
| 50 | 86 ± 5.59 |
| 100 | 77 ± 6.34 |
| 200 | 62 ± 4.06 |
| 400 | 14 ± 9.20 |
| 800 | 10 ± 4.63 |
| 1000 | 14 ± 6.60 |

6. **NE2-6**

| Conc: µg/mL | Mean ± SEM |
|---|---|
| Control (0.5%DMSO) | 100 ± 0.02 |
| 10 | 112 ± 6.87 |
| 50 | 4 ± 1.57 |
| 100 | 2 ± 0.47 |
| 200 | 2 ± 0.80 |
| 400 | 3 ± 0.59 |
| 800 | 18 ± 7.36 |
| 1000 | 22 ± 4.00 |

7. CT2-4

| Conc: µg/mL | Mean ± SEM |
|---|---|
| Control (0.5%DMSO) | 100 ± 0.00 |
| 10 | 81 ± 7.81 |
| 50 | 17 ± 9.99 |
| 100 | 2 ± 0.19 |
| 200 | 2 ± 0.39 |
| 400 | 1 ± 0.44 |
| 800 | 2 ± 0.39 |
| 1000 | 2 ± 0.40 |

8. CT2-10

| Conc: µg/mL | Mean ± SEM |
|---|---|
| Control (0.5%DMSO) | 100 ± 0.00 |
| 10 | 89 ± 6.11 |
| 50 | 44 ± 7.88 |
| 100 | 2 ± 0.54 |
| 200 | 1 ± 0.27 |
| 400 | 0 ± 0.32 |
| 800 | 1 ± 0.53 |
| 1000 | 1 ± 0.44 |

6. % Cell viability of *Streptomyces* sp. on RAW 264.7 macrophage cells induced by hydrogen peroxide ($H_2O_2$)

1. JA03

| Conc: µg/mL | Mean ± SEM |
|---|---|
| Control (0.5%DMSO) | 100 ± 0.01 |
| $H_2O_2$ (100µM) | 121 ± 0.59 |
| 10 | 106 ± 3.86 |
| 50 | 115 ± 4.94 |
| 100 | 98 ± 8.40 |
| 200 | 90 ± 8.31 |

2. MKP33

| Conc: µg/mL | Mean ± SEM |
|---|---|
| Control (0.5%DMSO) | 100 ± 0.01 |
| $H_2O_2$ (100µM) | 122 ± 5.90 |
| 10 | 113 ± 9.94 |
| 50 | 96 ± 3.42 |
| 100 | 85 ± 2.92 |
| 200 | 79 ± 4.89 |

**3. CT1-17**

| Conc: µg/mL | Mean ± SEM |
|---|---|
| Control (0.5%DMSO) | 100 ± 0.01 |
| $H_2O_2$ (100µM) | 121 ± 4.28 |
| 10 | 104 ± 1.88 |
| 50 | 112 ± 3.42 |
| 100 | 100 ± 5.49 |
| 200 | 69 ± 9.37 |

**4. NE1-12**

| Conc: µg/mL | Mean ± SEM |
|---|---|
| Control (0.5%DMSO) | 100 ± 0.03 |
| $H_2O_2$ (100µM) | 120 ± 1.14 |
| 10 | 125 ± 8.63 |
| 50 | 107 ± 4.69 |
| 100 | 109 ± 3.24 |
| 200 | 111 ± 5.79 |



**5. ET3-23**

| Conc: µg/mL | Mean ± SEM |
|---|---|
| Control (0.5%DMSO) | 100 ± 0.02 |
| $H_2O_2$ (100µM) | 120 ± 1.95 |
| 10 | 81 ± 4.80 |
| 50 | 67 ± 4.65 |
| 100 | 52 ± 0.72 |
| 200 | 26 ± 12.64 |

**6. NE2-6**

| Conc: µg/mL | Mean ± SEM |
|---|---|
| Control (0.5%DMSO) | 100 ± 0.01 |
| $H_2O_2$ (100µM) | 125 ± 2.90 |
| 1 | 105 ± 6.98 |
| 5 | 104 ± 6.12 |
| 10 | 98 ± 6.27 |
| 20 | 15 ± 6.72 |

**7. CT2-4**

| Conc: µg/mL | Mean ± SEM |
|---|---|
| Control (0.5%DMSO) | 100 ± 0.00 |
| $H_2O_2$ (100µM) | 121 ± 9.26 |
| 1 | 103 ± 6.58 |
| 5 | 87 ± 8.53 |
| 10 | 80 ± 7.63 |
| 20 | 25 ± 7.21 |



**8. CT2-10**

| Conc: µg/mL | Mean ± SEM |
|---|---|
| Control (0.5%DMSO) | 100 ± 0.00 |
| $H_2O_2$ (100µM) | 121 ± 4.06 |
| 1 | 105 ± 6.18 |
| 5 | 97 ± 6.07 |
| 10 | 87 ± 7.29 |
| 20 | 55 ± 10.29 |

**7. Intracellular ROS generation of crude extracts of *Streptomyces* sp. on RAW 264.7 macrophage cells induced by $H_2O_2$**

**1. JA03**

| Conc: µg/mL | Mean ± SEM |
|---|---|
| $H_2O_2$ (100µM) | 138 ± 8.31 |
| 0.1 | 105.42 ± 8.46 |
| 0.5 | 107.17 ± 5.81 |
| 1 | 95.71 ± 4.44 |
| 5 | 100.69 ± 9.01 |
| 10 | 93.91 ± 7.19 |
| 50 | 89.43 ± 9.80 |
| 100 | 68.59 ± 7.77 |
| 200 | 56.69 ± 3.39 |

**2. MKP33**

| Conc: µg/mL | Mean ± SEM |
|---|---|
| $H_2O_2$ (100µM) | 141 ± 2.52 |
| 0.1 | 92 ± 10.40 |
| 0.5 | 90 ± 5.37 |
| 1 | 80 ± 2.81 |
| 5 | 61 ± 4.63 |
| 10 | 54 ± 6.30 |
| 50 | 56 ± 5.00 |
| 100 | 40 ± 4.94 |
| 200 | 33 ± 6.56 |

**3. CT1-17**

| Conc: µg/mL | Mean ± SEM |
|---|---|
| $H_2O_2$ (100µM) | 147 ± 9.36 |
| 0.1 | 109.18 ± 2.30 |
| 0.5 | 95.67 ± 7.05 |
| 1 | 80.43 ± 9.37 |
| 5 | 69.37 ± 6.04 |
| 10 | 65.13 ± 9.50 |
| 50 | 70.14 ± 6.80 |
| 100 | 52.69 ± 4.32 |
| 200 | 49.85 ± 4.28 |

**4. NE1-12**

| Conc: µg/mL | Mean ± SEM |
|---|---|
| $H_2O_2$ (100µM) | 147 ± 8.35 |
| 0.1 | 90.58 ± 10.17 |
| 0.5 | 88.26 ± 2.85 |
| 1 | 79.68 ± 10.07 |
| 5 | 66.20 ± 10.11 |
| 10 | 58.95 ± 7.36 |
| 50 | 41.88 ± 4.91 |
| 100 | 38.55 ± 3.83 |
| 200 | 38.10 ± 4.31 |

5. ET3-23

| Conc: µg/mL | Mean ± SEM |
|---|---|
| $H_2O_2$ (100µM) | 147 ± 1.64 |
| 0.1 | 108.34 ± 8.27 |
| 0.5 | 88.92 ± 8.65 |
| 1 | 70.59 ± 2.00 |
| 5 | 78.51 ± 12.32 |
| 10 | 44.60 ± 9.44 |

6. NE2-6

| Conc: µg/mL | Mean ± SEM |
|---|---|
| $H_2O_2$ (100µM) | 131 ± 8.30 |
| 0.1 | 77 ± 9.61 |
| 0.5 | 80 ± 4.50 |
| 1 | 74 ± 7.33 |
| 5 | 66 ± 9.32 |
| 10 | 56 ± 4.28 |



7. CT2-4

| Conc: µg/mL | Mean ± SEM |
|---|---|
| $H_2O_2$ (100µM) | 140 ± 2.69 |
| 0.1 | 98 ± 4.69 |
| 0.5 | 87 ± 9.13 |
| 1 | 95 ± 14.70 |
| 5 | 86 ± 9.90 |
| 10 | 86 ± 9.85 |

8. CT2-10

| Conc: µg/mL | Mean ± SEM |
|---|---|
| $H_2O_2$ (100µM) | 149 ± 9.27 |
| 0.1 | 99 ± 1.82 |
| 0.5 | 101 ± 6.14 |
| 1 | 112 ± 6.67 |
| 5 | 95 ± 6.61 |
| 10 | 88 ± 6.86 |

**8. NO production of crude extracts of *Streptomyces* sp. on RAW 264.7 macrophage cells induced by lipopolysaccharides (LPS)**

1. JA03

| Conc: µg/mL | Mean ± SEM |
|---|---|
| LPS (100ng/ml) | 29.039 ± 0.28 |
| 0.05 | 16.46 ± 2.34 |
| 0.5 | 17.23 ± 1.86 |
| 5 | 17.71 ± 1.88 |
| 50 | 13.33 ± 0.99 |
| 100 | 10.68 ±2.43 |
| 200 | 3.92 ± 1.12 |

2. MKP33

| Conc: µg/mL | Mean ± SEM |
|---|---|
| LPS (100ng/ml) | 36.625 ± 0.56 |
| 0.05 | 23.14 ± 2.82 |
| 0.5 | 20.91 ± 3.41 |
| 5 | 16.36 ± 2.51 |
| 50 | 9.61 ± 1.84 |
| 100 | 4.19 ± 1.21 |
| 200 | 2.90 ± 1.02 |

3. CT1-17

| Conc: µg/mL | Mean ± SEM |
|---|---|
| LPS (100ng/ml) | 30.817 ± 0.53 |
| 0.05 | 22.65 ± 1.95 |
| 0.5 | 22.52 ± 0.63 |
| 5 | 22.72 ± 1.00 |
| 50 | 21.27 ± 2.21 |
| 100 | 15.04 ± 0.67 |
| 200 | 11.34 ± 0.43 |

**4. NE1-12**

| Conc: µg/mL | Mean ± SEM |
|---|---|
| LPS (100ng/ml) | 39.457 ± 0.45 |
| 0.05 | 25.79 ± 1.68 |
| 0.5 | 28.75 ± 1.14 |
| 5 | 25.35 ± 1.98 |
| 50 | 22.91 ± 1.47 |
| 100 | 16.39 ± 1.30 |
| 200 | 11.73± 0.75 |

**5. CT2-4**

| Conc: µg/mL | Mean ± SEM |
|---|---|
| LPS (100ng/ml) | 33.457 ± 2.22 |
| 0.01 | 16.910 ± 1.24 |
| 0.05 | 17.801 ± 1.05 |
| 0.5 | 18.373 ± 0.97 |
| 5 | 16.622 ± 0.52 |
| 10 | 15.696± 0.65 |



**6. CT2-10**

| Conc: µg/mL | Mean ± SEM |
|---|---|
| LPS (100ng/ml) | 29.808 ± 0.26 |
| 0.01 | 21.69 ± 1.03 |
| 0.05 | 17.33 ± 1.95 |
| 0.5 | 16.11 ± 1.34 |
| 5 | 15.27 ± 2.06 |
| 10 | 13.87 ± 1.78 |

# VITA

| | |
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