SCREENING AND PURIFICATION FOR INHIBITORS OF GSK3 AND RAS/RAF FROM ACTINOMYCETES AND *MYRMECODIA TUBEROSA*

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DECLARATION

I affirm that this thesis is the result of my own work except where material is taken from the work of others, in which case it is acknowledged.

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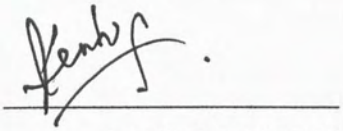

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ABSTRACT

Soil microbes especially actinomycetes are a main source of the production of bioactive metabolites through its secondary metabolisms. Therefore, soils are collected at the rain forest of Danum valley during the field trip to Danum Valley. The collected soil samples are used for the isolation and purification of actinomycetes using humic acid vitamin agar and oatmeal agar. Purified actinomycetes are fermented by using mannitol-peptone medium for the production of secondary metabolites. Besides that, one assign strain of actinomycetes, H7372, which is isolated by Cheah, is fermented using the KF-7L fermentor. Fermentation of the actinomycetes are ended after 120 hours and harvested by adding acetone at the proportion of 1:1 (v/v). Acetone extract obtained from the fermentation is used for the screening for bioactive metabolites against mammalian GSK-3β and Ras/Raf-1 protein interaction. Besides that, *Myrmecodia tuberosa*, which is a medicinal plant that found in the forest of Sabah is also tested. This medicinal plant is extracted using two different extraction methods, which are solid-liquid extraction system and liquid-liquid extraction system. From the liquid-liquid extraction, two different layers, which are the methanol aqueous and chloroform layer are used in the screening against mammalian GSK-3β and also Ras/Raf-1 protein interaction. In the screening of bioactive metabolites against mammalian GSK-3β, H10075 and H10079, which is the *gsk-3* null mutant transform with pKT10-GSK3β and Yep24-MCK1 respectively. Yeast two-hybrid screening system is used in the screening for inhibitors against Ras/Raf-1 protein interaction. Total of 7 of 94 new isolates of Danum Valley, which are H11695, H11722, H11821, H11853, H11855, H11856 and H11857, showing inhibition in the screening against GSK-3 β. While, 9 of 94 isolates, which are H11822, H11855, H11856, H11857, H11858, H11861, H11867, H11878 and H11879 showing inhibition against Ras/Raf-1. The medicinal plants extract did not show inhibition effects against both of screening system. The inhibitor can be distinguished into two types that are potential and toxic inhibitor. Potential inhibitors are the inhibitor that will inhibit the abnormal cells while permitting the growth of normal cells; meanwhile toxic inhibitors are inhibitor that will inhibit both of the normal and abnormal cell growth in the Ras/Raf-1 and GSK-3 pathway.

ABSTRAK

Bakteria tanah terutamanya aktinomycetes merupakan sumber utama dalam penghasilan bioaktif metabolisma melalui metabolisma sekundernya. Oleh itu, tanah dikumpul dari hutan hujan di Lembah Danum semasa lawatan ke Lembah Danum. Tanah yang telah dikumpulkan digunakan dalam pemencilan dan penulenan aktinomycetes dengan menggunakan humic acid vitamin agar dan oatmeal agar. Aktinomycetes yang telah ditulenkan digunakan dalam penapaian dengan menggunakan mannitol-peptone medium untuk penghasilan metabolisma sekunder. Selain itu, satu strain aktinomycetes yang diberikan, H7372, yang dipencilkan oleh Cheah, ditapaikan dengan menggunakan medium yang sama dalam KF-7L fermentor. Penapaian aktinomycetes akan berakhir selepas 120 jam dan akan dituai dengan penambahan aseton dalam nisbah 1:1 (v/v). Ekstrak aseton yang diperolehi daripada penapaian akan digunakan dalam penyaringan bioaktif metabolit dalam perencatan mamalia GSK-3β dan protein interaksi Ras/Raf-1. Selain itu, satu tumbuhan ubatan Sabah, *Myrmecodia tuberosa*, juga diuji. Tumbuhan ubatan ini diekstrak dengan menggunakan dua jenis kaedah pengekstrakan iaitu pepejal-cecair pengekstrakan dan cecair-cecair pengekstrakan. Melalui cecair-cecair pegekstrakan, dua lapisan yang berbeza diperolehi. Lapisan ini terdiri daripada ekstrak metanol akueus dan kloroform. Kedua-dua jenis lapisan ini digunakan dalam penyaringan perencat GSK-3β dan protein interaksi Ras/Raf-1. Dalam penyaringan perencat GSK-3β, dua jenis mutant yis digunakan, iaitu H10075 dan H10079 di mana yis ini merupakan transformasi daripada *gsk-3* null mutant dengan pKT10-GSK3β dan Yep24-MCK1 masing-masing. Sistem yis dua-hibrid digunakan dalam penyaringan perencat Ras/Raf-1 protein interaksi. Sebanyak 7 daripada 94 sampel iaitu H11695, H11722, H11821, H11853, H11855, H11856 dan H11857 menunjukkan kebolehannya dalam perencatan GSK-3β dan 9 daripada 94 sampel iaitu H11822, H11855, H11856, H11857, H11858, H11861, H11867, H11878 dan H11879 menunjukkan kebolehannya dalam perencatan interaksi protein-protein Ras/Raf-1. Manakala, ekstrak tumbuhan ubatan tidak menunjukkan sebarang perencatan ke atas sistem penyaringan yang digunakan. Terdapat dua jenis perencat iaitu

perencat toksik dan perencat potensi. Perencat toksik akan merencat pertumbuhan sel normal dan tidak normal manakala perencat potensi hanya akan merencat pertumbuhan sel tidak normal dalam tranduksi isyarat GSK-3 dan Ras/Raf-1.

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SYMBOL AND ABBREVIATION

°C	degree Celsius
dH_2O	distilled water
g	gram
mg	milligram
mL	milliliter
M	molar
mM	millimolar
µg	microgram
µm	micrometer
nm	nanometer
GSK-3	glycogen synthase kinase-3
ERK	extracellular signal-regulated kinase
IRS	insulin receptor substrate protein
PI3K	phosphatidylinositol 3-kinase
PIP3	phosphatidylinositol 3, 4, 5 trispohosphate
PIP2	phosphatidylinositol 4, 5 bispohosphate
PKB	protein kinase B

CHAPTER 1

INTRODUCTION

Natural products are always related with the discoveries of the new bioactive compounds that could serve as the novel drugs for diseases such as cancer, Alzheimer's disease and other infectious diseases. The earliest natural products chemotherapy that successfully isolated was the penicillin that was discovered by Sir Alexander Fleming in 1929, which was used for the treatment of bacterial infectious diseases such as staphylococcal infection (Ho, 2003).

Natural products can be found and isolated from plants, microorganisms, and animals. Among the sources of bioactive natural products, plants and microorganisms are the main source of the bioactive compounds and they are widely used in the research to discover new novel drugs.

In Malaysia, there are variety types of medicinal plants that can be found around the rain forest that have a high value and potentially serve as a medicine against diseases. These are including the *Eurycoma longifolia* and *Gingko biloba*, which are usually used

by the local people as the source of medicine to cure diseases. Besides that, *Myrmecodia tuberosa* or known as ant plants locally is believed to have a potential in producing of new novel drugs against non-infectious diseases.

In addition, microorganisms that isolated from rain forests of Malaysia have the potential serves as the novel drugs against the cancer cell. This is especially the actinomycetes member such as *Streptomyces*. Discovery of the *Streptomyces*, strain H7372, which was isolated by Cheah (2002) from the roots of *Bruguiera sp* growing in the mangrove in Kampung Termunong, Tuaran, is able to disrupt the Ras-Raf interaction in the yeast two-hybrid system. In addition, Mr. Lee Kun Hyung in Tokyo University also found that the crude extract from H7372 could decrease the amount of phosphorylated mammalian MEK 1/2 and ERK 1/2 with the Western blotting (Ho, 2003).

1.1 Objective

The main objective of this research is to screen and purify the bioactive compounds that can serve as inhibitors against glycogen synthase kinase-3 and ras/raf from actinomycetes that are isolated and purified from the Danum Valley rain forest, and *Myrmecodia tuberosa*.

CHAPTER 2

LITERATURE REVIEW

2.1 *Myrmecodia tuberosa*

Mutualism relationship between the ants and plants is one of a very important interaction in the rain forest. In the forest, the ants play an important role in keeping the lianas away, competitor plants and herbivores from growing pioneer tree (Widodo and Maryati, 1996).

Myrmecodia tuberosa is an example of a plant that shows the mutualism relationship between the ant and plants. *Myrmecodia tuberosa* is also known as ant plant by the local people in Borneo. Besides that, local residents in Sabah, especially native, are using this plant as medicine. Therefore, it is categorized as medicinal plants in Sabah. It is a myrmecophyte plant and categorized in the family of Rubiaceae. Picture of *Myrmecodia tuberosa* is shown in Figure 2.1 in the following pages.

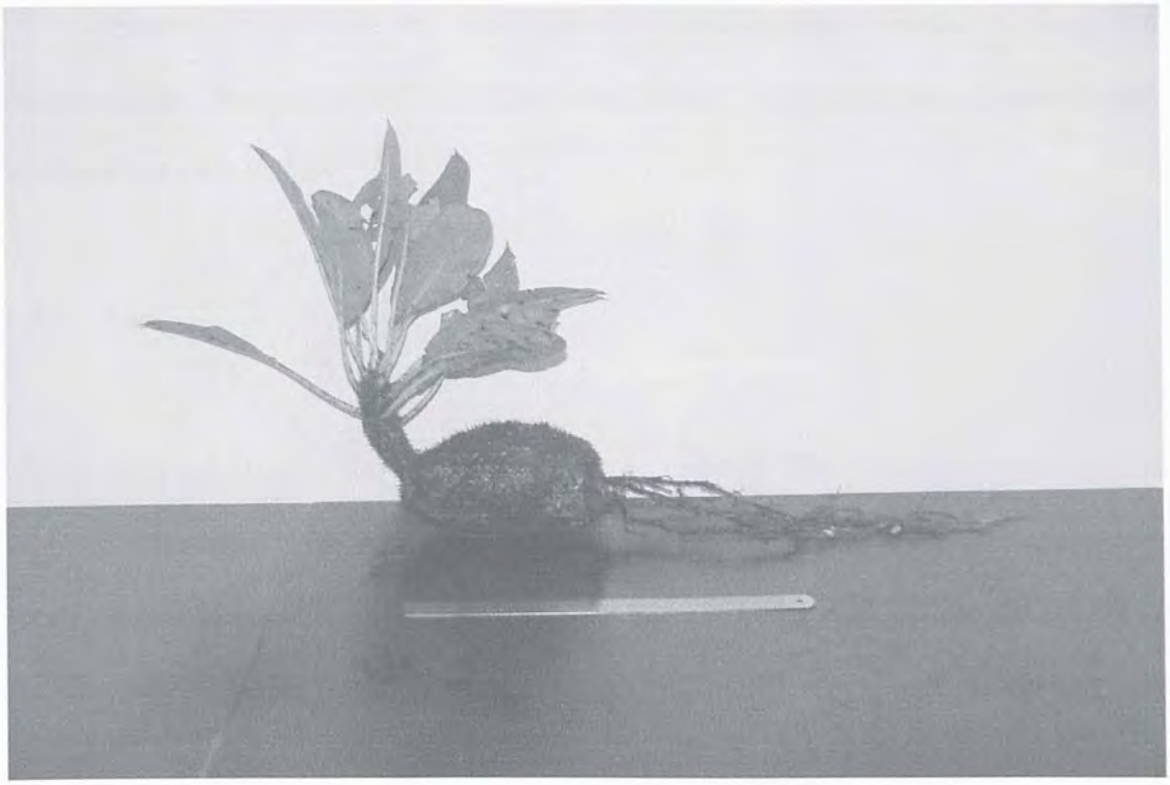

Figure 2.1 *Myrmecodia tuberosa*

2.1.1 Morphology

Myrmecodia tuberosa has a tuber with an oval shape and it is usually dark brown in color. The tuber of the plant is usually covered with spines, which are the modified roots that can be simple or branched.

Myrmecodia tuberosa tend to have only one unbranched stems with a large leaves. The stem of the plants is usually covered by the clypeoli, which is an unusual shield-like structures and the clypeoli is boarded with spines. (Huxley and Jebb, 1991)

Flowers of ant plant are usually found in between the clypeoli of the plant. Flowers of the plant are formed in hollow called alveoli and usually white in color while the fruits are yellow color.

2.1.2 Ecology

Myrmecodia tuberosa or known as ant plant is an epiphyte that found growing on trunks of the dipterocarps in the forest of Malaysia. The habitats where the plants are usually found are low in nutrients and bright where the sunlight easily passed through. Ant plants are found in Malaysia, and extended to Philippines, south to Cape York Peninsula in Queensland and east as far as Fiji, but mostly they are widely found with the highest diversity in New Guinea (Huxley and Jebb, 1991).

Since it is a myrmecophyte plants, the tuber of the ant plants is the nesting site for the ants. Besides as a nesting site for the ants, the plants also provide a protection for the ants for their predators. In return, the ants may act as a control against pest of the plants such as caterpillar and other herbivores (Widodo and Maryati, 1996).

In *Myrmecodia tuberosa*, there is no host specificity where it can be colonized by any species of the ants. Only the strong and dominant ants usually will colonize the chamber of the plant (Widodo and Maryati, 1996).

2.2 Actinomycetes

Actinomycetes are bacteria that belonging to the order *Actinomycetales*. Actinomycetes especially *Streptomyces* are very important in the medical viewpoint since they can produce the novel drugs against diseases (Madigan *et al.,* 2003).

2.2.1 Characteristic of Actinomycetes

Actinomycetes are gram-positive bacteria that contain a high composition of guanine and cytosine (GC). The DNA base compositions of the actinomycetes are within the range of 63-78% of GC and this shown that actinomycetes have the highest GC percentage comparing to others known bacteria (Madigan *et al*., 2003).

Actinomycetes are filamentous bacteria and their morphology resembles that of the filamentous fungi. However, the filaments of actinomycetes consist of the prokaryotic cells with the diameter of the filaments are much smaller than the fungi (Tortora *et al*., 2002). Mycelium is the network of filaments that will be formed by the actinomycetes as a result of a successful growth and branching (Madigan *et al*., 2003).

Actinomycetes will form spores when mature. The manner of the spore formation is usually used as a guideline to distinguish between the actinomycetes (Madigan *et al*., 2003). Besides that, cell wall of the actinomycetes also used to distinguish and separate them into subgroup.

2.2.2 *Streptomyces*

Genus *Streptomyces* is the best study actinomycetes. This bacterium is commonly isolated from the soil. *Streptomyces* usually produce a gaseous compound called geosmin that gives the fresh soil its typical musty odor (Tortora *et al.*, 2002).

Streptomyces spores called conidia are formed at the end of the aerial filaments and each of the conidia is capable to form a new colony as the spores drop to the suitable environment (Tortora *et al.*, 2002). Differences in the shape and arrangement of the aerial mycelia and spores-bearing structure become the most important criteria in distinguish and classifying the *Streptomyces* into the different group (Madigan *et al.*, 2003).

The pigmented conidia and sporophores of Streptomyces are the main contributors for the coloring of the mature Streptomyces. Different types of Streptomyces could have the different color of the conidia and sporophores, which become the unique characteristic of the Streptomyces. Besides that, this characteristic also makes the Streptomyces easily detected on the agar plate (Madigan *et al.*, 2003).

2.3 Secondary metabolite

2.3.1 Plants

Secondary metabolite compounds of plants are the compounds that biosynthetically derived from the primary metabolites but limited in the distribution in the plant kingdom and they are restricted to a particular taxonomic group. These compound do not have any role in the primary metabolism of a plant but usually have an ecological role, for example, they biochemical pathways for propagation and protection from the attack of others organisms such as herbivores (Baladrin *et al*., 1985).

Besides that, secondary metabolites compounds are the main source that gives the plant colors, flavors and smells. These will produce the source of drugs, insecticides, dyes, flavors and fragrances. Moreover, phytomedicine can be found with the medicinal plants. Thus, comparing to the primary metabolites, the secondary metabolites are considered as the specialty materials a fine chemicals since they can be used as the drugs, insecticides, dye and many others things (Mann *et al*., 1987).

2.3.2 Actinomycetes

Microorganisms and other cells could synthesize secondary metabolites when the cells and their environment are meet. The products of the secondary metabolites are not produced during the rapid growth of the cells. Secondary metabolites are differing

tremendously in the chemical structure and biological activity. For this, almost 600 different antibiotics have been identified (Bailey and Ollis, 1986).

Actinomycetes, especially *Streptomyces* are the largest producer of secondary metabolites that are valuable in pharmaceutical. Usually secondary metabolites are synthesized under the suboptimal growth condition nears the end of the exponential growth phase. Thus, during the fermentation of Streptomyces, the cells are grown until almost the end of the stationary phase to avoid contamination with the waste produce by the cells during the death phase. Screening is then performed to identify the novel biological active molecules formed by the cells during the fermentation (Higgs *et al.*, 2001).

2.4 Cell Cycle and Cancer Cell

In an organism, the rate of the cell division is a regulated process that is intimately associated with growth, differentiation and tissue turnovers. The division cycle of most cells consists of four processes that are cell growth, DNA replication, distribution of duplicated chromosomes to daughter cells and cell division (Cooper *et al.*, 2004).

Cells do not undergo division unless they receive signals that regulate them to enter the active segments of the cell cycle. Resting cells are said to be in the G_0 phase of the cell cycle. The division cycle of most of the cells are divided into four segments, which are M, G_1, S and G_2. M phase (mitosis) of the cycle is corresponding to mitosis,

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