SCREENING FOR MITOGEN-ACTIVATED PROTEIN KINASE KINASE 1 (MKK1) INHIBITOR IN FUNGI

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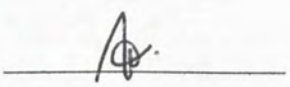

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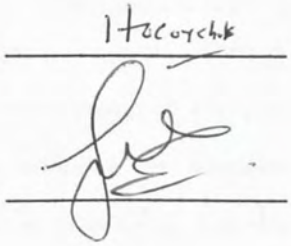
(PROFESSOR DR. HO COY CHOKE)	
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ABSTRACT

Microfungi of varied species were isolated from soil samples collected under leaf litters of trees in the Newberry research plot situated in Danum Valley Conservation Area. These fungi were isolated using potato-dextrose agar (PDA) with the addition of 0.75% chloramphenicol and sodium chloride, NaCl, (0.005%) which will selectively inhibit the growth of other microbes other than fungus. The fungi were then purified on the same media, but without the 0.75% chloramphenicol and 0.005% NaCl, to obtain pure single colony of the fungi. Morphological observations, fermentation for secondary metabolites and stocking in silica gel for long term storage of the fungi were made during this time. Acetone extracts of the fungi were then tested in the $MKK1^{p386}$ inhibitor screening system. The screening system utilizes mutant yeast $MKK1^{p386}$ which is under the control of the strong GAL1p. Overexpression of the mutant gene will cause cell growth arrest with the addition of galactose. So far, 119 fungal extracts (26 extracts were provided by Mr. Ong Si Mon, 36 extracts provided by Mr. Vun Su Chiun and 57 extracts isolated from my soil samples) have been tested and none have showed any activity on the system. There was no growth on the galactose plate around the disc extracts and no inhibition zone in the glucose plate discs extracts. All the 57 fungal isolates present various diversity. Sectoring has been observed in some of the fungal strain, H9434 (DV234-1) and H9473 (DV283-2), isolated from Danum Valley, suggesting genetic instability of the fungi.

ABSTRAK

Pelbagai variasi spesis kulat mikro telah dipencilkan dari sampel tanah yang dikutip dari bawah himpunan daun pelbagai jenis pokok, di dalam plot penyelidikan Newberry yang terletak di Kawasan Konservasi Lembah Danum. Kulat-kulat ini telah dipencilkan menggunakan kentang-dekstros agar (PDA) yang mengandungi 0.75% kloramfenikol dan 0.005% natrium klorida, NaCl, yang bersifat memilih dan akan menghalang pertumbuhan mikrob lain selain kulat. Kulat tersebut kemudiannya ditulenkan dengan media yang sama tanpa 0.75% kloramfenikol dan 0.005% natrium klorida untuk mendapatkan koloni tulen kulat. Koloni tulen kulat diperlukan untuk beberapa sebab; iaitu untuk dokumentasi lanjut berkenaan pemerhatian morfologi and selular, untuk simpanan dalam bentuk agar condong dan stok gel silika, dan akhir sekali difermentasikan untuk mendapatkan metabolit sekunder dan bagi pengekstrakan dengan aseton. Ekstrak aseton kulat tersebut kemudian diuji dalam sistem penyaringan perencat MKK1^{p386}. Sistem penyaringan ini menggunakan strain yis MKK1 ^{p386} mutan, yang dipengaruhi oleh GAL1p. Penzahiran melampau gen mutan ini akan menyebabkan pertumbuhan sel terhalang dengan penambahan galaktosa. Sebanyak 119 ekstrak kulat (26 ekstrak dibekalkan oleh En. Ong Si Mon, 36 ekstrak telah dibekalkan oleh En. Vun Su Chiun, dan 57 ekstrak dari sampel tanah yang telah saya pencilkan) telah diuji, tetapi tidak memberikan sebarang keputusan positif. Tiada sebarang pertumbuhan disekeliling kertas cakera ekstrak pada piring galaktosa dan tiada sebarang zon perencat disekeliling kertas cakera ekstrak pada piring glukosa. Kesemua 57 kulat yang dipencilkan mempamerkan kepelbagaian. Pengsektoran telah diperhatikan pada beberapa strain kulat iaitu H9434 (DV234-1) dan H9473 (DV283-2), yang telah dipencilkan dari sampel tanah Lembah Danum. Ini mencadangkan ketidakstabilan genetik kulat tersebut.

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cm	Centimeter
°C	Degree Celsius
g	gram
ha	hectar
L	Liter
km^2	Kilometer square
M	Molar
m	meter
mg	millligram
ml	milliliter
mm	millimeter
psi	pounds per square inch
rpm	rotation per minute
µl	Microliter or 10^{-6} liter

LIST OF ABBREVIATIONS

gbh	girth at breast height
ERK	extracellular signal regulated kinase
GGI	geranylgeranyl inhibitor
FTI	farnesyl transferase inhibitor
MAP	mitogen activated protein
Mapk	Mitogen-activated protein kinase
Mek 1/2	Mitogen-activated protein kinase kinase 1/2
MKK1	MAP kinase kinase 1
PDA	potato-dextrose agar
MSG5	MAP kinase phosphatases
RSK	ribosomal S6 kinase
Ser	Serine
SOS	son of sevenless

CONTENTS

CHAPTER 1

PREFACE

1.1 Introduction

This research is done mainly for the purpose of screening for Mitogen-activated Protein Kinase Kinase 1 (MKK1) inhibitor in filamentous microfungi isolated from soil samples of identified trees from Danum Valley Conservation Area, Sabah. Potential inhibitor of the MKK1 gene can be especially useful in cancer therapy, and also in the study of cell signaling.

Mitogen-activated protein kinase (MAPK) pathways are evolutionarily conserved signal transduction cascades connecting extracellular stimuli to a wide range of cellular responses. The MAPK cascades are sequential phosphorylation-mediated activation of three kinases, MAPK kinase kinase, MAPK kinase, and MAPK. Activation of MAPK requires phosphorylation of both threonine and tyrosine residues of a T*X*Y motif in the activation loop. Therefore, inactivation of MAPK can be achieved by dephosphorylation of either of the two phosphorylation sites. It has been demonstrated that three types of

phosphatases, protein-tyrosine phosphatase, serine/threonine phosphatase, and dual specificity phosphatase, are involved in negative regulation of MAPK from yeast to mammals (Hahn and Thiele, 2002).

Growth factor receptor tyrosine kinase trigger the proliferation of fibroblast and other cells via the activation of Ras, which is found constitutively in active form, 25% in all human cancer. Inhibitor of mitogen-activated protein kinase (MAPK) that lies downstream of ras therefore has potential to suppress the growth of many tumors. Indeed PD98059 which prevents the activation of the MAPK Kinase 1 (MKK1), reverse the phenotype of the Ras-transformed cell lines. Interestingly the PD 98059 binds only to the inactive form of MKK1, thus preventing it from being activated by the Raf gene (Cohen, 1999). Therefore, this research aims to screen microbial fungi for selective inhibitor of the MKK1 gene for cancer therapy and development of neurological protection intention.

1.2 Research Background

Soil samples for this research were collected from the forest near Danum Valley Field Centre. The Centre provides facilities for research, education, and wilderness recreation in the 438 km^2 forest of Danum Valley Conservation Area. Within its 438 square kilometers is to be found a rich diversity of animal and plant life. This area of forest is protected with a Class I Protection Forest Reserve and as such cannot be logged. About 90% of the Conservation Area is classified as lowland dipterocarp forest with the remaining 10% being low canopy, submontane forest mainly found on Mt. Danum at the

heart of the Conservation Area. The forest provides home to more than 120 mammals such as the endangered Sumatran rhino including ten primate species among which are the Orangutan and the Proboscis monkey. The valley is also home to over 275 bird species and numerous reptiles, amphibians, fishes, countless insects and other fascinating creatures (www.icsb-sabah.com.my).

Established research plots in the forest are but one of the research facilities provided by the Centre. My group trip of five undergraduate students, one post graduate student, under Prof. Ho's supervision, collected soil samples from under leaf litters of identified trees in the northern part of the established Newberry research plots.

Figure 1.1: Map of Sabah. The arrow is pointing to Danum Valley Conservation Area. (www.suteraharbour.com)

1.3 Research Objectives

The objectives for this research are:

1. To isolate microfungi in soil samples collected from the Newberry research plots situated in Danum Valley Conservation Area.

2. To purify, ferment and extract secondary metabolites from isolated fungi.

3. To screen the fungal extracts for bioactive compounds, especially MKK1 protein inhibitor to eukaryote's signal transduction as potential drug for cancer therapy.

CHAPTER 2

LITERATURE REVIEW

2.1 Fungi

The oldest fungal fossils are from about 600 million years ago, from northern Russia, but molecular data suggest that fungi may have been present 1 billion years ago. Fungi are eukaryotes that live nearly everywhere – in soil, in and on plants, insects, in water, in dung and in the intestines of diverse animals. As decomposers, fungi release carbon, oxygen, nitrogen and phosphorus from dead plants and animals in soil, recycling them back into plants. They are so vital to the plant that their diversity in the soil largely determines the spectrum of species comprising plant communities which in turn, affects the types of animals present. Mycologist-biologists who studied fungi estimate that they have identified only about 5% of the million or more species that exists (Lewis *et al.*, 2004).

Yeasts are single-celled forms that reproduce by budding; molds (filamentous fungi) are characterized by the development of hyphae, which result in the colony

characteristics seen in the laboratory. Hyphae elongate by a process known as apical elongation, which requires a careful balance between cell wall lysis and new cell wall synthesis. Because molds are often differentiated on the basis of conidiogenesis, structures such as conidiophores and conidiogenous cells must be carefully evaluated. Some molds produce special sac-like cells called sporangia, the entire protoplasm of which becomes cleaved into spores called sporangiospores. Sporangia are typically formed on special hyphae called sporangiophores.

A number of medically important fungi express themselves phenotypically as two different morphologic forms, which correlate with the saprophytic and parasitic modes of growth. Such fungi are called dimorphic fungi. Some researchers restrict the term to pathogens that grow as a mold at room temperature in the laboratory and as budding yeast or as spherules either in tissue or at 37°C. In contrast, others use dimorphic for any fungus that can exist as two different phenotypes, regardless of whether it is pathogenic. The term "dimorphic" is used to describe fungi that typically grow as a mold in vitro and as either yeast cells or spherules *in vivo.* Examples of medically important dimorphic fungi include *Blastomyces dermatitidis* (hyphae and yeast cells) and *Coccidioides immitis* (hyphae and spherules).

In mycology, fungi are classified on the basis of their ability to reproduce sexually, asexually, or by a combination of both. Asexual reproductive structures, which are referred to as anamorphs, are the basis for one of the sets of criteria. Because the criteria are based upon asexual morphologic forms, this system does not reflect

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