CHEMICAL AND BIOLOGICAL PROFILING OF WEEDS AND MEDICINAL PLANTS TARGETING PROTEIN KINASE AND PHOSPHATASES IN SIGNAL TRANSDUCTION IN CANCER

AZLINAH BINTI MATAWALI

THESIS SUBMITTED IN FULFILLMENT FOR THE DEGREE OF MASTER OF PHILOSOPHY

SCHOOL OF SCIENCE AND TECHNOLOGY
UNIVERSITI MALAYSIA SABAH
2013

DECLARATION

I hereby declare that the material in this thesis is my own except for quotations, excerpts, equation summaries and references which have been duly acknowledged.

07 September 2012

Azlinah Binti Matawali

PS2008-8418

CERTIFICATION

NAME : **AZLINAH BINTI MATAWALI**

MATRIC NO. : **PS2008-8418**

TITLE : **CHEMICAL AND BIOLOGICAL PROFILING OF BIOACTIVE WEEDS AND MEDICINAL PLANTS TARGETING PROTEIN KINASE AND PHOSPHATASES IN SIGNAL TRANSDUCTION IN CANCER.**

DEGREE : **MASTER OF PHILOSOPHY (BIOTECHNOLOGY)**

VIVA DATE : **26 NOVEMBER 2012**

DECLARED BY

1. **SUPERVISOR** Signature
   Assoc. Prof. Dr. Jualang Azlan Gansau

2. **CO-SUPERVISOR 1**
   Assoc. Prof. Dr. Lee Ping Chin

3. **CO-SUPERVISOR 2**
   Assoc. Prof. Dr. How Siew Eng

ABSTRACT

CHEMICAL AND BIOLOGICAL PROFILING OF WEEDS AND MEDICINAL PLANTS TARGETING PROTEIN KINASE AND PHOSPHATASES IN SIGNAL TRANSDUCTION IN CANCER

A total of 162 methanolic extracts of plant collected from Sabah were studied and screened for novel bioactive compounds against protein kinase and phosphatases involved in signal transduction in cancer. The targeted protein screening systems were kinase (MKK1) and phosphatase (MSG5 and PP1) which using different strain of mutated yeast namely $MKK1^{P386}$, $MKK1^{P386}$-MSG5, PAY704-1 and PAY700-4. Screening of crude methanolic extracts showed 13 potential extracts against PP1 protein which classified as inhibitor to GLC7 (UMS71, UMS91 and UMS990); inhibitor sensitive to glc7-10 catalytic domain change (UMS80) and inhibitor insensitive to glc7-10 catalytic domain change (UMS70, UMS108, UMS901, UMS908, UMS963, UMS974, UMS975, UMS984 and UMS993). Meanwhile, about 21 and 26 crude methanolic extracts including UMS643 were found as toxic against MKK1 and MSG5 screening assay, respectively. Four extracts (UMS71, UMS91, UMS108 and UMS643) had been selected to further separate by using liquid-liquid extraction methods and subsequently re-tested against all screening assay. However, only UMS71 and UMS91 showed consistent inhibitions against PP1 screening assay. The potential extracts partitions are Chloroform (CE), Hexane (HE) and Ethyl acetate (EAE) from UMS71 and Chloroform (CE) from UMS91. They were found to be inhibitor insensitive to *glc7-10* catalytic domain change with the inhibitory zones ranged between 7.33±1.15mm until 9.5±0.70mm. Thus, both UMS71 (*Chromoleana odorata*) and UMS91 (*Mikania micrantha*) had been chromatographed through Thin Layer Chromatography (TLC) and Column Chromatography. Each column fraction was screened against PP1 screening assay and the results showed 8 potential fractions from CE of UMS71. Fraction 2 (F2) was classified as inhibitor to *GLC7* and exhibited strongest inhibitory zones ranged between 8.00±0.00mm until 15.0±0.0mm. As for UMS91, Fraction 2 (F2) of CE also showed activities during PP1 screening assay. This fraction was classified as inhibitor to GLC-7 with the range between 13.5±0.7mm until 14.0±1.4mm. Phytochemical test had been conducted on UMS71 dan UMS91 extracts whereas both UMS71.CE (Fraction F1-F10) and UMS91.CE (Fraction F1-F5) were scanned through UV/Vis spectroscopy. The presence of alkaloid, flavonoid, tannin, saponin and triterpenoid were observed on both samples. However, only selected fraction (UMS71.CE.F2) which possessed the most consistent and strongest antiphosphatase activities had been analysed for compounds identification by using GC-MS. The analysis revealed the presence of 10 compounds. The dominant phytocomponents in the extract fraction are hexachloro-ethane, n-nonylaldehyde, methyl-4-oxooctanoate, longiverbenone, 2-butenal,2-methyl-4-(2,6,6-trimethyl-1-cyclohexen-1-yl), neophytadiene, phytol, dihydro-neoclovene, aromadendrene and

2,6-ditert-butylquinone. Fraction UMS71.CE.F2 had also chosen to undergo the enzymatic analysis in order to determine the specificity of inhibitions against protein phosphatase type-1 (PP1). Spectrophotometric method was used to assay for the enzyme activity and both maximum enzyme velocity (V_{max}) and Michealis-Menten (K_m) constants were evaluated and compared for normal and inhibited reactions. The K_m and V_m for substrate (DiFMUP) were 0.125mM and 125 while the $K_{m'}$ and $V_{m'}$ at 300µg/µl were 0.60mM and 200.0, respectively. Other biological activities such as *in-vitro* cytotoxicity and antimicrobial test of UMS71 also had been reported. However, cytotoxicity test was only conducted at CE partitions level were found to exhibits cytotoxic activities against HeLa cervix adenocarcinoma (IC_{50} value 39.00±1.00µg/ml) cancer cell lines. Meanwhile, antibacterial test carried out for UMS71.CE.F2 showed week inhibitory activities on *S. pneumonia* (11.67±2.08mm), *S. aureus* (10.33±0.58mm), *P. aeruginosa* (10.67±0.58mm), E*. coli* (8.00±0.00mm) and *S. typhii* (8.00±0.00mm) when compared to ampicillin as control positive.

Keyword: Kinase, Phosphatases, Signal transduction, Cancer, *Chromolaena odorata*

ABSTRAK

Sebanyak 162 ekstrak metanol daripada tumbuhan yang didapati dari Sabah telah dikaji bagi menentukan kehadiran sebatian bioaktif terhadap protein kinase dan fosfatase yang lazimnya terlibat dalam transduksi isyarat pada mekanisme kanser. Ujian penyaringan ini memfokuskan kepada perencat kinase (MKK1) dan perencat fosfatase (MSG5 dan PP1) menggunakan strain yis yang telah dimutankan iaitu MKK1^{P386}, MKK1^{P386}-MSG5, PAY704-1 dan PAY700-4. Penyaringan ekstrak metanol kasar menunjukkan sebanyak 13 ekstrak telah merencatkan protein PP1 yang mana dapat dikelaskan kepada kumpulan perencat terhadap GLC7 (UMS71, UMS91 dan UMS990); perencat sensitif kepada perubahan domain katalisis glc7-10 (UMS80) dan perencat tak sensitif kepada perubahan domain katalisis glc7-10 (UMS70, UMS108, UMS901, UMS908, UMS963, UMS974, UMS975, UMS984 dan *UMS993). Sebanyak 21 ekstrak metanol kasar didapati bersifat toksik kepada protein MKK1, manakala 26 ekstrak pula didapati toksik kepada protein MSG5 termasuklah UMS643 yang menunjukkan sifat toksik kepada kedua-dua protein tersebut. Oleh yang demikian, empat ekstrak kasar (UMS71, UMS91, UMS108 and UMS643) telah dipilih untuk pemisahan menggunakan teknik pemisahan cecair-cecair. Ujian penyaringan semula bagi semua pecahan ekstrak menunjukkan hanya UMS71 dan UMS91 sahaja memberikan perencatan yang konsisten terhadap ujian PP1; iaitu pada pecahan ekstrak daripada klorofom (CE), heksana (HE) dan etil asetat (EAE) daripada ekstrak UMS71 serta pecahan klorofom (CE) daripada ekstrak UMS91. Pecahan-pecahan ini merupakan perencat tak sensitif kepada perubahan domain katalisis glc7-10 dengan julat zon perencatan sebanyak 7.33±1.15mm sehingga 9.5±0.70mm. Sehubungan itu, ekstrak UMS71 (Chromoleana odorata) dan UMS91 (Mikania micrantha) telah dikromatografikan menggunakan teknik Kromatografi Lapisan Nipis (TLC) dan Kromatrografi Turus. Setiap fraksi yang terhasil telah diuji semula terhadap ujian penyaringan PP1. Terdapat 8 fraksi yang berpotensi pada CE ekstrak UMS71 telah dikenalpasti termasuklah fraksi 2 (F2) yang merupakan perencat terhadap GLC7. Fraksi ini mencatatkan zon perencatan yang paling besar iaitu pada julat 8.00±0.00mm sehingga 15.0±0.0mm. Bagi ekstrak UMS91, hanya fraksi 2 (F2) pada CE ekstrak yang bersifat perencat terhadap GLC7 dengan julat perencatan sebanyak 13.5±0.7mm sehingga 14.0±1.4mm. Ujian fitokimia telah dilakukan ke atas semua ekstrak daripada UMS71 dan UMS91 manakala pecahan ekstrak UMS71.CE (Fraksi F1-F10) dan UMS91.CE (Fraksi F1-F5) telah diimbas menggunakan spektroskopi UV/Vis. Ujian fitokimia mengesahkan kehadiran alkaloid, flavonoid, tannin, saponin dan triterpenoid pada kedua-dua sampel tersebut. Walaubagaimanapun, hanya fraksi terpilih (UMS71.CE.F2) telah dianalisis menggunakan GC-MS. Hal ini kerana fraksi ini mempunyai kadar perencatan antifosfatase yang lebih konsisten dan bersaiz besar berbanding ekstrak lain. Data menunjukkan kehadiran 10 jenis sebatian pada pecahan ekstrak tersebut iaitu hexachloro-ethane, n-nonylaldehyde, methyl-4-oxooctanoate, longiverbenone, 2-butenal,2-methyl-4-(2,6,6-trimethyl-1-*

cyclohexen-1-yl), neophytadiene, phytol, dihydro-neoclovene, aromadendrene dan 2,6-ditert-butylquinone. Di samping itu, fraksi UMS71.CE.F2 juga telah diuji pada ujian analisa enzim bagi mengesahkan kespesifikasiannya terhadap protein fosfatase jenis-1 (PP1). Kaedah spektrofotometrik dilakukan untuk menentukan nilai maksimum kealiran enzim (V_{max}) dan pemalar Michealis-Menten (K_m) sama ada dengan kehadiran perencat atau sebaliknya. Nilai K_m dan V_m bagi substrat (DiFMUP) pada reaksi normal adalah masing-masing sebanyak 0.125mM and 125, manakala nilai $K_{m'}$ and $V_{m'}$ pada kepekatan 300ug/ul adalah sebanyak 0.60mM and 200.0. Aktiviti biologi lain seperti ujian sitotoksik secara in-vitro dan ujian antimikrobial juga telah dilaporkan pada sampel UMS71. Ujian sitotoksik yang hanya dilakukan menggunakan pecahan ekstrak CE menunjukkan aktiviti ketoksikan pada sel kanser HeLa cervix adenocarcinoma (nilai IC_{50} 39.00±1.00µg/ml). Ujian antibakteria pada fraksi UMS71.CE.F2 pula telah menunjukkan perencatan lemah terhadap S. pneumonia (11.67±2.08mm), S. aureus (10.33±0.58mm), P. aeruginosa (10.67±0.58mm), E. coli (8.00±0.00mm) and S. typhii (8.00±0.00mm) berbanding ampicillin yang bertindak sebagai kawalan positif.

Kekunci : Kinase, Fosfatase, Transduksi isyarat, Kanser, Chromolaena odorata

ACKNOWLEDGEMENT

In the name of Allah, the Most Gracious and the Most Merciful.
All praises to Allah for the strengths and His blessing. The completion of this thesis was made possible through the invaluable contribution of a number of individual. Foremost, my utmost gratitude to my supervisor Assoc. Prof. Dr. Jualang Azlan Gansau for his patience and guidance during the research and writing of this thesis. I would also like to thank my thesis committee; Assoc. Prof. Dr. Lee Ping Chin and Assoc. Prof. Dr. How Siew Eng for their encouragement and insightful comments.

I would like to express my appreciation to MOSTI for the scholarship, our collaborators Dr. Latifah Saiful Yazan (UPM), Mr. Didi Erwandi Mohd Haron (UM), Mr. Julius Kulip and Mr. Johnny Gisil (IBTP, UMS) and also staffs of Klias Peat Swamp Forest, Klias, Sabah. I expand my thanks to all lecturers and staffs from Biotechnology programme especially Assoc. Prof. Dr. Zaleha Abd Aziz for their constant moral supports and encouragement.

I am indebted to my fellow labmates; Ainil Farhan, Nurul Ain and Jury Tise Taunson for the sleepless nights we were working together. I also owe my gratitude to numerous undergraduates that had been helping me completing this research. Thanks for helping me get through the difficult times and for all the emotional support, comraderie, sincere encouragement and inspiration for these past few years.

Sincere thanks also to my colleagues especially Makdi, Noorhanis, Norhaniza, Ozayanna, Bong, Gabriella, Adznila, Fauze and Fitri Rozlianah for their kindness during my study. Words also fail me to express my appreciation to Fadzillah for always beside me during the happy and hard moments. Thanks for the friendship and memories.

My deepest gratitude also goes to my father, brothers and sister for their endless supports, unflagging love, prayers and encouragement. To those who indirectly contributed in this research, your kindness means a lot to me. Thank you.

Azlinah Matawali
7 September 2012

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ABBREVIATIONS

NCR	National Cancer Registry
MAPK	Mitogen Activated Protein Kinase
MAPKK	Mitogen Activated Protein Kinase Kinase
MAPKKK	Mitogen Activated Protein Kinase Kinase Kinase
MKK1	MAPK Kinase 1
MSG5	multicopy supressor of *gpa1*
ERK	Extracellular regulated kinase
p38	Mammalian MAPK family
JNK1	Mammalian MAPK family
p54α, p54β, p54γ	Mammalian MAPK family
MKK1^{P386}	hyperactive mutation form of *MKK1*
PP1	Protein phosphatase type 1
PP2	Protein phosphatase type 2
MKB	MAPK-binding
DSP	dual-specificity phosphatase
ATP	Adenine Triphosphate
YPD	Yeast Peptone Dextrose
YPD+1M sorbitol	Yeast Peptone Dextrose + 1 M Sorbitol
PAY700-4	PP1 temperature sensitive yeast strain
PAY704-1	PP1 wild type yeast strain
TLC	Thin Layer Chromatography
GS	Gas chromatography
MS	Mass Spectroscopy
U.S	United States
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
Formazan	1-[4,5-dimethylthiazol-2-yl]-3,5-diphenylformazan
C5	five carbon
IAW	Invasive alien weed
C. odorata	*Chromolaena odorata* (L.) King and Robinson
C. hirta	*Clidermia hirta* (L.) D. Don
PBS	Phosphate buffered saline solution
M. micrantha	*Mikania micrantha*
ITBC	Institute of Tropical Biology and Conservation
UMS	University Malaysia Sabah
BORH number	BORNEENSIS number
G. linearis	*Gleichenia linearis* (Burm.) Underw
ES	Enzyme-Substrate
U.S.A	United States of America
DMF	N,N-dimethylformamide
DiFMUP	6,8-difluoro-4-methylumbelliferyl phosphate
ME	Crude Methanolic Extract
HE	Hexane Extract
EAE	EtnylAcetate Extract

CE	Chloroform Extract
CME	Chloroform:Methanol Extract
BE	Buthanol Extract
AE	Aqueous Extract
3T3	Normal cell line
HeLa	Cervix adenocarcinoma
CaOV3	Ovarian cancer
A549	Lung cancer
Ext	Excitation
Ems	Emission

LIST OF SYMBOLS

K_m	Michealis-Menten constant
V_{max}	Maximum observable velocity
β	Beta
γ	Gamma
α	Alpha
^0C	Degree celcius
K_1	Rate constants for formation of ES
ES	Enzyme-Substrate complex.
K_{-1}	Rate constant of dissociation of ES
K_{cat}^1	Catalytic rate constant
R_t	Reaction rate
$[S]_t$	Instantaneous substrate concentration
mm	milimitres
m	metres
cm	centimetres
%	Percentage
(v/v)	Volume per volume
<	Less than
mg	Miligram
ml	Mililitre
-	Negative
+	Addition
rpm	Revolution per minutes
±	Plus minus
kDa	Kilo Dalton
U	Unit
X	Times
CO_2	Carbon dioxide
g	Gravity
µl	Microlite
m/z	Mass per charge ratio

LIST OF APPENDIX

LIST OF FIGURES

LIST OF PHOTO

CHAPTER 1

INTRODUCTION

Cancer is the accumulation of mutations that causes dysregulation in cell functions. It is the leading cause of death worldwide and accounted for 13% of total deaths, which is equal to 7.6 million deaths in 2008 (World Health Organization, 2011). However, the numbers predicted to continue arise over 11 million in 2030. Five major cancers that recorded are lung, stomach, colorectal, liver and breast cancer (World Health Organization, 2011). In Malaysia, cancer is still one of the main health problems. National Cancer Registry (NCR) has reported 70 000 new cancer cases among Malaysians in Peninsular Malaysia between 2003 and 2005 (Lim *et al.,* 2008).

Signal transduction is a process of conveying signals from receptor upon reversible phosphorylation process that involves both kinases and phosphatases. Human genome has been reported to encode approximately 2000 protein kinases and 500 protein phosphatases (Cohen, 1999). Central building blocks in the intracellular signalling networks are the Mitogen-activated protein kinase (MAPK) pathway (Shaul and Seger, 2007). This pathway is activated by dual specificity MAPK kinases (MAPKKs) which are themselves activated by MAPKK kinases (MAPKKKs) (Treisman, 1996). Regulation of the MAPKs activity is vital in signal transduction because it controls cellular processes. MAPKs itself have been linked with development and progression of several cancers; such as prostate cancer, breast, leukaemia and skin cancers (McCubrey *et al.,* 2007; Arnoidussen and Saatcioglu, 2009; Inamdar *et al.,* 2010). Hence, this pathway served as potential target in combating cancer diseases.

Previously, few kinase inhibitors were successfully introduced as cancer drugs such as Gleevac which had been approved for combating chronic myeloid leukemia (Noble *et al.,* 2004). Few others kinase inhibitors that currently available in market are such as m-amsacrine (m-AMSA), doxorubicin, vincristine (VCR) and choline phosphotransferase (CPT) (Yu, 1998). Meanwhile, numerous phosphatase inhibitors also had been continuously introduced as cancer drugs such as okadaic acid and tautomycetin (Shenolikar, 1994). However, some of these kinase and phosphatases inhibitors were found to encounter several issues such as drug-resistance problems, negative side effect issues, poor bioavailability and drug metabolism during early clinical trials and some drugs had problems in drug delivery. Thus, the searching for the new and novel anti kinase and phosphatase drug was urgently needed especially by manipulating the richness of our natural product sources.

Natural product offers broad spectrum of resources in drug discoveries. Approximately 23 new drugs derived from natural products that were used as treatments of various disorders since 2001 until 2005 (Lam, 2007). Plants have been widely used as therapeutic remedies. Plant-derived anticancer drugs have an impressive number in present clinical use and undergoing trials. However, competitions against new promising bioactive compounds derived from other natural sources such as terrestrial microbes and marine organisms forces the discovery of new and novel plant-derived anticancer agents. Challenges in overcoming the problems of drug target specificity, side effects and failure during clinical trials lead us to screen for new plant-derived novel inhibitors that effectively hit specific signal transduction involves in cancer.

Therefore, this study was conducted in order to search for the new plant-derived kinase and phosphatase inhibitors found in Sabah. The objectives of this research are as follows;

a) To collect, extract and screen the plant crude extracts against protein kinase (*MKK1*) and protein phosphatases (*MSG5* and *glc7*).
b) To fractionate and isoalate the bioactive compound(s) from selected samples against protein kinases or protein phosphatases.
c) To conduct enzymatic analysis towards selected potential plant extracts and subsequently determine the value of kinetic constant (K_m and V_{max}).
d) To study both *in vitro* cytotoxicity test and antimicrobial assay of the selected potential plant extracts.