

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



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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

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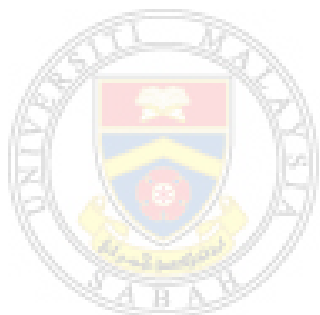
## ABSTRACT

### 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<sup>P386</sup>, MKK1<sup>P386</sup>-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  $7.33 \pm 1.15$ mm until  $9.5 \pm 0.70$ mm. 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  $8.00 \pm 0.00$ mm until  $15.0 \pm 0.0$ mm. 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  $13.5 \pm 0.7$ mm until  $14.0 \pm 1.4$ mm. 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 phytochemicals 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 300 $\mu$ g/ $\mu$ l 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<sub>50</sub> value 39.00 $\pm$ 1.00 $\mu$ g/ml) cancer cell lines. Meanwhile, antibacterial test carried out for UMS71.CE.F2 showed week inhibitory activities on *S. pneumonia* (11.67 $\pm$ 2.08mm), *S. aureus* (10.33 $\pm$ 0.58mm), *P. aeruginosa* (10.67 $\pm$ 0.58mm), *E. coli* (8.00 $\pm$ 0.00mm) and *S. typhii* (8.00 $\pm$ 0.00mm) when compared to ampicillin as control positive.

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



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## 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  $7.33 \pm 1.15 \text{ mm}$  sehingga  $9.5 \pm 0.70 \text{ mm}$ . Sehubungan itu, ekstrak UMS71 (*Chromolaena odorata*) dan UMS91 (*Mikania micrantha*) telah dikromatografikan menggunakan teknik Kromatografi Lapisan Nipis (TLC) dan Kromatografi 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  $8.00 \pm 0.00 \text{ mm}$  sehingga  $15.0 \pm 0.0 \text{ mm}$ . Bagi ekstrak UMS91, hanya fraksi 2 (F2) pada CE ekstrak yang bersifat perencat terhadap GLC7 dengan julat perencatan sebanyak  $13.5 \pm 0.7 \text{ mm}$  sehingga  $14.0 \pm 1.4 \text{ mm}$ . 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 diimbangi 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*



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## LIST OF ABBREVIATIONS

<b>NCR</b>	National Cancer Registry
<b>MAPK</b>	Mitogen Activated Protein Kinase
<b>MAPKK</b>	Mitogen Activated Protein Kinase Kinase
<b>MAPKKK</b>	Mitogen Activated Protein Kinase Kinase Kinase
<b>MKK1</b>	MAPK Kinase 1
<b>MSG5</b>	multicopy supressor of <i>gpa1</i>
<b>ERK</b>	Extracellular regulated kinase
<b>p38</b>	Mammalian MAPK family
<b>JNK1</b>	Mammalian MAPK family
<b>p54<math>\alpha</math>, p54<math>\beta</math>, p54<math>\gamma</math></b>	Mammalian MAPK family
<b>MKK1<sup>P386</sup></b>	hyperactive mutation form of <i>MKK1</i>
<b>PP1</b>	Protein phosphatase type 1
<b>PP2</b>	Protein phosphatase type 2
<b>MKB</b>	MAPK-binding
<b>DSP</b>	dual-specificity phosphatase
<b>ATP</b>	Adenine Triphosphate
<b>YPD</b>	Yeast Peptone Dextrose
<b>YPD+1M sorbitol</b>	Yeast Peptone Dextrose + 1 M Sorbitol
<b>PAY700-4</b>	PP1 temperature sensitive yeast strain
<b>PAY704-1</b>	PP1 wild type yeast strain
<b>TLC</b>	Thin Layer Chromatography
<b>GS</b>	Gas chromatography
<b>MS</b>	Mass Spectroscopy
<b>U.S</b>	United States
<b>MTT</b>	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
<b>Formazan</b>	1-[4,5-dimethylthiazol-2-yl]-3,5-diphenylformazan
<b>C5</b>	five carbon
<b>IAW</b>	Invasive alien weed
<b><i>C. odorata</i></b>	<i>Chromolaena odorata</i> (L.) King and Robinson
<b><i>C. hirta</i></b>	<i>Clidemia hirta</i> (L.) D. Don
<b>PBS</b>	Phosphate buffered saline solution
<b><i>M. micrantha</i></b>	<i>Mikania micrantha</i>
<b>ITBC</b>	Institute of Tropical Biology and Conservation
<b>UMS</b>	University Malaysia Sabah
<b>BORH number</b>	BORNEENSIS number
<b><i>G. linearis</i></b>	<i>Gleichenia linearis</i> (Burm.) Underw
<b>ES</b>	Enzyme-Substrate
<b>U.S.A</b>	United States of America
<b>DMF</b>	N,N-dimethylformamide
<b>DiFMUP</b>	6,8-difluoro-4-methylumbelliferyl phosphate
<b>ME</b>	Crude Methanolic Extract
<b>HE</b>	Hexane Extract
<b>EAE</b>	EthylAcetate Extract

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



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## LIST OF SYMBOLS

$K_m$	Michealis-Menten constant
$V_{max}$	Maximum observable velocity
$\beta$	Beta
$\gamma$	Gamma
$\alpha$	Alpha
$^{\circ}C$	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
$\pm$	Plus minus
kDa	Kilo Dalton
U	Unit
X	Times
CO <sub>2</sub>	Carbon dioxide
g	Gravity
$\mu$ l	Microlite
m/z	Mass per charge ratio



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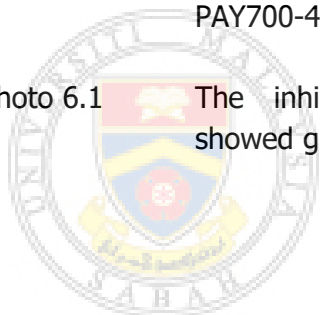
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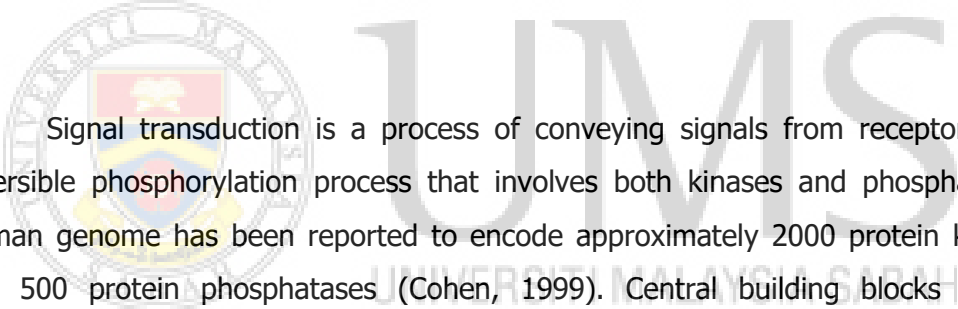
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## CHAPTER 1

### 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).



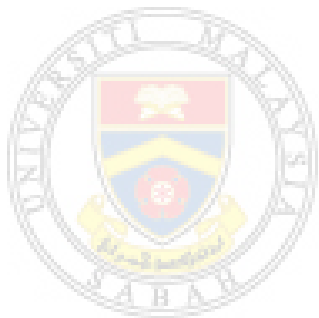
Signal transduction is a process of conveying signals from receptor upon reversible phosphorylation process that involves both kinases and phosphatases. Human genome has been reported to encode approximately 2000 protein kinases and 500 protein phosphatases (Cohen, 1999). Central building blocks in the 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 isolate 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.



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