PRIMARY SCREENING FOR NATURAL INHIBITORS AGAINST EUKARYOTIC SIGNAL TRANSDUCTION FROM SELECTED MEDICINAL PLANTS IN SABAH, MALAYSIA

Jualang Azlan Gansau, Goh Keng Sean & How Siew Eng

School of Science & Technology,
Universiti Malaysia Sabah, 88999 Kota Kinabalu, Sabah, Malaysia.

ABSTRACT. *Studies on eukaryotic signal transduction pathways have led to a variety of potential molecular targets for cancer therapy including Mitogen-activated Protein Kinase Kinase 1 (MKK1), Glycogen Synthase Kinase 3β (GSK-3β) and Type 1 Protein Phosphatase (PP1). The protein kinases and phosphatases in the signal transduction pathways play a vital role in mitogen activated protein (MAP) kinase signal transduction, tumorigenesis, apoptosis, and cancer metastasis. In this study, genetically engineered yeast strains were used as a model in the search for potential inhibitors against these signalling elements. Eleven species of medicinal plants were selected from various locations in Sabah, Malaysia, extracted and tested against MKK, GSK-3β and PP1. Preliminary results showed the presence of potential MKK1 inhibitors in the crude extracts of Alphitonia excelsa, Cordyline terminalis and Tinospora crispa. However, no inhibitor was found against GSK-3β and PP1. The crude extracts of Cordyline terminalis was further fractionated using a bioassay guided fractionation and four partially pure active fractions were isolated.*

KEYWORDS. Inhibitor, Kinase, Phosphatase, Signal transduction, Medicinal plants

INTRODUCTION

The enormous natural resources on earth enable mankind to explore for drug discovery. These include microorganisms, marine sponges, and plants, ranging from fungi to higher flowering plants (Garcia, 1978; Garcia-Hansel *et al.,* 2000; Aloot *et al.,* 2001). Interestingly, one out of four commercial drugs available is medicinal plant based (Berg, 1997). Thus, medicinal plant is a good source for scientists to discover new drugs for the treatment of diseases. Furthermore, there is a high correlation between the application of traditional medicines and the current therapeutic use of medicinal plants in drug development (Farnsworth, 1988).

Cancer is still the leading cause of global death which in turn caused by the genetic defects or mutation occurs in the cell itself. There were a total of 7.6 million deaths worldwide caused by various types of cancer, by year 2030 the global death caused by cancer will reach up to 11.3 million, an increase of 3.7 million deaths in 25 years time (Boerma and Shibuya, 2007). This alarming rate has driven scientists and researchers to investigate for target-based inhibitors, including inhibitors targeting the signal transduction involves in cancer cells development.

Misregulation of signalling molecules is one of the main causes of mutation and cancer. These signalling molecules are mainly protein kinases and phosphatases which involve in the expression of growth and proliferation of cells. When the elements in signal transduction mutated, an over expression of these signaling molecules occurs, which lead to uncontrollable growth and proliferation of cells and eventually cause the cancer (Lobbezzoo *et al.,* 2003).

Therapies for cancer have targeted proteins though the signal transduction and cell proliferation mechanisms have yet to been understood fully (Levitzki, 2003). The inhibitors can be pharmacological probes or affinity ligands targeting toward eukaryotic protein kinases and phosphatases (Mackintosh and Mackintosh, 1994). Although these inhibitors can be developed synthetically, the natural resources on earth provide us with an enormous amount of natural products that are potential inhibitors against cancer. The secondary metabolites and phytochemicals in plants are one of the main natural resources humankind can explore for the discovery of potential anti-cancer drugs.

These inhibitors or also known as signal transduction modulators are able to interfere with the signal transduction cascade by (1) blocking cell surface receptors, (2) inhibition of general factor (GF) receptor tyrosine kinase, and (3) inhibit effects of downstream genes (e.g. MAPKs). Therefore, it is important to develop signal transduction modulators target the cancer cell but cause no harm to other normal and healthy cells in the body. The objective of this study is to screen for potential plants species inhibiting kinases and phosphatases such as Protein Kinase Kinase 1 (MKK1), Glycogen Synthase Kinase 3β (GSK-3β) and Type 1 Protein Phosphatase (PP1)].

MATERIALS AND METHODS

Plant Collection

Eleven samples of plant species (Table 1) were collected from various locations in Sabah, Malaysia. Plant materials were oven dried at 40^{o}C for two weeks and blended to powder form for efficient extraction purpose.

Extraction and Purification Procedure

Methanol was used to extract the bioactive compounds from the plant samples by ratio of 1:10 (w/v) plant material to solvent. The bioactive crude extract was then analysed using thin layer chromatography (TLC) and fractionated using column chromatography (Farnsworth, 1986; Harborne, 1998). Silica gel (Merck, 0.040-0.063 mm) was used as a packing material in column chromatography with EtOAc:MeOH (35:65) as a solvent system. The bioactive fractions were analysed using Agilent 1100 series RP-HPLC equipped with a DAD detector and a Zobax Eclipse XDB C18 column (5μm, 4.6×150 mm).

Table 1. Plant species.

Code	Plant Species	Common Name	Family	Medicinal Value/Treatment for
E01	*Neobalanocarpus Heimii*	Cengal Chengal	Dipterocarpaceae	Antioxidant property (Norizah *et al*., 2002)
E02	*Alphitonia excelsa (Leaves)*	Paku Dita / Red Ash	Rhamnaceae	Abdominal distress (Perera *et al*., 1993) Stomach upset, sore eyes, headaches, bites and stings and washing (Allan & Adkins, 2005)
E03	*Alphitonia excelsa (Bark)*			
E04	*Gynura procumbens*	Akar Sebiak	Asteraceae	Kidney problems (Wiart, 2000)
E05	*Elephantopus mollis*	Soft Elephantsfoot	Asteraceae	Stomach ache, wound, cuts (Fasihuddin & Hasmah, 1993)
E06	*Cordyline terminalis*	Andong / Ti Plant	Laxmanniaceae	Dysentery, tuberculosis, measles (Fasihuddin & Hasmah, 1993)
E07	*Tinospora crispa*	Bakawali / Petawali	Menispermaceae	Hypertension, diabetes, typhoid fever (Fasihuddin & Hasmah, 1993)
E08	*Coelogyne hirtella*	Orchids	Orchidaceae	Arthritis, diarrhoea, headache, cough, digestion diseases, heal wound & incision (Kong *et al*., 2003)
E09	*Cymbidium bicolor*			
E10	*Jatropha curcas L.*	Daun Tangan-Tangan	Euphorbiaceae	Skin diseases, wound, rheumatic (Fasihuddin & Hasmah, 1993)
E11	*Duranta repens*	Daun Bilah / Golden Dewdrop	Verbenaceae	Malaria (Anis *et al*., 2001)

Preparation of Yeast Strains

SC minus uracil, Yeast Cultivation Media and Yeast Peptone Dextrose media were used for GSK-3β, MKK1 and PP1 screening system; and the yeast strains were H10075 (pKT10-GSK-3β), H10068 ($MKK1^{P386}$) and H10017/H10018, respectively (Table 2). The crude methanolic plant extracts were tested for inhibitory activity using paper disc diffusion method (Farnsworth, 1986).

Screening for GSK-3β Inhibitors

The yeast strain H10075 was grown aerobically with shaking at 180 rpm for 48 h at 37°C in SC-Ura liquid medium adjusted to pH 5.6. SC-Ura agar adjusted to pH 7.2 was autoclaved and stabilized at 48°C for an hour before adding the appropriate yeast strain (100 µL culture/25-mL medium) and pouring into Petri dishes. The methanolic extracts (20 µL) were applied onto 6-mm diameter paper discs (Whatman No. 3; Whatman, Brentford, UK) and left to evaporate in a laminar flow cabinet for 5 min. Each extract was tested on two different screening plates, which were incubated at 25°C and 37°C for 72 h (Andoh *et al.,* 2000; Ho, 2003).

Screening for MKK1 Inhibitors

The yeast strain H10068 was grown aerobically with shaking at 220 rpm for 48 h at 28 °C in a yeast fermentation liquid medium adjusted to pH 7.2. A volume of 2.0 mL of yeast culture suspended in phosphate buffer solution (PBS) was pipetted into 100 mL of screening medium. After the screening medium (yeast fermentation agar) was divided into Part A (2 flasks A1 & A2) and B (2 flasks, each added with glucose and galactose, B1 & B2) and autoclaved separately, the flasks were transferred into a water bath at about 50 °C to maintain its temperature. In the meantime, the yeast strain H10068 cultured in the broth was centrifuged at 4000 rpm for 10 minutes to obtain a pellet. The pellet was washed twice with 10 mL of phosphate buffer solution (PBS) and then suspended in 5 mL of PBS for every 25 mL of fermentation media. Subsequently, the medium in A1 and B1 flasks were mixed thoroughly and 2.0 mL of the suspended yeast strain in PBS was pipetted aseptically to the screening medium and mixed gently. The same procedure was repeated for medium in A2 and B2 flasks. The screening medium was then poured into four petri dishes (25 ml each) and left to solidify at room temperature. The methanolic extracts (20 µL) were applied onto 6-mm diameter paper discs (Whatman No. 3; Whatman, Brentford, UK) and left to evaporate in a laminar flow cabinet for 5 min. Each extract was tested on two different screening plates (Glucose and Galactose plate), which were incubated at 28°C for 5 days. Observations were made and recorded based on the growth zones around the paper discs in the galactose plate (Watanabe *et al.,* 1995; Ho, 2003).

Table 2. Yeast strains used for the screening systems.

Strains	Genotype	Series used in UMS	Reference/Source
YTA003W-pKT10-GSK3β	*MATα his3 leu2 ura3 trp1 ade2 mck1::TRP1 mds1::HIS3 mrk1 yol128C::LEU2* [pKT10-GSK3b]	H10075	Andoh *et al.* (2000)
MKK1^{P386}	*MAT**a**/MATa ura3/ura3 leu2/leu2 trp1/trp1 his4/his4 can1/can1* [pNV7-MKK1^{P386}]	H10068	Watanabe *et al.* (1995)
PAY704-1	*Matα ade2-1 his3-11 leu2-3,112trp1-1 ura3-1 can1-100 ssd1-d2 glc7::LEU2 trp1::GLC7:: TRP1 Gal^{+}*	H10018	Andrews & Stark, (2000)
PAY700-4	*Mat**a** ade2-1 his3-11 leu2-3,112trp1-1 ura3-1 can1-100 ssd1-d2 glc7::LEU2 trp1::glc7-10:: TRP1 Gal^{+}*	H10017	Andrews & Stark, (2000)

Screening for PP1 inhibitors

The yeast strains PAY700-4 (glc7-10) and PAY704-1 (GLC7wild-type) were grown aerobically with shaking at 220 rpm for 72 h at 28 °C in a YPD liquid medium adjusted to pH 7.2. The pH adjusted YPD agar, either supplemented or not supplemented with 1 mol L^{-1} of sorbitol, was autoclaved and stabilized at 48°C for an hour followed by addition of the appropriate yeast strain (100 µL culture/25-ml medium) and pouring into Petri dishes to give four combinations of yeast cells and medium. The extracts (20 µL) were applied onto 6-mm diameter paper discs (Whatman No. 3; Whatman, Brentford, UK) and left to evaporate in a laminar flow cabinet for 5 min. Each extract was tested on the four different screening plates, which were incubated at 25 and 37°C for 5 days (Andrews & Stark, 2000; Ho, 2003).

RESULTS AND DICUSSION

Evaluation of Biological Activities of the Crude Extracts

The summary of biological activities of the crude plant extracts tested against kinases and phosphatase are summarized in Table 3. Results obtained from 3 trials showed that no plant extracts exhibited inhibition activity against GSK-3β and PPI. However, the crude extracts of *Alphitonia excelsa* (E02), *Cordyline terminalis* (E06) and *Tinospora crispa* (E07) are potential candidates of MKK1^{P386} inhibitors, while crude extracts of *Alphitonia excelsa* (E03), *Cymbidium bicolor* (E09) and *Duranta repens* (E11) exhibited inconsistent inhibition. The largest inhibition was found in the crude extract of *Cordyline terminalis* (18.3±2.6 mm) followed by the leaf extract of *Alphitonia excelsa* (14.1±2.0 mm), and stem extract of *Tinospora crispa* (11.7 ±2.3mm) (Table 4, Figure 1). In MKK1^{P386} inhibitor screening system, yeast cells utilize glucose and grow as normal wild type strains on the glucose plate. On the galactose plate, the presence of galactose induces the GAL1 promoter and hence results in the overexpression of MKK1^{P386}, thus causing growth inhibitory effect on the yeast cells. As a result, if there is an enzyme inhibitor (plant extract) that inhibits MKK1^{P386}, the yeast can grow on the galactose plant because it will arrest the Pkc pathway at Bck1, MKK1 or Mpk1 (Watanabe *et al.,* 1995; Ho, 2003).

Table 3. Summary of biological activities of methanolic crude plant extracts screened for anticancer.

Extract	Anti Kinase and Phosphatase		
	GSK-3β	MKK1^{P386}	PP1
E01	No Activity	No Activity	No Activity
E02	No Activity	**Potential**	No Activity
E03	No Activity	Inconsistent	No Activity
E04	No Activity	No Activity	No Activity
E05	No Activity	Inconsistent	No Activity
E06	No Activity	**Potential**	No Activity
E07	No Activity	**Potential**	No Activity
E08	No Activity	No Activity	No Activity
E09	No Activity	Inconsistent	No Activity
E10	No Activity	No Activity	No Activity
E11	No Activity	Inconsistent	No Activity

Note:
Concentration of extract : 100 mgmL^{-1}
Volume of sample on disc : 20 µL
Diameter of paper disc : 6 mm
Negative control : Methanol

Note: E01 - *Neobalanocarpus Heimii,* E02 - *Alphitonia excelsa (Leaf),* E03 - *Alphitonia excelsa (Bark),* E04 - *Gynura procumbens,* E05 - *Elephantopus mollis,* E06 - *Cordyline terminalis,* E07 - *Tinospora crispa,* E08 - *Coelogyne hirtella,* E09 - *Cymbidium bicolor,* E10 - *Jatropha curcas L.,* E11 - *Duranta repens.*

Glucose Plate **Galactose Plate**

 Starting from upper disc clockwise :E01, E02, E03, E04, E05 & E06
 Centre disc :Negative control (99.8% MeOH)

Glucose Plate **Galactose Plate**

 Starting from upper disc clockwise :E07, E08, E09, E10 & E11
 Centre disc :Negative control (99.8% MeOH)

Figure 1. The activity of plant crude extracts against MKK1^{P386} screening system.

Table 4. Screening of crude plant extracts for MKK1^{P386} inhibitors.

Extract	Glucose		Galactose		Remarks
	Plate	Inhibition $\mu \pm \sigma$ (mm)	Plate	Growth* $\mu \pm \sigma$ (mm)	
E01	✓	0	×	0	No Activity
E02	✓	0	×	14.1 ± 2.0	Potential
E03	✓	0	×	7.3 ± 6.4	Inconsistent
E04	✓	0	×	0	No Activity
E05	✓	0	×	8.0 ± 7.0	Inconsistent
E06	✓	0	×	18.3 ± 2.6	Potential
E07	✓	0	×	11.7 ± 2.3	Potential
E08	✓	0	×	0	No Activity
E09	✓	0	×	7.0 ± 6.1	Inconsistent
E10	✓	0	×	0	No Activity
E11	✓	0	×	6.7 ± 5.8	Inconsistent

Abbreviation:
✓: Growth of yeast on whole plate
×: No growth of yeast on whole plate
* : Growth of yeast around paper disc

Note:
Concentration of extract : 100 mgmL^{-1}
Volume of sample on disc : 20µL
Diameter of paper disc : 6 mm
Negative control : Methanol

Note: E01 - *Neobalanocarpus Heimii,* E02 - *Alphitonia excelsa (Leaf),* E03 - *Alphitonia excelsa (Bark),* E04 - *Gynura procumbens,* E05 - *Elephantopus mollis,* E06 - *Cordyline terminalis,* E07 - *Tinospora crispa,* E08 - *Coelogyne hirtella,* E09 - *Cymbidium bicolor,* E10 - *Jatropha curcas L.,*E11 - *Duranta repens.*

The methanolic extract of *Cordyline terminalis* (E06) was further fractionated using column chromatography with EtOAc:MeOH (35:65) as a solvent system, which was pre-determined through thin layer chromatography (silica gel) analysis. A total of 34 fractions were obtained and pooled into 9 fractions according to the Rf value of the fractions (pre-determined qualitatively using thin layer chromatography). The 9 fractions obtained after pooling were screened for biological activity against MKK1^{P386} with a total of 3 trials. The pooled fractions 6, 7, 8 and 9 (P6, P7, P8 and P9) gave positive results with pooled fraction 6 (P6) showing the strongest biological activity. The inhibition was 11.3±1.1, 13.0±0, 12.8±0.4 and 11.3±2.4, respectively (Table 5 and Figure 2).

The chemical profiles of the positive pooled fractions from the column chromatography were analysed using a reverse phase analytical column. The results indicated that all the active pooled fractions (P6, P7, P8 and P9) comprised of similar chemical profiles, thus there were combined and represented in Figure 3.

Table 5. Screening of E06 fractions against MKK1^{P386} .

Fraction	Glucose		Galactose		Remarks
	Plate	Inhibition μ ± σ (mm)	Plate	Growth* μ± σ (mm)	
P1	✓	0	×	0	No Activity
P2	✓	0	×	0	No Activity
P3	✓	0	×	0	No Activity
P4	✓	0	×	0	No Activity
P5	✓	0	×	0	No Activity
P6	✓	0	×	13.3 ± 1.1	Potential
P7	✓	0	×	13.0 ± 0.0	Potential
P8	✓	0	×	12.8 ± 0.4	Potential
P9	✓	0	×	11.3 ± 2.4	Potential

Abbreviation:
✓: Growth of yeast on whole plate
×: No growth of yeast on whole plate
* : Growth of yeast around paper disc

Note:
Concentration of extract : 20 mgmL^{-1}
Volume of sample on disc : 20μL
Diameter of paper disc : 6 mm
Negative control : Absolute Methanol

Glucose Plate **Galactose Plate**

Starting from upper disc clockwise : P1, P2, P3, P4, P5, P6, P7, P8 & P9
Centre disc : Negative control (Absolute Methanol)

Figure 2. Activity of E06 fractions against MKK1^{P386} screening system.

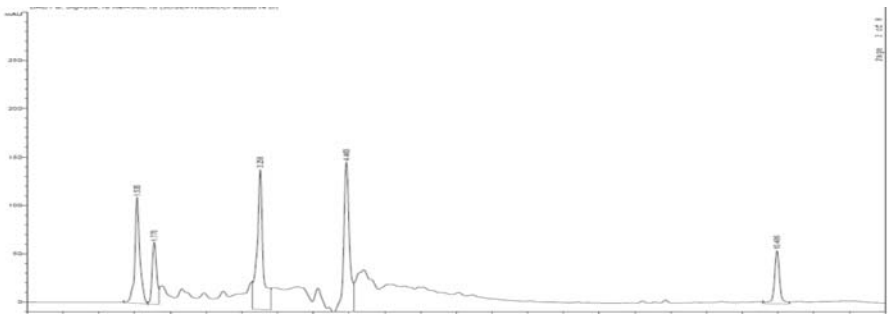

Figure 3. Major peaks detected at 254 nm for the bioactive fraction against MKK1^{P386}.

CONCLUSION

Out of 11 selected plants species, only the crude extracts of *Alphitonia excelsa* (E02), *Cordyline terminalis* (E06) and *Tinospora crispa* (E07) exhibited potential inhibition against MKK1, but none of the samples inhibited GSK-3β and PP1. The largest inhibition effect was found in the crude extract of *Cordyline terminalis* (E06). Further fractionation and purification of E06 extract using a bioassay-guided approach has yielded a partially pure fraction containing 5 major compounds when analysed using a RP-HPLC. Future work could be done to further purify these fractions to identify the bioactive compounds.

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