Inhibitory effect of semi-purified extracts obtained from Potato Tree (Solanum erianthum) against protein kinase and phosphatases.

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Abstract. Kinase and phosphatase are two types of protein which copiously involved in the signal transduction cascade. Misleading of these signaling processes will cause cancer development and other related diseases in human. Therefore, over expression of these signaling proteins might be decreased by the presence of potential inhibitor. In the present study, an attempt was made to verify the potential of Solanum erianthum collected from Sabah, Malaysia against proteins in signal transduction involved in cancer pathway. Leaves of S. erianthum were extracted using methanol. Extracts were tested against protein phosphatase type 1 (PP1), MAPK kinase (MKK1) and MAPK kinase phosphatase (MSG5); which using PAY704-1 and PAY700-4, MKK1P386 and MKK1P386-MSG5 yeast strains, respectively. The results revealed that methanolic extracts of S. erianthum exhibited toxic activities against all assays. Bioassay-guided fractionation of S. erianthum showed positive activities from CHCl3 fraction (CE) against PP1 protein. Chromatographic separation later confirmed column fractions F1 and F2 of S. erianthum as PP1 inhibitor. In-vitro cell growth inhibition assay of this plant sample showed moderate activities against HeLa, CaOV3 and MCF7 cell lines.

1. Introduction

Cancer is still the main factor in both morbidity and mortality rate worldwide with constantly increasing number of cancer cases either in economically developed countries and developed countries [1]. In Malaysia, about 18,219 cases of cancer were identified [2]. Boik [3] reported disruption in signal transduction as part of the cancer causes. Signal from extracellular environment was transmitted through chains of specific proteins and resulted in various cell response such as proliferation, differentiation and cell development [4]. Phosphorylation activity which carried out by protein kinases mainly involved addition of a phosphate group to the target protein [5]. Meanwhile, dephosphorylation was done by protein phosphatases that act as negative regulator during each phosphorylation process [6-9].

About 50% from total protein undergoing phosphorylation with estimated 518 kinases and 156 phosphatases in human genome [10]. Thus, abnormal oncogenic activation will cause genetic and epigenetic changes; either by increasing specific activity of the kinases, overexpression, or loss of negative
regulation [10]. Protein kinases such as MKK1 and ERK2 was found actively expressed in renal carcinoma cell which is usually involved in 80-85% of kidney cancer [11]. Both protein kinases and phosphatases also involved in DNA damage response (DDR) mechanisms. Dephosphorylation inactive DDR factors during normal cell growth [12]. PP1 acting as negative regulator which inhibited protein serine/threonine kinase, therefore causes development and proliferation of cancer cell [13]. Thus, inhibiting these two proteins might serve as golden opportunities in combating cancer development.

*Solanum* (Solanaceae) genera was used traditionally for various ailment. *Solanum erianthum* for instance was used as folk medicine to cure malaria, leprosy, venereal diseases, stimulate the liver functions and to treat haemorrhoids, scrofula, headache and toothache [14]. The leaves were also used for expelling all impurities through the urine, in treatment of leucorrhea and as an abortifacient. Other *Solanum* sp. such as *S. trilobatum* L. was used to cure of asthma, *S. nigrum* L. for stomachache and wounds healing and *S. surattrense* Burm. F. for toothache [15]. Previous studies also showed biological activities of *S. erianthum* such as antifungal and antitrichomonal [16-17]. Chen [18] reported solavetivone isolated from root of *S. erianthum* possessed good anti-inflammatory activity.

Few studies had been conducted on plant inhibitory effect to cell signalling proteins especially kinase and phosphatases. Preliminary data from two weed species collected from Sabah, namely *Mikania micrantha* and *Chromolaena odorata* were reported to harbour potential as protein phosphatase type-1 inhibitor [19-20]. However, no study has been done in searching for anti-kinase and anti-phosphatase activities of *Solanum erianthum*. Therefore, the aim of this study was to evaluate the anti-kinase and anti-phosphatase activities of the plant extracts and subsequently determine their cytotoxic activities against several cancer cell lines.

2. Materials and methods

2.1. Plant material, extraction and fractionation

The leaves of *Solanum erianthum* were collected from Kampung Salimandut, Kota Marudu, Sabah. Voucher specimen was deposited at BORNENSIS, Institute for Tropical Biology and Conservation, Universiti Malaysia Sabah (BORH number 0971). Dried leaves powder was soaked in methanol (1:10, w/v) for three days, filtered, dried in vacuo and freeze dried. Polarity of the extraction solvent would affect the extraction yield. Methanol was used in this study because active compounds shown to be better extracted with methanol and also tend to give better inhibitory effects compared to other solvents [21-22]. Crude methanolic extract were then further fractioned using liquid-liquid extraction using six different solvents; chloroform, buthanol, hexane, ethyl acetate, chloroform-methanol (4:1) and aqueous [23]. Potential chloroform partitionate were eluted in column chromatographic following gradient elution methods with flow rate 10 ml/min by using methanol:chloroform (3:17) (v/v), methanol:chloroform (7:3) (v/v) and methanol (99.9%) (v/v) as solvent system yielding 10 pooled fractions named as F1-F10.

2.2. Microorganisms

Antikinase and antiphosphatase screening systems based on mutant *Saccharomyces cerevisiae* strains collected from Prof. Minoru Yoshida (University Tokyo, Japan) and Prof. Michael J. Stark (University of Dundee, Scotland). The yeast strains genotype and procedures for MKK1, MSG5 and PP1 assay were based on [19].

2.3. Anti-kinase (MKK1\textsuperscript{P386}) and anti-phosphatase (MKK1\textsuperscript{P386}_MSG5 and PP1) screening system

The *Saccharomyces cerevisiae* strains genotype and procedures for MKK1, MSG5 and PP1 assay were referring to [19].
2.4. Cell culture ad in-vitro cell viability

The cancer cell lines namely HeLa (cervical cancer), CaOV3 (ovarian cancer) and MCF-7 (breast cancer-receptor negative) were maintained in RPMI-1640 medium containing 10% fetal bovine serum and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). The cells were incubated at 37°C under 5% CO2 in a humidified atmosphere. Cell number and viability were determined by staining the cells with trypan blue dye. The cells were subcultures when reach confluent at 99%.

Approximately 1.0 x 10⁵ of cells/ml were treated with various concentrations (μg/ml) of *Solanum erianthum* until 50% of cell underwent apoptosis. After the treatment, cells were harvested by centrifugation (450 xg, 10 minutes, room temperature) and washed with phosphate buffered saline (PBS) twice before analysis. About 20 μl of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) solution was added into the cell suspension and left for 4 hours at 37°C in dark condition. The plate was spun and 50 μl of media was sucked out. Subsequently, the formazan salt was dissolved by 100 μl of DMSO and left for 10 minutes at room temperature. The optical density (OD) was measured by using an ELISA reader (Sunrise, Tecan) at 570 nm test wavelength and 630 nm reference wavelength. Concentration that inhibits 50% of the cell growth compared to the untreated sample (IC₅₀) was determined by the absorbance (OD) versus concentration curve. The test was done in triplicates.

3. Results and discussion

3.1. Anti-kinase activity

The MKK1 anti kinase assay had been extensively studied by Matawali *et al.*, [19]. MKK1^{P386} coupled with GAL1 promoter will be cultivated on two different media; glucose and galactose. Induction of this promoter by galactose caused overexpression and later halted the yeast growth. Hence, inhibitor to this protein kinase might able to reverse the reaction by allowing the growth of yeast on galactose plate. Potential inhibitor for this kinase protein then can be easily detected by the ability to rescue the strains from this toxicity. However, there are also possibilities for the growth arrest by another protein in Pkc1 pathway, for instance Bck1. Thus, the experiments had been later expanded to another screening method; MSG5 screening assay. Result showed toxic activity *S. erianthum* extract against MKK1 screening system (Table 1). Further separation using liquid-liquid separation revealed only toxic activity of CME fraction. Toxic effect of this fraction can be observed at as lower as 10 mg/ml of fraction concentration.
### Table 1. Screening results of *S. erianthum* against MKK1 screening system.

| Sample            | Fraction            | Concentration (mg/ml) | MKK1 Glucose | Galactose | Remarks       |
|-------------------|---------------------|-----------------------|--------------|-----------|---------------|
|                   |                     |                       | x ± SD (mm)  | x ± SD (mm) |               |
|                   | Crude methanolic extract | 100                   | 7.50 ±0.71   | NG        | Toxic         |
|                   | CE                  | 100                   | G            | NG        | No activity   |
|                   | BE                  | 100                   | G            | NG        | No activity   |
|                   | HE                  | 100                   | G            | NG        | No activity   |
|                   | EAE                 | 100                   | G            | NG        | No activity   |
| *S. erianthum*    | AE                  | 100                   | G            | NG        | No activity   |
|                   |                     | 100                   | 11.0 ± 0.00  | NG        | Toxic         |
|                   |                     | 70                    | 11.0 ± 0.00  | NG        | Toxic         |
|                   |                     | 50                    | 10.8±0.29    | NG        | Toxic         |
|                   |                     | 30                    | 10.1±0.25    | NG        | Toxic         |
|                   |                     | 10                    | 9.0±0.00     | NG        | Toxic         |
|                   |                     | 1                     | G            | NG        | No activity   |

*C, BE, HE, EAE, AE and CME marked as chloroform extract, butanol extract, hexane extract, ethyl acetate extract, aqueous extract and chloroform-methanol extract respectively, while absolute methanol work as the control of the study.*

*G and NG marked as growth of yeast and no yeast growth, respectively.

* x ± SD stand for mean ± standard deviation.

#### 3.2. Anti-phosphatase activity

Both MSG5 and PP1 assay also had been further discussed by [19]. Overexpression of MSG5 masked the toxicity caused by hyperactivation of Mpk1 pathway in MKK1 assay. In MSG5 assay, potential inhibitor will be detected by ability to arrest growth of yeast on galactose media caused by suppressing activity towards overexpression of MSG5. MKK1 inhibitor candidates validated when the extract tested positive to both MKK1 and MSG5 screening assay.

Results in Table 2 shows that only crude methanolic extract of *S. erianthum* has toxic effect against MSG5 screening system. Further separation shows only CME fraction showed stable toxic activity with inhibition zone in both glucose and galactose plates for MSG5 assay at as lower as 10 mg/ml of fraction concentration. Meanwhile, CE and BE fractions promoted inconsistent toxic activity against MSG5 assay.
### Table 2. Screening results of *S. erianthum* against MSG5 screening system

| Sample                | Fraction | Concentration (mg/ml) | MSG5       | Remarks       |
|-----------------------|----------|-----------------------|------------|---------------|
|                       |          |                       | Glucose    | Galactose     |               |
|                       |          |                       | $\bar{x}$ ± SD (mm) | $\bar{x}$ ± SD (mm) |               |
| Crude methanolic extract | 100      | 7.00 ± 1.41           | 10.00 ± 0.00 | Toxic         |
| CE                    | 100      | 9.50 ± 0.58           | 11.00 ± 0.00 | Inconsistent/Toxic |
| BE                    | 100      | 7.00 ± 0.00           | 7.00 ± 0.00  | Inconsistent/Toxic |
| HE                    | 100      | G                     | G          | No activity   |
| EAE                   | 100      | G                     | G          | No activity   |
| S. erianthum           | AE       | 100                   | G          | G             | No activity   |
|                       |          | 100                   | 10.88 ± 1.44 | 10.50 ± 1.29 | Toxic         |
|                       |          | 70                    | 10.63 ± 0.48 | 10.13 ± 0.25 | Toxic         |
|                       |          | 50                    | 10.25 ± 0.29 | 10.00 ± 0.00 | Toxic         |
|                       |          | 30                    | 10.13 ± 0.25 | 9.88 ± 0.25  | Toxic         |
|                       |          | 10                    | 9.38 ± 0.25  | 9.00 ± 0.71  | Toxic         |
|                       |          | 1                     | G          | G             | No activity   |

*CE, BE, HE, EAE, AE and CME marked as chloroform extract, buthanol extract, hexane extract, ethyl acetate extract, aqueous extract and chloroform-methanol extract respectively, while absolute methanol work as the control of the study.

*G and NG marked as growth of yeast and no yeast growth, respectively.

*$\bar{x}$ ± SD stand for mean ± standard deviation.

On the other hand, crude methanolic extracts of *S. erianthum* showed toxic effect against PP1 assay (Table 3). PP1 inhibitor extract should allow to inhibit wild type yeast growth on YPD medium at 37 ºC. Liquid-liquid separation from the crude extract showed only chloroform partitionate (CE) promote potential inhibitory against PP1 assay. PP1 inhibition can be observed as low as 10 mg/ml of fraction concentration. CME fraction however, promoted toxic activity, while no activity was observed for BE fraction of *S. erianthum* against PP1 assay. CE fractions of *S. erianthum* was chosen to be further fractioned through column chromatography. About 10 fractions collected and Results showed fraction 1 (CE.F1) and fraction 2 (CE.F2) of *S. erianthum* showed potential activities as inhibitor for Mpk1 cascade. The inhibition zone was detected for PAY704-1 growth on YPDS at 37 ºC. Meanwhile, Fraction 9 (CE.F9) showed toxic activity.

#### 3.3. Cytotoxic assay

The chloroform extract (CE) of *S. erianthum* was evaluated for cytotoxicity against HeLa, CaOV3 and MCF-7 cells (Figure 1). The extract showed moderate activity against HeLa ($53.67\pm 3.2146 \mu g/ml$), CaOV3 ($83.67\pm 1.5275 \mu g/ml$) and MCF-7 ($82.00\pm 3.4641 \mu g/ml$) cell lines. There are quite less data reported about bioactivities of *S. erianthum* against cancer cell lines. Volatile oil of *S. erianthum* demonstrated potent inhibitory activity against human breast ductal carcinoma and human prostatic carcinoma cell lines [24]. A study conducted by Chen [18] isolated few compounds from volatile oil of *S. erianthum*. However, none of
the compounds can inhibit human lung squamous carcinoma (CH27), human hepatocellular carcinoma (Hep 3B), human oral squamous carcinoma (HSC-3) and human melanoma (M21) cell lines.

Figure 1. Chloroform extracts of *S. erianthum* against cancer cell lines.

*IC₅₀ (μg/ml) of HeLa, CaOV3 and MCF-7 are 53.67±3.21, 83.67±1.53, 82.00±3.47, respectively.*

*HeLa, CaOV3 and MCF-7 marked as cervical cancer, ovarian cancer and breast cancer-receptor negative, respectively.*
Table 3. Screening results of *S. erianthum* against PP1 screening system

| Sample | Fraction | Concentration (mg/ml) | PAY704-1 (wild type) | PAY700-4 (Mutant) | Remarks |
|--------|----------|-----------------------|----------------------|-------------------|---------|
|        |          | x ± SD (mm)           | YPD                  | YPD+S             |         |
|        |          |                       | YPD                  | YPD+S             |         |
|        |          |                       | 28° C 37° C          | 28° C 37° C       |         |
| Crude methanolic extract | 100 | 8.50 ±0.71 | G | 9.00 ±0.00 | G | G | NG | G | G | Toxic |
| CE     | 100      | 13.00± 1.41 | G | G | G | G | NG | G | G | Inhibitor* |
|        | 70       | 12.25± 3.10 | G | G | G | G | NG | G | G | Inhibitor* |
|        | 50       | 10.75± 2.22 | G | G | G | G | NG | G | G | Inhibitor* |
|        | 30       | 9.25± 2.22 | G | G | G | G | NG | G | G | Inhibitor* |
|        | 10       | 8.50± 1.73 | G | G | G | G | NG | G | G | Inhibitor* |
|        | 1        | G | G | G | G | G | NG | G | G | No activity |
| CE.F1  | 10       | 13.33± 1.51 | G | G | G | G | NG | G | G | Mpk1 cascade inhibitor |
| CE.F2  | 10       | 12.00± 2.65 | G | G | G | G | NG | G | G | Mpk1 cascade inhibitor |
| CE.F3  | 10       | G | G | G | G | G | G | NG | G | G | No activity |
| CE.F4  | 10       | G | G | G | G | G | G | NG | G | G | No activity |
| Sample Code | Concentration | Growth | NG | G | G | G | G | G | G | Activity  |
|-------------|---------------|--------|----|---|---|---|---|---|---|-----------|
| CE.F5       | 10            | G      | G  | G | G | G | NG| G | G | No activity |
| CE.F6       | 10            | G      | G  | G | G | G | NG| G | G | No activity |
| CE.F7       | 10            | G      | G  | G | G | G | NG| G | G | No activity |
| CE.F8       | 10            | G      | G  | G | G | G | NG| G | G | No activity |
| CE.F9       | 10            | 12.33±3.79 | G  | 10.67±2.31 | 11.67±1.53 | 11.33±2.89 | NG| 9.33±1.15 | 9.33±2.89 | Toxic |
| CE.F10      | 10            | G      | G  | G | G | G | NG| G | G | No activity |

| Sample Code | Concentration | Growth | NG | G | G | G | G | G | G | Activity  |
|-------------|---------------|--------|----|---|---|---|---|---|---|-----------|
| BE          | 100           | G      | G  | G | G | G | NG| G | G | No activity |
| HE          | 100           | G      | G  | G | G | G | NG| G | G | No activity |
| EAE         | 100           | G      | G  | G | G | G | NG| G | G | No activity |
| AE          | 100           | G      | G  | G | G | G | NG| G | G | No activity |

| Concentration | Growth | NG | G | G | G | G | G | G | G | Activity  |
|---------------|--------|----|---|---|---|---|---|---|---|-----------|
| 100           | 10.50±0.71 | 14.50±3.10 | 8.50±1.53 | 8.50±0.71 | 12.50±0.71 | NG| 9.50±0.71 | 11.50±0.71 | Toxic |
| 70            | 9.50±0.71  | 14.25±2.22 | 8.00±1.53 | 8.00±1.15 | 11.50±0.00 | NG| 8.50±0.71 | 11.25±2.22 | Toxic |
| 50            | 8.25±0.71  | 13.75±2.22 | 7.00±1.00 | 7.75±0.50 | 10.00±0.00 | NG| 8.00±0.00 | 8.25±0.71 | Toxic |
| 30            | 7.50±0.71  | 11.25±2.22 | 7.00±1.00 | 7.00±0.00 | 8.25±0.71 | NG| 7.00±0.00 | 7.00±0.00 | Toxic |
| 10            | 7.00±0.71  | 9.50±1.73  | 6.75±0.50 | 7.50±0.71 | NG| G | G | G | Toxic |
| 1             | G           | G      | G  | G | G | G | NG| G | G | No activity |

*CE, BE, HE, EAE, AE and CME marked as chloroform extract, butanol extract, hexane extract, ethyl acetate extract, aqueous extract and chloroform-methanol extract respectively, while absolute methanol work as the control of the study.
*G and NG marked as growth of yeast and no yeast growth, respectively.
*x ± SD stand for mean ± standard deviation.
4. Conclusion

In conclusion, preliminary results show potential activities of *S. erianthum* for PP1 assay and toxic activities for MKK1 and MSG5 screening assays. Bioassay guided fractionation of this sample shows positive activities as PP1 inhibitor and moderate cytotoxicity test against cancerous cell lines tested. These findings suggested the need for further isolation, characterization and elucidation of the active constituent(s) as anti-kinase, anti-phosphatase and as anticancer agents.

5. References

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