Mallotus Mollissimus and Solanum Erianthum Exhibit Antikinase, Antiphosphatase and Anti-Cancer Properties

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Abstract:
Cancer is a leading cause of death worldwide and caused by dysregulated signal transduction from kinase and phosphatases. Inhibitors of kinase and phosphatase have demonstrated anticancer properties. Therefore, this study aimed to investigate the antikinase, antiphosphatase and cytotoxic properties of Mallotus mollissimus (M. mollissimus) and Solanum erianthum (S. erianthum). Toxic activities against PP1, MKK1 and MSG5 assays were demonstrated by S. erianthum methanol extract. Bioassay-guided fractionation of the methanolic extracts showed that chloroform fraction (CE) of M. mollissimus exhibited toxic activity against PP1. Meanwhile, CE of S. erianthum showed positive activity on PP1 assay. Column chromatography separation of the CE has revealed that fractions F1 and F2 of M. mollissimus are toxic against PP1. Meanwhile, F1 and F2 CE fractions of S. erianthum were positive against PP1 and F9 fraction showed toxic activity in PP1 assay. Chloroform extracts of both plants exhibit cytotoxicity activity against HeLa, CaOV3 and MCF7 cell lines. This study demonstrated the potential of M. mollissimus and S. erianthum extracts in antikinase, antiphosphatase and anti-cancer activities which warrant further purification and identification.

Keywords:
Cytotoxicity, kinase, Mallotus mollissimus, phosphatase, Solanum erianthum.

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INTRODUCTION

Cancer is a leading cause of death worldwide. It has been a global burden with the increasing number of cancer cases either in economically developed countries and developed countries [1]. There are approximately 18,219 cancer cases were reported in Malaysia [2]. The abnormality of signal transduction is one of the causes of cancer [3, 4]. Signal transduction involves protein kinases and phosphatase carries out phosphorylation and dephosphorylation of protein, respectively [5-9]. Roughly 80% of renal cell carcinoma have high expression of MKK1 and ERK2 protein kinase [10]. Meanwhile, in DNA damage response protein phosphatase 1 acts as a negative regulator to inhibit the serine/threonine kinase phosphorylation process which causes cancer development and cellular growth [11].

*Mallotus* (Euphorbiaceae) and *Solanum* (Solanaceae) genera were used for a wide array of traditional medical applications. Leaves and stem bark of *M. peltatus* (Geist) Muell. Arg. var *acuminatus* was used to cure intestinal ailments, skin infections and trematodic infection [12]. Leaves of *M. macrostachyus* were used as an antidote against snake-poison [13]. Studies have demonstrated that several genera of *Mallotus* such as *M. japonicus*, *M. peltatus*, *M. macrostachyus* and *M. philippinensis* exhibit strong antioxidant, antimicrobial and anti-inflammatory activities, antiproliferative effects against human breast cancer, wound healing and traditional treatments [12, 14-20].

The medicinal potential of *Mallotus mollissimus* which is indigenous to Malesia and Queensland, and *Solanum erianthum* which is indigenous to the tropical and sub-tropical America are poorly reported [21, 22]. Specifically, the antikinase and antiphosphatase activities of *M. mollissimus* and *S. erianthum*. Hence, this study investigated the antikinase and antiphosphatase activities of *M. mollissimus* and *S. erianthum* extracts followed by anti-cancer study against cancer cell lines.

MATERIALS AND METHODS

Plant Sample and Processing

Leaves of *Mallotus mollissimus* (*M. mollissimus*) and *Solanum erianthum* (*S. erianthum*) were collected from Kampung Seri Aman and Kampung Salimandut, Kota Marudu, Sabah, respectively. The specimens were deposited at BORNEENSIS, Institute for Tropical Biology and Conservation (Voucher Specimen No. BORH 0969 and BORH 0971). *M. mollissimus* and *S. erianthum* leaves were dried and immersed in methanol (1:10 w/v) for 72 hours. The concentrated methanolic extracts were further fractioned using liquid-liquid extraction to yield chloroform (CE), chloroform-methanol (CME), and butanol (BE) fractions [23]. CE of *M. mollissimus* was then eluted using silica gel column chromatography with 0.040-0.063mm and 230-400 mesh with solvent system consists of hexane:ethyl acetate (2:1) (v/v) to yield 13 pooled fractions (F1-F13). Meanwhile, *S. erianthum* was further separated by gradient elution with methanol: chloroform (3:17 v/v), methanol: chloroform (7:3 v/v) and methanol (99.9% v/v) yielding 10 pooled fractions (F1-F10).

Microorganisms

MAPK Kinase (MKK1), MAP Kinase Phosphatase (MSG5) and Protein Phosphatase 1 (PP1) yeast screening system were performed to determine the antikinase and antiphosphatase properties of plant extracts [24-26]. The yeast strains used were MKK1\textsuperscript{P386}, MKK1\textsuperscript{P386-MSG5}, PAY704-1 (wild type) and PAY704-1 (mutant type) strains. There were kind gifts of Prof. Minoru Yoshida from University Tokyo, Japan and Prof. Michael J. Stark from the University of Dundee, Scotland.

Antikinase (MKK1\textsuperscript{P386}) Screening System

The screening media consist of Part A and Part B were prepared separately [24, 27]. A final volume of 100 mL consists of Part A and Part B were mixed and 2 mL of yeast culture was added. Then, the media were poured into sterile Petri dishes and left to solidify. Sterile paper discs with a diameter of 6 mm were impregnated with 20 μL of extracts (100mg/mL). Discs were transferred onto the screening media. Each extract was tested on glucose and galactose plate and incubated at 28°C for 5 days. The test was done in triplicates. The diameter of inhibition zones (x ± s mm) in assays was recorded.

Antiphosphatase (MKK1\textsuperscript{P386-MSG5 AND PP1}) Screening System

The screening media for MKK1\textsuperscript{P386-MSG5} screening system consists of Part A and Part B were prepared separately [24, 28]. Both (100 mL) Part A and Part B were mixed thoroughly and 2 mL of the yeast culture
(dissolved in PBS buffer) was added into the screening medium. Then, media were poured into sterile Petri dishes and left to solidify. Sterile paper discs with 6 mm diameter were impregnated with 20 µL of extracts (100mg/ml). Discs were transferred onto the screening media. Each extract was tested on a glucose and galactose plate and incubated for 5 days at 28°C. The test was done in triplicates.

PP1 screening assay uses two different types of media namely, YPD (Yeast Peptone Dextrose) and YPD+1M sorbitol media [26]. Screening media was mixed with 100µl of each yeast strain and poured into Petri dishes. Sterile paper discs (6 mm diameter) were impregnated with 20 µL of extracts (100mg/ml). The disc was transferred onto the screening media and incubated at 28°C, 37°C for 5 days. The screening test was done in triplicates. The diameter of inhibition zones (x ± s mm) in both screening systems were recorded.

**Cell Cytotoxic Assay**

Chloroform extracts (CE) of *M. mollissimus* and *S. erianthum* were screened for cytotoxic activity against Henrietta Lacks (HeLa), cellosaurus cell line (CaOV3) and Michigan Cancer Foundation-7 (MCF-7) cancer cell. Approximately, 1.0 x 10^5 of cells/ml were treated with various concentrations (µg/mL) of *M. mollissimus* and *S. erianthum* chloroform extracts until cell death of 50% occurs. Next, the cells were subjected to centrifugation (450 x g for 10 mins) and washed twice with phosphate-buffered saline (PBS). Approximately, 20 µL of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) solution was added into the cell suspension and incubated at 37°C, 5% CO₂ for 4 hours. The plate was spun at 450xg for 10 minutes and 50 µL of media was aspirated. 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. The 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.

**RESULTS**

**Antikinase Activity**

The crude methanolic extracts of *M. mollissimus* did not exhibit any kind of activity as in MKK1 and MSG5 (Table 1). Whereas there is toxic activity shown by crude methanolic extracts of *S. erianthum* in MKK1 with growth in glucose plate (7.50±0.71) and no growth in galactose plate. Moreover, MSG5 screening system indicated the crude methanol extracts also contains toxic activity as there were growth in glucose (7.00±1.41) and galactose-containing (10.0±0) plate.

Different fractions (chloroform fraction, chloroform-methanol fraction and butanol fraction) obtained from the liquid-liquid portioning *S. erianthum* were tested against MSG5 screening system observed that chloroform fraction did not have toxic activities up to 70 mg/ml, but there were inconsistent toxicities observed at 100 mg/ml at 9.50±0.58 and 11.0±0 in glucose and galactose plates, respectively (Table S1). Interestingly, the chloroform-methanol fraction of *S. erianthum* shows toxic activities against MKK1 and MSG5 from 10 mg/ml to 100 mg/ml (Table 2).

**Antiphosphatase Activity**

Crude methanolic extracts of *M. mollissimus* and *S. erianthum* demonstrated toxic activities in PP1 screening systems (Table 1). Bioassay-guided fractionation showed that the chloroform fractions shows toxic activity from 10 mg/ml to 100 mg/ml (Table S2). CE.F1 and CE.F2 also shows toxic activity at 10 mg/ml. *S. erianthum* shows highly potential activity in the chloroform fraction, CE.F1, CE.F2, CE.F9 and chloroform-methanol fraction (Table S2). The chloroform fraction exhibited as an inhibitor whereas chloroform-methanol fraction exhibited toxic activities from 10 mg/ml up till 100 mg/ml. CE.F1 and CE.F2 fractions demonstrated Mpk1 activity at 10 mg/ml.

**Cytotoxic Activities against 3T3, HeLa, CaOV3 and MCF-7 Cell Lines**

We observed that chloroform extracts of *M. mollissimus* and *S. erianthum* exhibit cytotoxic activities against HeLa, CaOV3 and MCF-7 cell lines ranges from 53.67±3.21 µg/ml – 141.0±2.65 µg/ml of IC₅₀ (Table 3). However, only chloroform extracts of *M. mollissimus* shows cytotoxic activities against 3T3 cell line of up to 98.83±3.55 µg/ml of IC₅₀.

**DISCUSSION**

**Antikinase Activity**

MKK1 and MSG5 assays were performed to study the antikinase activity. MKK1 assay uses MKK1P386 yeast strain that was able to grow on glucose medium but not on galactose. Hence, the growth of yeast can be
compared between these two media. Yeast cells have a stunted growth through overexpression of MKK1$^{386}$ when promoted by a strong \textit{GAL1} promoter.
Table 1: Screening Results $[x \pm s \ (mm)]$ of Crude Methanolic Extract of *M. mollissimus* and *S. erianthum* against MKK1, MSG5, PP1 Screening System

| Crude methanol extract (100mg/mL) | MKK1 | MSG5 | PAY704-1 | PP1 | PAY700-4 | Toxicity |
|----------------------------------|------|------|----------|-----|----------|----------|
| Glucose                          | Galactose | Toxicity | Glucose | Galactose | Toxicity | YPD | YPD+S | YPD | YPD+S | 28-C | 37-C | 28-C | 37-C | 28-C | 37-C | 28-C | 37-C |
| *M. mollissimus*                 | G    | NG   | No activity | G    | G      | No activity | 10.0±2.83 | 10.0±2.83 | 10.0±2.83 | 10.0±2.83 | NG | 10.0±1.41 | 17.0±4.24 | Toxic |
| *S. erianthum*                  | 7.50±1.71 | NG   | Toxic | 7.00±1.41 | 10.6±0 | Toxic | 8.50±0.1 | G    | 9.0±0   | G    | G    | NG    | G    | G    | Toxic |

Notes: G = Growth, NG = No Growth.

Table 2: Screening Results of Liquid-Liquid Partition Extracts of *S. erianthum* against MKK1 Screening System

| Sample | Fraction | MKK1 |
|--------|----------|------|
|        |          | Concentration (mg/ml) | Glucose $x \pm s$ (mm) | Galactose $x \pm s$ (mm) | Remarks |
| *S. erianthum* | Chloroform-methanol extract (CME) |           |                       |                      |         |
|        | 100      | 11.0±0.00 | No growth | Toxic |
|        | 70       | 11.0±0.00 | No growth | Toxic |
|        | 50       | 10.8±0.29 | No growth | Toxic |
|        | 30       | 10.3±0.25 | No growth | Toxic |
|        | 10       | 9.0±0.00  | No growth | Toxic |
|        | 1        | Growth    | No growth | No activity |

Table 3: Biological Activity Chloroform Extracts of *M. mollissimus* and *S. erianthum* against Cancer Cells

| Sample         | Plates | 3T3* | HeLa* | CaOV3* | MCF-7* |
|----------------|--------|------|-------|--------|--------|
| *Mallotus mollissimus* | 1      | 90.1±4.010 | 77.50±0.50 | 141.00±2.946 | 63.00±1.323 |
|                | 2      | 98.8±3.547 | 75.33±1.155 | 137.67±0.577 | 56.6±4.042 |
| *Solanum erianthum* | 1      | VT | 53.67±3.215 | 83.67±1.528 | 82.00±3.454 |
|                | 2      | VT | 59.33±3.055 | 86.50±8.322 | 115.67±3.786 |

*3T3= normal cells, HeLa= cervical cancer, CaOV3= ovarian cancer, MCF-7= breast cancer-receptor negative.
The presence of galactose induced the GAL1 promoter which resulted in the overexpression of MKK1<sup>P386</sup> that inhibit the growth of yeast cells. Hence, the presence of potential inhibitor that inhibits MKK1<sup>P386</sup> causes cell growth due to the inhibition of Pkc pathway at Bck1, MKK1 or Mpk1 [24]. MSG5 screening assay was conducted to specify the targets of MKK1 activities<sup>22</sup>. Results showed only S. erianthum extract exhibit toxicity against MKK1 screening system and no activity for M. mollissimus extract (Table 1). Subsequent liquid-liquid separation revealed CME fraction of S. erianthum contains toxic activity where an inhibition zone was observed in the glucose plate for MKK1 assay (Table 2). This fraction exhibits a toxic effect to a low as 10 mg/ml of concentration.

**Antiphosphatase Activity**

MSG5 inhibitor screening uses a mutant yeast (MKK1<sup>P386</sup>_MSG5) that carries MSG5 plasmid producing MKK1<sup>P386</sup>_MSG5 yeast strain. MSG5 is a protein phosphatase and its substrates are MAP kinase, family members. Overexpression of MSG5 suppresses the toxic effect caused by hyperactivation of the Mpk1 pathway by dephosphorylating and inactivating Mpk1 MAPK. Hence, the uninduced GAL1 promoter causes the mutant yeast to grow on a glucose medium. However, yeast strain could also grow on galactose plate because of the overexpression of MSG5 will inhibit Mpk1 which consequently suppresses the growth inhibition due to overexpression of MKK1<sup>P386</sup> [24, 28]. If the inhibitory activities of the extract on both MKK1 and MSG5 assay, thus it can be interpreted that the inhibition of specific on MKK1. Results in Table 1 shows that only crude methanolic extract of S. erianthum has a toxic effect against MSG5 screening system.

Further separation shows only CME fraction exhibit stable toxic activity with inhibition zone in both glucose and galactose plates for MSG5 assay at as lower as 10 mg/ml concentration. Meanwhile, CE and BE fractions promoted inconsistent toxic activity against MSG5 assay (Table S1).

Antiphosphatase activity was performed using PP1 screening assay targeting type-1 protein serine/threonine phosphatase (PP1). GLC7 in Saccharomyces cerevisiae encodes the catalytic subunit of type I protein serine/threonine phosphatase (PP1). Saccharomyces cerevisiae with temperature-sensitive glc7 allele (glc7-10) which exhibits a G<sub>2</sub>/M arrest at the restrictive temperature. This gene causes kinetochore functions to defect at 37°C. Microtubule-binding activity decreases in the presence of ATP presence at both 37°C and 26°C. Depletion of ATP by apyrase restored the microtubule-binding activity in wild type strain. However, this activity was not restored in glc7-10 at 37°C and marginally restored in glc7-10 at 26°C [26, 29]. At restrictive temperature, the activities of PP1 mutant (glc7-10) were able to be mimic by a potential inhibitor. glc7-10 causes cell cycle arrest and impairment of cell wall integrity at 37°C, but cellular growth and membrane integrity are rescued by adding 1M of sorbitol. Therefore, a potential inhibitor that acts on wild-type GLC7 should demonstrate similar properties with the rescue of cell proliferation and membrane integrity by 1M of sorbitol. Hence, no inhibition zone at the wild type strain with 1M of sorbitol [26, 29]. PP1 assay shows that crude methanolic extracts of both tested species exhibit toxicity (Table 1).

Further tests on methanol fractions of both species have resulted that only CE of M. mollissimus S promote potential inhibitory against PP1 assay at as low as 10mg/ml concentration (Table S2). The inhibitory activity of CE fraction is due to the ability to inhibit a normal but reversible GLC7 which could be rescued by sorbitol that would not affect mutant PAY700-4 which carries glc7-10 allele [26]. This is shown by the inhibition growth of PAY704-1 which is only at YPD medium 37°C. CE fraction of M. mollissimus and CME fraction of S. erianthum exhibit toxicity. No activity was observed for CME and BE fractions for M. mollissimus and BE fraction of S. erianthum in PP1 assay, respectively.

CE fractions of M. mollissimus and S. erianthum were subjected to further column chromatography fractionation and later tested against PP1 screening system (Table S2). We observed that CE.F1 and CE.F2 of M. mollissimus were toxic to PP1 due to the presence of inhibition zones on both yeast PAY704-1 and PAY700-4 at 27°C. In contrast with that, 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°C. Meanwhile, Fraction 9 (CE.F9) showed toxic activity. Other fractions did not show any activity (data not shown).

**Cytotoxic Assay**

Cytotoxicity of M. mollissimus and S. erianthum chloroform extract against HeLa, CaOV3 and MCF-7 cells were evaluated (Table 3). Extracts of M.
mollissimus were moderately toxic against HeLa (77.50 µg/ml ± 0.50) and MCF-7 (63.00 µg/ml ± 1.323) and exhibited weak activity with the IC₅₀ values of 141.00 µg/ml ± 2.646 against CaOV3 cells. Meanwhile, S. erianthum, extract showed moderate activity against Hela, CaOV3 and MCF-7 with IC₅₀ of 53.67 µg/ml ± 3.215, 83.67 µg/ml ± 1.528, and 82.00 µg/ml ± 3.464, respectively. This shows that extracts of M. mollissimus and S. erianthum were able to exhibit anticancer activity.

CONCLUSION

This study demonstrated activities of S. erianthum in PP1 assay, whereas toxic activities were observed in MSG5 and MKK1 assays. M. mollissimus only showed toxic activities for antiphosphatase assays. Cytotoxicity test showed that both plants extracts showed potential anticancer activities. These findings highlighted the potential of S. erianthum and M. mollissimus extracts which indicated subsequent characterization and elucidation of the active constituent(s) could serve as antikinase, antiphosphatase and anticancer agents.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

SUPPLEMENTAL DATA

The supplemental data can be downloaded from the journal website along with the article.

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