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Blumea balsamifera (L.) DC. Elicit Anti-Kinase, Anti-Phosphatase and Cytotoxic Activities against Acute Promyelocytic Leukemia Cells (HL-60)

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ABSTRACT

Blumea balsamfera (L.) DC. (*B. balsamfera*) extract has been shown to exhibit many biological activities. However, the anti-kinase, anti-phosphatase and cytotoxic activities of *B. balsamfera* are not well understood. Therefore, this study aimed to investigate the anti-kinase and anti-phosphatase activities using MKK1, MSG5 and PP1 screening systems. Cytotoxic activity was evaluated using acute promyelocytic leukemia cell lines (HL-60). Methanol extracts of *B. balsamfera* were partitioned into hexane (HE), chloroform (CE), chloroform-methanol (CME), butanol (BE) and aqueous fractions (AQE). Only the CE fraction demonstrated toxic activity against PP1 screening system. Other fractions did not show activity in PP1 screening. CE fractions were further fractionated using silica gel chromatography and a further 11 fractions were obtained. Fraction 2 (CE.F2) showed activity against PP1 and was further fractionated and tested. CE.F2.F6.F3 fraction tested positive against PP1. Inhibition of PP1 by the F2.F6.F3 fraction was further confirmed using an enzymatic reaction and the V_{max} and K_m constants were 124.999 µmol/ml.min and 204.624 µM, respectively. A Lineweaver-Burk plot outcome of F2.F6.F3 revealed decreasing of K_m and V_{max} values which supported the inhibition of PP1 activities. Cytotoxic activities against HL-60 were observed for the CE, CE.F1, CE.F2 and CE.F7 fractions. We have demonstrated that *B. balsamfera* and its specific fractions exhibited anti-kinase and anti-phosphatase activities. These substances have the potential to be used as treatment agent for acute promyelocytic leukemia.

Keywords: *Blumea balsamifera*, Anti-kinase, Antiphosphatase, Acute promyelocytic leukemia.

Introduction

Blumea balsamifera (L.) DC. (*B. balsamifera*) is a medicinal plant traditionally used to reduce high fever and has wound healing effects on open cuts or wounds in humans.[1] An infusion and decoction of the plant's leaves are also used as a stomachic, carminative, diaphoretic, expectorant, and vermifuge agent, and for rheumatism, hypertension and sudorific purposes.[1-6] Additionally, a decoction of the leaves is used in bath water for women during post-natal period for remedial purposes.[1,2] The recent increasing interest in using traditional medicinal plants has led to the interest in investigating selected the biological activities of *B. balsamifera*.

Kinase and phosphatase are two types of proteins that are involved in cell signal transduction.[7-10] In signal transduction, kinase acts as a phosphotransferase in protein phosphorylation by adding a phosphate group to the target protein, whereas phosphatase acts by removal of the phosphate group in dephosphorylation.[8,10-12] However, abnormality in signal transduction has been reported in the development of cancer and other diseases.[13] For example, serine/threonine kinase and phosphatase is a crucial surveillance mechanism in DNA damage response (DDR) to maintain cellular genomic integrity.[14]

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In the development of cancer, phosphatase causes uncontrolled cell proliferation that leads to increasing cellular DNA damage. Therefore, by inhibiting phosphatase activity in cancer cells, these cells go into cell cycle arrest.[14]

Mitogen-activated protein kinase kinase 1 (MKK1), extracellular receptor kinase 2 (ERK2), and MAPK phosphatase (MKP) expressions are involved in 80-85% of renal cell carcinoma (RCC), leukemia and cancers of the liver, colon and prostate.[15,16] MKP-1 was reported to mediate resistance to chemotherapeutic agents such as cisplatin in ovary and lung cancer via the ERK pathway and inhibit cancer apoptosis via the MAPK pathway.[17,18] Hence, discovering inhibitors of mitogen-activated protein kinase (MEK) could provide additional treatment options in cancer cell therapy.

HL-60 cells were derived from a patient with acute promyelocytic leukemia. The isolated cells possess the ability to differentiate to a variety of different cell types of the myelomonocytic lineage, such as granulocytes, monocytes, macrophage-like cells and eosinophils.[19] Differentiation of HL-60 occurs due to the presence of several induction agents such as methylprednisolone, chitosan oligomer, and arsenic sulphide.[20-22] However, there are conditions where HL-60 suffer uncontrolled differentiation, such as in the presence of protein phosphatase. Therefore, this led to the present study to investigate the anti-kinase and anti-phosphatase as well as the cytotoxic activities of *B. balsamifera* towards HL-60.

Materials and Methods

Plant material, extraction and fractionation

B. balsamifera leaves were collected from Kampung Seri Aman, Kota Marudu, Sabah in December 2010. The samples were identified by a plant taxonomist, Mr. Julius Kulip from the Institute for Tropical Biology and Conservation, Universiti Malaysia Sabah. A voucher specimen was deposited at the herbarium (Voucher No. BORH 0970).

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B. balsamifera leaves were dried and 10 g of the powdered leaves were soaked in 100 mL of methanol for three days. The extract was filtered and concentrated to 10% (v/v) from the initial volume. Fractionation was performed on the concentrated methanol extract using the liquid-liquid extraction method to yield hexane (HE), chloroform (CE), chloroform-methanol (CME), butanol (BE) and aqueous (AQE) fractions.[23] All fractions were tested against anti-kinase and anti-phosphatase screening systems.

Chloroform extract (CE) that exhibited type-1 protein serine/threonine phosphatase (PP1) inhibitor activity was further separated by column chromatography (30 × 3cm i.d) using silica gel (0.040-0.063 mm, 230-400 *mesh* ASTM, Merck) with a methanol: chloroform (1:19) solvent system. The column chromatography yielded 11 fractions (999.CE.F1, 999.CE.F2, 999.CE.F3, 999.CE.F4, 999.CE.F5, 999.CE.F6, 999.CE.F7, 999.CE.F8, 999.CE.F9, 999.CE.F10 and 999.CE.F11).

Fraction 999.CE.F2 that exhibited potential inhibition properties towards PP1 was further purified using column chromatography and a hexane: ethyl acetate (2:3) solvent system. This purification yielded eight other fractions (999.CE.F2.F1, 999.CE.F2.F2, 999.CE.F2.F3, 999.CE.F2.F4, 999.CE.F2.F5, 999.CE.F2.F6, 999.CE.F2.F7, 999.CE.F2.F8). Next, amongst these eight fractions, fraction 999.CE.F2.F6 exhibited potential inhibition activity in the PP1 assay. The fraction were further fractionated by column chromatography with hexane: ethyl acetate (1:1) as the solvent system and 7 fractions were yielded (999.CE.F2.F6.F1, 999.CE.F2.F6.F2 999.CE.F2.F6.F3, 999.CE.F2.F6.F4, 999.CE.F2.F6.F5, 999.CE.F2.F6.F6 and 999.CE.F2.F6.F7). All fractions were tested for anti-kinase and anti-phosphatase activities.

Anti-kinase and anti-phosphatase screening (MKK1, MSG5 and PP1)
Microorganisms

For the anti-kinase and anti-phosphatases screening, a mutant yeast strain *Saccharomyces cerevisiae*, $MKK1^{P386}$, was used for MKK1, $MKK1^{P386}$_MSG5 for MSG5 screening, PAY700-4 (mutant type) and PAY704-1 (wild type) for PP1 screening.[24-26] Yeast strains were kind gifts from Prof. Minoru Yoshida (University Tokyo, Japan) and Prof. Michael J. Stark (University of Dundee, Scotland).

Screening systems

$MKK1^{P386}$ and $MKK1^{P386}$-MSG5 mutant yeasts were cultured for 2 days in a 28°C shaking incubator at 220 rpm and subjected to centrifugation at 13,000 rpm for 5 mins at 4°C followed by resuspension in PBS (phosphate buffered saline).[24,27] Next, 2 ml of this suspension was added to each 100 ml of media containing Part A (general media) and Part B (either glucose or galactose) at equal volumes.

Yeast strains, PAY704-1 (wild type) and PAY700-4 (mutant) were cultured and incubated in yeast extract-peptone-dextrose (YPD) broth for 3 days, 28°C at 220 rpm. For the screening assay, 100µl of the yeast cultures were added into every 25ml of screening media, respectively (YPD or YPD + Sorbitol and in either 25°C or 37°C).[26]

Screening for inhibitors against MKK1, MSG5 and PP1 were performed using disc diffusion assay. Media were inoculated with the specific yeast used with the respective screening tests. Next, discs impregnated with the extracts were placed on the agar surface. Approximately 20 µl of each sample extracts was pipetted on the disc at a final concentration of 100 mg/ml. The plates were then incubated at 28°C for 5 days for both MKK1 and MSG5 screening systems. For the PP1 screening system, both PAY704-1 and PAY700-4 plates were incubated at 28°C and 37°C for 5 days.

Protein phosphatase-1 (PP1) and enzymatic assay

PP1-inhibition activity of the fraction UMS999.CE.F2.F6.F3 was performed using the EnzChek phosphatase Assay kit (Molecular Probes, E12020) with DiFMUP (6,8-difluoro-4-methylumbelliferyl phosphate) as a substrate. The fraction was dissolved in 1 × NEB buffer to 10ug/ul. The fraction was diluted with PP1 master mix (0.1 U, New England Biolabs, P0754L) and DiFMUP to a final volume of 100 ul of solution per well. The DiFMUP product (6,8-difluoro-4-methylumbelliferone) was measured using Fluoroskan FL (ThermoFisher, USA) at approximately 358/455 nm every minute for 30 minutes.

Measurement of anti-phosphatase activity

Activity of the PP1 enzyme was assayed by a Lineweaver-Burk plot. The assay was performed using varied concentrations of DiFMUP (50 mM, 100 mM, 125 mM, 150 mM and 175 mM). The kinetic constants (Km and Vmax) were determined by the reciprocal plot of the reaction rate versus the reciprocal of substrate concentration.

Determination of PP1 mode of inhibition, Km and Vmax values

The mode of inhibition on the enzyme was assayed by the Lineweaver-Burk plot. The assay has varied concentrations of DiFMUP (50 mM, 100 mM, 125 mM, 150 mM and 175 mM) and inhibitor (3 µg/µL, 4 µg/µL, 5 µg/µL, 6.5 µg/µL, and 8 µg/µL). The kinetic constants (K_m and V_{max}) were determined by the reciprocal plot of reaction rate versus the reciprocal of substrate concentration.

Cytotoxic assay
Cell line

Human acute promyelocytic leukemia cell line, HL-60 (American Tissue Culture Collection, Virginia, USA) were cultured in RPMI 1640 medium (Invitrogen Corp., USA) supplemented with 10% (v/v) fetal bovine serum.

In vitro cell proliferation assay

To investigate cellular toxicity, 1.5×10^4 cells/well was plated into 96-well plates with 80 µL of the medium. Next, the cells were treated with each extract (CE, CE.F1, CE.F2 and CE.F7) at a concentration of 20 µg/mL and 100 µg/ml. After 48 hours of treatment, the medium and extracts were aspirated and replaced with a new medium containing MTT dye solution. Medium and excess MTT were aspirated and formed formazan was solubilized with 100 µl of DMSO. Absorbance, as a measurement of viable cell number was taken at 570 nm with a Spectramax Microplate reader (Molecular Devices, USA).

Results and Discussion

The present study was carried out to test crude methanolic extract and a fractionated chloroform extract for anti-kinase, anti-phosphatase, enzymatic and cytotoxic activities.

Anti-kinase and anti-phosphatases activities

Crude methanolic extracts of leaves of *B. balsamifera* show toxic activity towards the PP1 screening system (Table 1). However, the same extract did not exhibit any activity against MKK1 and MSG5 screening systems (data not shown). Therefore, further investigation of PP1 activities and fractionation of the chloroform extract was further investigated. Fractionation of crude methanolic extract demonstrated that the resulting chloroform extract (CE) exhibited the toxic activity in the PP1 screening system. The minimum inhibitory concentration determination of the CE fraction showed toxic activities at the concentration of 30 mg/ml and was potentially toxic at 10 mg/ml (Table 1).

In an anti-phosphatase screening assay, PP1 was targeted by a temperature-sensitive and wild-type *Saccharomyces cerevisiae*. A PP1 potential inhibitor found in the CE fraction of the present study can mimic the kinetochore functions in temperature-sensitive *Saccharomyces cerevisiae* and cause cell cycle arrest.[26,28] A potential inhibitor that acts on the wild-type *GLC7* produced the same observations. Therefore, the results indicated that the CE fraction contains PP1 inhibitors.

Further column chromatography separation of the CE fraction yielded 11 fractions that were then tested against the PP1 screening system (Table 2). Only Fraction 1 (F1) and Fraction 2 (F2) showed potential inhibitory activity, whereas Fraction 3 (F3) and Fraction 7 (F7) showed toxic activity. Other fractions did not exhibit any kind of activity. Potential activity of the fraction can be seen with an inhibition zone of yeast growth on YPD media at 37˚C for the wild type, PAY704-1. Meanwhile, toxic activity with an inhibition zone

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was observed in YPD and YPD+S medium for both types of strains at 28˚C.[26] F2 demonstrated the greatest potential activity against PP1 and was further chromatographed followed by screening of the PP1 activity which showed that F2.F6. exhibited toxic activity. F2.F6 was further subjected to chromatography and F2.F6.F3 was found to possess a potential as an inhibitor in the PP1 screening system.

PP1 enzymatic assay of F2.F6.F3

The mode of phosphatase inhibition of the fraction was investigated using the Lineweaver-Burk plot. From the plot, the phosphatase inhibition activity by UMS999.CE.F2.F6.F3 increased with increasing concentration (Table 3). V_{max} and K_m constants for PP1 determined from the Lineweaver-Burke plot was 124.999 µmol/mL.min and 204.624 µM, respectively. From the results, decreasing V_{max} value with increasing inhibitor concentration indicated decreasing PP1 activity in phosphorylation of the DiFMUP substrate.

In this study, the K_m value obtained was not synchronized with the increasing fraction concentration. This is because the K_m assumption value was not stable and is difficult to determine by the manual plotting.[29] Moreover, the Lineweaver-Burke plot also has an exaggerating error for small rates, and minimizing error for large rates, which causes difficulty in the determination of the K_m value.[29,30] Therefore, the specific K_m and V_{max} values with the absence and presence of UMS999.CE.F2.F6.F3 demonstrated that the fraction is a mixed competitive inhibitor against phosphatase 1.

Cell cytotoxicity

The extracts of *B. balsamifera* (UMS999.CE, UMS999.CE.F1, UMS999.CE.F2 and UMS999.CE.F7) were evaluated for cytotoxicity towards the HL-60 cell line, a type of acute promyelocytic leukemia cells at two different doses, 20 ug/mL and 100 ug/mL. All extracts and fractions were cytotoxic towards HL-60 at both concentrations tested (Table 4).

Treatment with fraction UMS999.CE exhibited the highest HL-60 cell viability percentage at a concentration of 100 µg/ml (2.0% ± 0.178) and 20 µg/mL (52.5% ± 2.692). Further fractionation of CE, yielded UMS999.CE.F2 which exhibited the strongest cytotoxic activity against HL-60 at 100 µg/mL (0.6% ± 0.479) and 20 µg/mL (2.05% ± 0.307). The differences in of cytotoxic activities were observed in UMS999.CE crude and UMS999.CE.F2 fractions and were supported by a past study which found that the partial inhibitory concentration (IC_{50}) value of crude extracts (20 µg/ml) was higher than the pure compounds (4 µg/mL).[31]

B. balsamifera from other countries had demonstrated toxicities against cancer cells such as the Yoshida sarcoma, NCI-H187 lung cancer and oral cancer cells.[3,6] Hasegawa *et al.* (2006), also demonstrated the synergistic effect of dihydroflavonol BB-1 isolated from *B. balsamifera* by sensitizing leukemia cells with a tumor necrosis factor-related apoptosis-inducing ligand.[32] This synergistic effect has also been studied in HL-60 cells but no significant sensitization effect was observed.[32]

Table 1: Screening results of UMS999.CE against the PP1 screening system. The assay revealed potential inhibitory effects of the fractions categorized into toxic, potential, or no activity

Concentration (mg/ml)	Yeast based screening system								Remarks
	PAY704-1				PAY700-4				
	YPD Inhibition $\bar{x} \pm s$ (mm)		YPD+S Inhibition $\bar{x} \pm s$ (mm)		YPD Inhibition $\bar{x} \pm s$ (mm)		YPD+S Inhibition $\bar{x} \pm s$ (mm)		
	28°C	37°C	28°C	37°C	28°C	37°C	28°C	37°C	
100	6.50 ± 0.71	14.25 ± 2.22	G	7.50 ± 0.6	7.00 ± 0.00	NG	G	8.00 ± 0.0	Toxic
70	6.50 ± 0.71	15.25 ± 3.10	G	8.00 ± 1.2	7.00 ± 0.00	NG	G	8.00 ± 0.0	Toxic
50	G	14.75 ± 2.22	G	7.75 ± 0.5	7.00 ± 0.00	NG	G	7.00 ± 0.0	Toxic
30	G	12.25 ± 2.22	G	6.75 ± 0.5	7.00 ± 0.00	NG	G	7.00 ± 0.0	Toxic
10	G	7.50 ± 1.73	G	G	G	NG	G	G	Potential
1	G	G	G	G	G	NG	G	G	No activity

Note: G = Yeast growth, NG = No yeast growth.

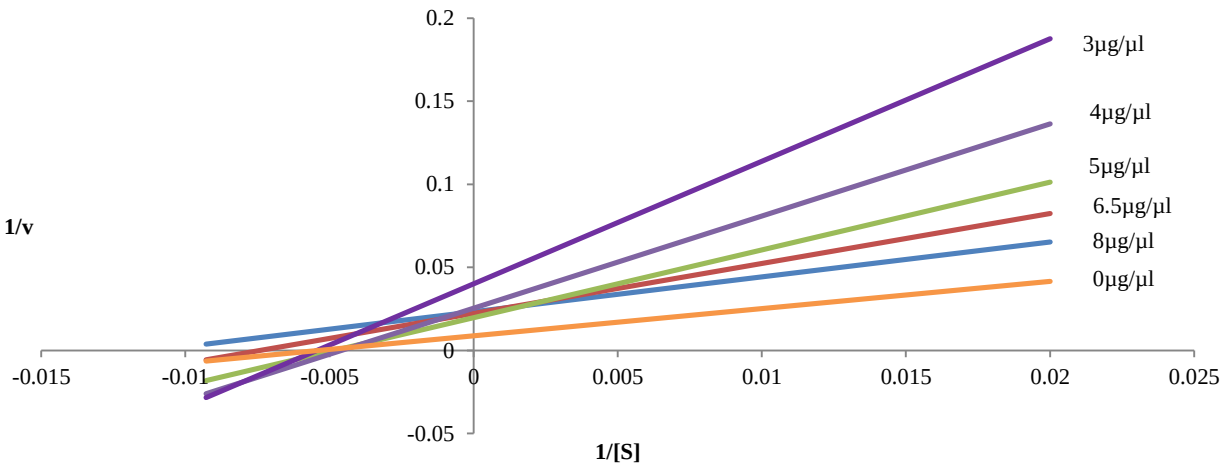

Figure 1: Lineweaver-Burk plot for inhibition of PP1 by UMS999.CE.F2.F6.F3 fraction as the inhibitor.

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Table 2: Results for all fractions of UMS999.CE, as produced from column chromatography against the PP1 screening system. The assay revealed potential inhibitory effects of the fraction. The effects were categorized as toxic, potential, no activity or Inhibitor *Mpk1* cascade

Fraction	Yeast based screening system								Remarks
	PAY704-1				PAY700-4				
	YPD Inhibition $\bar{x} \pm s$ (mm)		YPD+S Inhibition $\bar{x} \pm s$ (mm)		YPD Inhibition $\bar{x} \pm s$ (mm)		YPD+S Inhibition $\bar{x} \pm s$ (mm)		
	28°C	37°C	28°C	37°C	28°C	37°C	28°C	37°C	
CE.F1	G	8.00 ± 1.41	7.00 ± 0.00	7.50 ± 0.71	G	NG	G	10.50 ± 0.71	Potential
CE.F2	G	7.00 ± 0.00	8.00 ± 0.00	G	G	NG	G	11.50 ± 3.53	Potential
CE.F3	7.50 ± 0.71	10.00 ± 1.41	9.00 ± 1.41	9.00 ± 1.41	8.50 ± 0.71	NG	7.50 ± 0.71	13.00 ± 1.41	Toxic
CE.F7	8.00 ± 0.00	9.00 ± 2.83	8.00 ± 0.00	G	8.50 ± 0.71	NG	7.00 ± 0.00	14.50 ± 0.71	Toxic
CE.F2.F6 (10 mg/mL)	Partial 8.00 ± 0.00	G	Partial 7.00 ± 0.00	10.50 ± 0.71	8.00 ± 0.00	NG	Partial 8.00 ± 0.00	Partial 7.00 ± 0.00	Toxic
CE.F2.F6.F3 (1 mg/mL)	G	G	G	Partial 11.50 ± 0.71	G	NG	G	G	Inhibitor *Mpk1* cascade

Note: G = Yeast growth, NG = No yeast growth.

Table 3: K_m and V_{max} values for PP1 in the absence and presence of the isolated fraction (UMF999.CE.F2.F6.F3)

Constant values	UMS999.CE.F2.F6.F3 Concentrations					
	0 µg/µL	3 µg/µL	4 µg/µL	5 µg/µL	6.5 µg/µL	8 µg/µL
K_m (µM)	204.624	155.10	136.58	214.53	221.80	184.28
V_{max}	124.999	52.63	45.45	52.63	40.00	25.00

Table 4: Biological activity of *B. balsamifera* extracts against HL60 cells. Percentage (%) of cell viability post-treatment with different doses of extract is tabled.

Extracts code	% Viability with different concentration of extract	
	100 µg/mL	20 µg/mL
UMS999.CE	2.0 ± 0.178	52.5 ± 2.692
UMS999.CE.F1	0.4 ± 0.115	40.9 ± 2.755
UMS999.CE.F2	0.6 ± 0.479	2.0 ± 0.307
UMS999.CE.F7	0.4 ± 0.242	38.8 ± 7.744

Conclusion

In conclusion, we have demonstrated the potential activities of *B. balsamifera* extract as a PP1 inhibitor using mutant yeasts in the screening assay. The outcome was supported by the kinetic enzyme assay. The potential fraction of *B. balsamifera* also exhibited cytotoxicity against HL-60, acute promyelocytic leukemia cells. Hence, the identification of pure compounds from this fraction warrants future research, while the pure compound(s) could produce alternative treatments for acute promyelocytic leukemia cell.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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