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Biological activities of *Chromolena odorata* (L.) King and Robinson (Asteraceae) collected from Sabah, Malaysia as protein phosphatase type-1 inhibitor

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ABSTRACT

Chromolena Odorata has been traditionally used as wound healer in local community. The present study investigated the anti-kinase and anti-phosphatases activities on methanolic *C. odorata* extract. Mutant yeast strains used are MKK1^{P386}, MKK1^{P386}_MSG5, PAY704-1 and PAY700-4. Bioassay guided fractionation of *C. odorata* revealed positive activities of hexane, ethyl acetate and chloroform partitions. Column chromatography of all partitionates later confirmed fraction F2 from chloroform extract had most favorable activity with inhibitory zone ranged between 7±0.0 mm until 15±0.0 mm. Kinetic analysis including maximum enzyme velocity (V_m) and Michealis-Menten constant (K_m) were evaluated and compared for both normal and inhibited reactions. Enzyme activity with DiFMUP as substrate showed fraction F2 act as PP1 enzyme inhibitor with the K_m value 0.60 mM and V_m value 200 mM/min as compared to the normal enzymatic reaction. Results provided unveil the potential of *C. odorata* as an effective therapeutic agent.

Keywords: *Chromolena Odorata*, Pokok kapal terbang, Sabah, Protein phosphatase type-1.

INTRODUCTION

Drug discoveries in cancer treatment by targeting cell signaling pathway had become part of prominent research in science. Protein phosphorylation/dephosphorylation is a post translational modification (PTM) of amino acids that involves delicate interplay between protein kinases and protein phosphatases [1]. Human genome encodes approximately 518 protein kinases where about two-thirds of them are serine/threonine kinases, and 156 protein phosphatases [2].

Abnormalities from the control of Mitogen Activated Protein Kinase (MAPK) signalling pathway had been detected in various types of cancers as Extracellular signal Regulated Kinase (ERK) pathway had been reported to be involved in tumorigenesis including cancer cell proliferation, migration and invasion; hence accounted for one-third of all human cancers [3, 4]. Some of the components from Ras/Raf/MEK/ERK signalling cascades also had been reported to be frequently mutated and aberrantly expressed in human cancer such as acute myelogenus leukemia, acute lymphocytic leukemia, breast cancer and prostate cancer [5]. Abnormal activation of this pathway was detected in human cancer mainly due to mutation at upstream membrane receptors. Thus ERK signalling pathway is considered as promising therapeutic target for the development of chemotherapeutic drugs.

On the other hand, protein phosphatases act as negative regulators as they reverse the protein kinases actions. One of the major classes of serine/threonine phosphatases is Protein phosphatase type 1 (PP1), which has been found in most eukaryotic cells [6]. PP1 take part on enormous cellular functions for instance in cell adhesion, synaptic plasticity, cell cycle, protein synthesis, muscle contraction, transcription and carbohydrate and lipid metabolism [7, 8, 9]. Thus, targeting in both protein kinases and phosphatases might serve as new focused in cancer therapeutics development.

Chromolaena odorata (L.) King and Robinson is a perennial weed of plantation crops and cleared land from family Asteraceae (Compositae). This invasive alien plant species is formerly known as *Eupatorium odoratum or* locally known as *Pokok Kapal Terbang* or *Pokok Malaysia*. The invasiveness of this plant often causes chaos in natural ecosystem. It was declared as 'Category 1' weed under the Conservation of Agricultural Reasources Act (CARA) and the National Environment Management Biodiversity Act (NEMBA) on Alien and Invasive Species List in South Africa [10]. *Chromolaena odorata* gave negative

impacts on natures and society due to its high-density invasions [11]. Despite that, various recent researches had proved the importance of *C. odorata* as medicinal plant [12-18]. Furthermore, Omokhua [10] on his report stated quite thorough discussions on both biotypes; including their differences in anatomy, ethnopharmacological usage, phytoconstituents and other biological activities. In this study, we made an attempt to further strengthen the ethnomedicinal properties of *C. odorata*, specifically regarding to the inhibitions of protein kinase or phosphatases involved in signal transduction in cancer.

Therefore, the objective in the present study was to investigate the potential of *C. odorata* as kinase or phosphatases inhibitor as well as to evaluate the kinetic constants (Km and Vmax) of PP1 activity inhibited by *C. odorata*.

MATERIALS AND METHOD

Plant material, Extraction and Fractionation

The leaves of *C. odorata* were collected, washed, dried and ground into powders. The sample extracted with absolute methanol at 100 mg/ml stock concentration. Further liquid-liquid extraction and column chromatography separation were based on [19]. In column chromatography, the mobile phase during separation of hexane partition was methanol and chloroform (ratio 1:19 (v/v)), ethyl acetate partition using methanol and ethyl acetate (ratio 1:19 (v/v)) and chloroform extract using chloroform 99.8% (v/v).

Anti-kinase ($MKK1^{P386}$) and anti-phosphatase ($MKK1^{P386}$_MSG5 and PP1) Screening System

Kinase and phosphatase screening assay were conducted using mutant yeast strain obtained 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 [20].

Kinetic properties of Protein phosphatase type-1

Enzymatic assay of *C. odorata* was carried out using protein phosphatase type-1 (PP1) enzyme (New England Biolabs, P0754L). The assay kit was EnzChek® Phosphatase assay kit (E12020, Molecular Probes) with DiFMUP (6,8-difluoro-4-methylumbelliferyl phosphate) as the substrate. The DiFMUP product (6,8-difluoro-4-methylumbelliferone) was measured using Fluroskan® Ascent FL (Fisher Scientific), ext/ems maximum at 355/480 nm once per minute for 60 minutes. All data computation was performed by using Ascent Software version 2.6. Kinetic parameters were determined from the Lineweaver-Burke graph plot. The V_{max} and K_m value for both normal and inhibited reactions were compared.

RESULTS AND DISCUSSIONS

In this study, *Saccharomyces cerevisiae* had been used as model organism in protein kinase and phosphatases assay namely as MKK1, MSG5 and PP1. MAPK activation pathway appear to have been conserved across evolution; as the kinase that comprise this pathway have been identified in organism ranging from mammal to *S. cerevisiae* [21-22]. In most eukaryotes, multiple genes were responsible in encoding PP1c isoform. However, only *Glc7* in *Saccharomyces cerevisiae* might able to encode PP1c [23]. PP1 which was found identical between human and *S. cerevisiae* had been reported to be extremely conserved throughout evolution even greater than tubulin or histone H2A which were well known as most slowly evolving proteins [9, 24, 25].

The principals for MKK1, MSG5 and PP1 assay had been thoroughly discussed on [20]. Results showed no significant activities of *C. odorata* during MKK1 and MSG5 screening assay. However, crude methanolic extract of this sample portrayed a potential inhibitor role during PP1 screening assay (Table 1). The extract had been observed as inhibitor to *GLC-7* which able to inhibit the catalytic domain that reduced the function of Glc7 protein in the cell integrity pathway irrespective to the mutation of the *glc7-10*.

The liquid-liquid separations confirmed three more sub partitions to have potential role as PP1 inhibitor. Hexane, Ethyl acetate and Chloroform (HE, EAE and CE) partitionates showed inhibitory zones on wild type strain, using YPD media at 37^0C. This made the extracts as inhibitor insensitive to glc7-10 catalytic domain change. This type of inhibitor is able to inhibit the normal Glc7 that is reversibly by 1M sorbitol or not without affecting the mutant Glc7 because *glc7-10* allele. The existence of such inhibitor however is unlikely.

CE extract had been subjected to column chromatography to yield another 10 column fractions coded as F1 until F10. These fractions had been re-tested on PP1 screening assay and data showed 8 of the fractions gave potential results, whereas another two are toxic and no activity, respectively. Among the fractions, F2 considered as inhibitor to *GLC-7* while the rest are considered as inhibitor insensitive to glc7-10 catalytic domain change.

Moreover, F2 had been subjected to undergo enzymatic analysis to determine specificity against PP1. The kinetic analysis of F2 had used DiFMUP as substrates as it is among the most efficient and versatile substrate in kinetic assay [26]. PP1-DiFMUP complex yielded reaction product 6,8-difluoro-4-methylumbelliferone which was detected spectrophotometrically at ext/ems 355/480 nm. A linear Lineweaver-Burke plot can be generated in order to get Km and Vm values. The kinetic constant Vm represents the maximum forward velocity of the reaction while Km signifies the substrate concentrations at half of Vm. The lower value of Km suggests that PP1 enzyme has higher affinity for the DiFMUP [27]. Based from the tabulated data on Table 2, kinetic constant V_m and K_m were generally found as inversely proportional to the inhibitor's concentrations. Fraction F2 gradually suppressed PP1 enzymatic activities which indicated by decreasing Vm value. This was found in sync with the value of K_m as higher extract concentrations had decreased the substrate concentrations at half of V_m. Thus, K_{cat} value for the reaction is 0.97 min^{-1} at 500 µg of *C. odorata* applied. These data showed the decreasing activity of the PP1 enzyme related to the concentrations applied. Hence, from this study it was proposed that the decreasing enzymatic activities of PP1 enzyme were due to the inhibitory activities caused by *C. odorata* extract.

The investigations on *C. odorata* had been started since decades ago. This plant had potentials to treat burns, wounds, skin infections, analgesic, anti-inflammatory, anti-pyretic, antioxidant, anti-staphylococcal, anti-spasmodic, anti-protozoal, anti-trypanosomal, anti-bacterial, anti-fungal, anti-hypertensive, diuretic, hepatotropic agent [28-31]. Phytochemicals studies had confirmed this plant is rich with terpenoids, alkaloids, tannins, flavonoids and other phenolic compounds [13, 32]. In cancer research, *C. odorata* had been acknowledged to possessed cytotoxic activities against LLC and HL-60 cancer cell lines [33] but this paper first reported the potential of *C. odorata* as PP1 inhibitor.

Table 1: Potential activities of *C. odorata* during PP1 screening assay

Sample	Extract	Yeast-based screening method								Remarks
		Glc-7								
		PAY704-1				PAY700-4				
		YPD	YPD+1S	YPD	YPD+1S	YPD	YPD+1S	YPD	YPD+1S	
		28^0C	28^0C	37^0C	37^0C	28^0C	28^0C	37^0C	37^0C	
UMS71	CM	0	0	12.0±0.0	8.0±1.4	0	α	0	8.0±0.0	Potential
UMS71	HE	0	0	9.0±1.00	0	0	0	α	0	Potential
	EAE	0	0	7.33±1.15	0	0	0	α	0	Potential
	CE	0	0	8.3±2.31	0	0	0	α	0	Potential
	M:CE	0	0	0	0	0	0	α	0	No activity
	BE	0	0	0	0	0	0	α	0	No activity
	AME	0	0	0	0	0	0	α	0	No activity
UMS71.HE	F1	0	0	0	0	0	0	α	0	No activity
	F2	0	0	0	0	0	0	α	0	No activity
	F3	0	0	0	0	0	0	α	0	No activity
	F4	0	0	0	0	0	0	α	0	No activity
UMS71.EAE	F1	0	0	0	0	0	0	α	0	No activity
	F2	0	0	0	0	0	0	α	0	No activity
	F3	0	0	0	0	0	0	α	0	No activity
	F4	0	0	0	0	0	0	α	0	No activity
	F5	0	0	0	0	0	0	α	0	No activity
	F6	0	0	0	0	0	0	α	0	No activity
UMS71.CE	F1	0	0	11±0.0	0	0	0	α	0	Potential
	F2	0	0	15±0.0	8±0.0	0	0	α	11±0.0	Potential
	F3	0	0	13±0.0	8±0.0	0	0	α	0	Potential
	F4	0	0	10±0.0	0	0	0	α	0	Potential
	F5	0	0	9±0.0	0	0	0	α	0	Potential
	F6	0	0	7±0.0	0	0	0	α	0	Potential
	F7	0	0	0	0	0	0	α	0	No activity
	F8	0	0	11±0.0	0	0	0	α	0	Potential
	F9	0	0	11±0.0	0	0	7±0.0	α	0	Toxic
	F10	0	0	8±0.0	0	0	0	α	0	Potential

Notes:
CM= Crude methanolic extracts, HE=Hexane extracts, EAE=Ethyl acetate extracts, CE=Chloroform extracts, C:ME=Chloroform methanol extracts, BE=Buthanol extracts, AME=Aqueous methanol extracts, F= Column chromatography fraction
UMS71 : *Chromolena Odorata*
Concentrations of stocks extracts : 2mg/ml
Diameter of paper disc : 6mm

Table 2: Kinetic analysis of PP1 using *C. odorata* as inhibitor

Kinetic constant	Normal reaction (DiFMUP)	Inhibited reaction (UMS71.CE.F2) (µg/well)		
		300	400	500
K_m (mM)	0.125	0.600	0.214	0.258
V_m (mM/min)	125.00	200.00	71.43	32.26
K_{cat} (min^{-1})	37.53	6.00	2.14	0.97

CONCLUSIONS

Preliminary data on *C. odorata* which commonly acknowledged as major invasive weeds species had demonstrates its potential as protein phosphatase type-1 inhibitor. To the best of our knowledge, there are still no investigation reported for their potential as kinase or phosphatases inhibitors involved in cancerous signal transduction cascade. Instead of the negative impacts brought by the colonization of this species, *C. odorata* had proven its worthy as traditional medicinal sources. Further investigations on its compound isolations and cell based assay studies are highly recommended.

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