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PROTEOME PROFILE OF OVARIAN CANCER CELL LINE A2780 TREATED WITH 9-METHOXYCANTHIN-6-ONE FROM *Eurycoma longifolia*

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Abstract

Proteomics is a useful tool for the delineation of the ovarian cancer cell line proteomes expressed under different conditions, enabling these to be interrelated to biological pathways or other aspects of cellular physiology. Since all drug targets are protein in nature, we would like to investigate the differential in protein expression of ovarian cancer cell line A2780 in reaction against 9-methoxycanthin-6-one compound from *Eurycoma longifolia*. Compound 9-methoxycanthin-6-one was reported in our previous study as having anti-proliferative effects in ovarian cancer cell line. Therefore, in this paper, we extended our study to observe further the modes of action of this compound on ovarian cancer cell lines. Ovarian cancer is the leading cause of mortality in gynaecological malignancies worldwide. It is one of the highest causes of death among female population in Malaysia. In this study, 2-Dimensional Electrophoresis (2-DE) profiling of non-treated and treated ovarian cancer cell line A2780 with 9-methoxycanthin-6-one compound from *E. longifolia* were performed to observe the in-vitro changes in protein expressions towards understanding the mechanism of action. Differential analyses were performed and subsequently, 15 proteins were selected for proteome identification. Our finding discovered that protein families that affected after ovarian cell line A2780 was treated with 9-methoxycanthin-6-one compound were enzymes, kinase, phosphatase and translation regulator with various molecular functions are amongst the families of proteins involved as adaptive stress response of ovarian cancer cells against 9-methoxycanthin-6-one compound.

INTRODUCTION

In Malaysia, a total of 115238 new cancer cases were registered in 2012-2016 and it was noted that 55.3% were cancer among females. Of the figure, ovarian cancer is identified among the 10 most common cancer in females [1] and a leading cause of mortality of women in urbanised countries [2]. Although great efforts in ovarian cancer therapy have been made in the past 4 decades, the overall cure rate still less than 40% across all stages. The long-term survival of ovarian cancer patients is still poor [3, 4]. *E. longifolia* is a popular medicinal plant in Southeast Asia and widely used as an aphrodisiac and also a traditional medication for various diseases from microbial infections such as dysentery and parasitic infection such as malaria. Over the years, *E. longifolia* have been studied for anti-proliferative potential [5]. Our previous study also discover anti-proliferative activity of compound 9-methoxycanthin-6-one in ovarian cancer cell line [6]. Pharmacological potential of this species were reported in many papers and promising results were demonstrated [7, 8]. Appreciating the fact of its pharmacological potential, anti-proliferative screening of bioactives isolated from this species was conducted and 9-methoxycanthin-6-one compound is identified as active in inhibiting proliferation of ovarian cancer cell lines [9]. In order to understand this compound's actions against ovarian cancer cells, proteomics study was conducted. Proteomics ability for large-scale qualitative and quantitative protein

analyses are known and this field is a popular approach for cancer research [10, 11]. As proteins are very important parts of living organisms, they are the main components of the physiological metabolic pathways of cells as they provide structure, catalysing almost all the reactions that take place in a living cell, transporting and storing materials, and controlling and defending living systems [12]. For that they are called as the workhorses of every living thing. Proteomics, via 2-Dimensional Electrophoresis (2-DE) is a tool for protein profiling since it has the opportunity to diagnose the quantitative and qualitative differences in the protein samples simultaneously. In this study, 2-DE profiling of non-treated and 9-methoxycanthin-6-one compound treated A2780 ovarian cancer cell line were performed to observe the changes in protein expressions towards the understanding of 9-methoxycanthin-6-one compound mechanism of action.

MATERIALS AND METHODS

Anti-ovarian Cancer Activity: Cell Culture

The A2780 ovarian carcinoma cell line [European Collection of Cell Cultures (ECACC)] and SKOV-3 ovarian carcinoma and WRL-68 normal liver cell lines [American Type Culture Collections (ATCC), USA were sub-cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS). The cell lines were further maintained in DMEM until it reached at sub-confluent level [13]. Each of the cell line was seeded approximately between 4000 to 6000 cells/ well in the 96-well plate and incubated overnight in 5% carbon dioxide in the air and pH7.4 conditions. Each cell line was then treated with 0.032, 0.16, 0.8, 4 and 20 µg/mL of 9-methoxycanthin-6-one and cisplatin as a control drug comparison study. All samples were assayedin triplicate.

Determination of IC_{50} Values

At the end of 72 hr the incubation period, the treatments were stopped and Sulphorhodhamine B assay was performed [14]. Generally, the living cells in each well were fixed with ice-cold 50% trichloroacetic acid (TCA), stained with 0.4% of Sulphorhodamine (SRB) and finally 10 mM Trizma pH 10.5 was added and mixed well with gentle agitation on a microtiter plate shaker (IKA, USA). The optical density (OD) of the treated and untreated cells was then measured at 492 nm using microplate reader (Tecan Infinite, Switzerland). The percentage of cells viability (%) was then calculated according to the formula:

$$Percentage\ of\ Cells\ Viability = \frac{OD_{492nm}\ treated}{OD_{492nm}\ non-treated} \times 100$$

Dose-response curves were plotted from the results of Percentage of Cells Viability versus concentrations (µg/mL) of the compound and drug tested. The IC_{50} value was obtained from this curve in which it was the concentration of sample tested that causes 50% inhibition on the cells population. The experiments were repeated at least in triplicate. All data were analysed as mean ± SEM based on 3 independent experiments. Cisplatin, one of the common chemotherapy drugs was included in this experiment for comparison study.

Protein Sample Preparation

The ovarian cancer cells A2780 (~10 million/T-75 Flask) were treated with 9-methoxycanthin-6-one compound of its IC_{50} value and incubated at 37 ºC, pH 7 with 5% CO_2 in air. Cells were sedimented by centrifugation and the pellet was homogenised in lysis buffer containing 6 M urea, 2M Thiourea, 4% CHAPS, 65mM dithiothreitol (DTT) and protease inhibitor using an ultrasonic cell disrupter (Branson, USA). The supernatant was removed by centrifugation at 13000 g for 30 min at 4 ◦C. Protein concentration was quantified using the 2-D Quant Kit (Amersham Biosciences, Uppsala, Sweden). The quantification protocol was performed as described by the manufacturer using Bovine Serum Albumin (BSA) as the protein standard. A standard calibration curve of absorbance values over several protein concentrations was constructed.

Two-Dimensional Gel Electrophoresis (2DE)

Separation of protein via 2-DE was performed as described by our previous method [15]. Briefly, 200 µg/µL of protein were mixed with rehydration buffer and then loaded onto IPGStrips of linear pH gradient 3-10 (GE Healthcare, Sweden). After IPG Strips equilibration, electrophoresis was carried out at 25 mA/gel for at 600V until the bromophenol blue front reached the edge of the gels. The proteins were visualized with Coomassie Brilliant Blue-G250 (CBB-G250) stain [16]. Digital images of the analytical gels were acquired and analysed quantitatively for differentially expressed proteins using ImageMaster 2D Platinium 7.0 analysis software (GE Healthcare, Sweden). Three independent 2-DE gels were performed for each sample. Differences of ≥2 in expression (ratio %V) between matched spots were considered significant whenever a spot group passed statistical analysis (anova, $p < 0.05$) and a second manual verification of the spots in the gel images

Protein Identification

Protein identification was performed using MALDI-TOF-MS/MS (Australian Proteomics Analysis Facilities (APAF),

Australia) and the results were matched with Mascot database. In summary, the protein of interest was plugged from 2-DE gels and subjected to trypsin digestion. MALDI

mass spectroscopy was performed using the 4800 plus MALDI TOF/TOF Analyser (AB Sciex).

RESULTS AND DISCUSSION

In vitro Anti-Ovarian Cancer Activity

A2780 and SKOV-3 ovarian carcinoma cell lines were used as ovarian cancer models in this study to investigate whether 9-methoxycanthin-6-one may exert anti-proliferative effects on ovarian cancer. 9-methoxycanthin-6-one is an alkaloid compound that was reported to be detected and present in the roots of *Eurycoma longifolia* [16, 17].

Determination of IC_{50} Values

The anti-proliferative effects of 9-methoxycanthin-6-one was evaluated based on IC_{50} values (Table 1) against ovarian cancer cell lines. The results shown that 9-methoxycanthin-6-one exerted almost the same anti-proliferative effects as cisplatin. Kardono *et al.* 1991 had reported that 9-methoxycanthin-6-one were active when treated against epidermoid (KB), fibrosarcoma, melanoma, lung, breast and colon cancer cell lines with IC_{50} ranges from 2.1 to 4.5 µg/mL. These current results had shown that 9-methoxycanthin-6-one was active in inhibiting the cancer cells proliferation in ovarian cancer cell lines (A2780 and SKOV-3). It is also noted that a previous study also reported that significant anti-proliferative effects on ovarian cancer cell line Caov-3 at IC50 3.2 ug/mL [17]. Cisplatin is known to cause cancer cell death by binding to DNA and damaging it. However, the molecular target of 9-methoxycanthin-6-one involved in the cell death mechanism remains to be explored. Proteomics analysis is one of the tools that has been used to map out the biochemical pathways and potential protein targets in drug discovery [18,19].

Table 1. Anti-proliferative activities (IC_{50} values) of 9-methoxycanthin-6-one against ovarian cancer cell lines

Compound	Cell Lines (IC_{50} values (µg/mL)	
	A2780	SKOV-3
9-methoxycanthin-6-one	2.02 ± 0.05	2.99 ± 0.06
Cisplatin (control drug)	2.35 ± 0.06	1.49 ± 0.09

The samples were assayed in triplicate and results represent mean ± SEM in at least 3 independent experiments

Differential in Protein Expressions Analysis

The 2-DE-based proteomic profiling approach was employed to analyse the differential expressed proteins from 9-methoxycanthin-6-one compound treated and non-treated ovarian cancer cell line A2780. At least 922 spots were detected in CBB G-250 stained gel. Fifteen differentially expressed proteins between treated and non-treated ovarian cancer cell line (selection criteria >2.0-fold increase or decrease in terms of protein spot intensity, ANOVA $p<0.05$) were detected where 3 were found up-regulated and 12 were down-regulated. Their positions on the 2-DE map are annotated in Figure 1. These proteins were sent for protein identification by MALDI-TOF-MS/MS analysis.

Protein Identification

The list of identified proteins and their functions were tabulated in Table 2. To understand the biological relation of the identified proteins with the progression of treated and non-treated ovarian cancer A2780 cell line, 15 proteins were classified according to their protein families (Figure 2).

Protein families is a group of proteins that share common origin, reflected by their functions and similarities in sequence and structure. The protein classification will aid understanding on global relationship between protein sequence, structure and functions [20] of the differentially expressed proteins after the treatment of 9-methoxycanthin-6-one compound on ovarian cancer cell line. From the results, 7 proteins were grouped under enzyme family, 1 protein kinase, 1 protein phosphatase, 1 protein translation regulator and 5 proteins that may not involve in metabolic pathways [21]. From the experiment data, most of identified protein in this family involved in cell metabolism such as glycolytic pathway, protein salvage pathways and amino acid metabolism pathways [22]–[30] and non-metabolism such as cell proliferation, signalling and transport [31]–[38]. Enzymes have both biological and chemical attributes. Enzyme family is identified from their ability to catalyse biochemical reactions. They modulate metabolic pathways and networks collectively and perform their molecular function in a particular cell compartment [39]. This family plays biggest role in cancer metabolism and explained by Warburg effect where mutated forms of so-called proto-oncogene and activated transcription factors have been

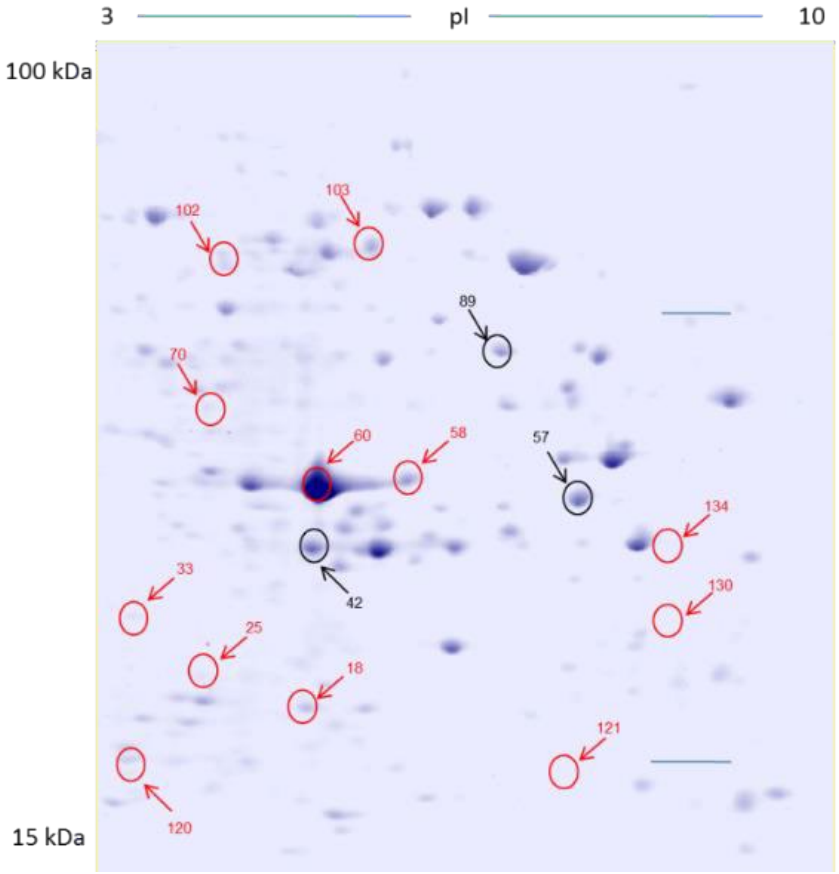

Figure 1. Representative of 2-DE gel of proteins separated from 9-methoxycanthin-6-one treated ovarian cancer A2780 cell line (Note: Red font represents down-regulated protein and Black font represents up-regulated protein).

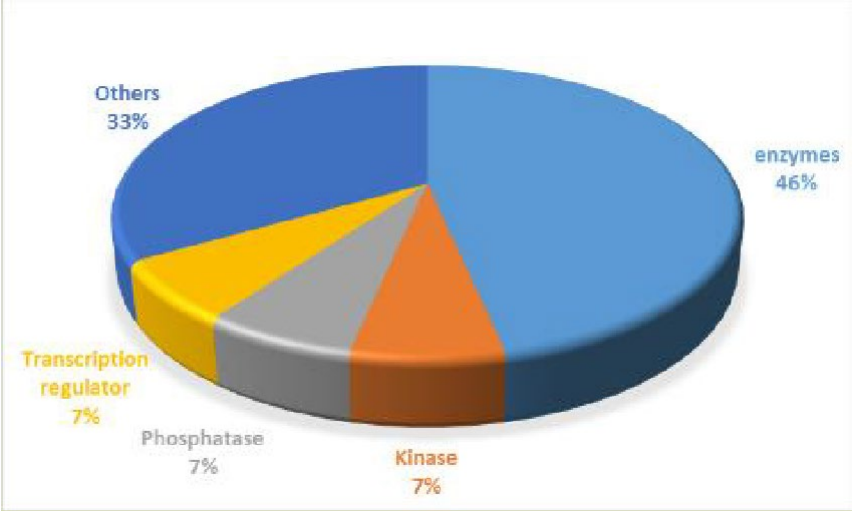

Figure 2. Protein classification according to the families. Proteins are classified into groups where the function and its properties are known. Thus, its roles in cell functions can be proposed based on the group to which it is predicted belong.

Table 2. List of identified proteins and information of proteins identified by MALDI-TOF-MS/MS. Accession number, gene name and protein name were referred to the Swiss-Prot/ Mascot Database.

No	Spot No	Protein Name	Accession No[a]	Theoritical pI[a]	mW (Da)[b]	Sequence coverage (%)	Mascot score	Protein abundance	Family	Molecular function[c]
1	18	Triosephosphate isomerase (TPI1)	P60174	5.65	30772	59%	556	Down-regulated	Enzyme	Isomerase
2	25	Phosphoglycerate mutase 1 (PGAM1)	P18669	6.67	28786	57%	583	Down-regulated	Phosphatase	Hydrolase
3	33	Purine nucleoside phosphorylase (PNP)	P00491	6.45	32097	42%	93	Down-regulated	Enzyme	Transferase
4	42	Nuclear valosin-containing proteinlike (NVL)	O15381	6.11	94991	28%	64	Up-regulated	Other	Ribosome biogenesis
5	57	Malate dehydrogenase 2(MDH2)	P40926	8.92	35481	39%	278	Up-regulated	Enzyme	Oxidoreductase
6	58	Glyceraldehyde-3-phosphate dehydrogenase (GADPH)	P04406	8.57	36030	22%	239	Down-regulated	Enzyme	Oxidoreductase
7	60	Annexin A2 (ANXA2)	P07355	7.57	38580	43%	431	Down-regulated	Other	RNA-binding
8	70	Capping protein (actin filament), gelsolin-like (CAPG)	P40121	5.82	38474	29%	167	Down-regulated	Other	Actin capping
9	89	Acetyl-CoA acyltransferase 2 (ACAA2)	P42765	8.32	41898	28%	128	Up-regulated	Enzyme	Transferase
10	102	Aldehyde dehydrogenase 1 family, member A1 (ALDH1A1)	P00352	6.3	54827	30%	233	Down-regulated	Enzyme	Oxidoreductase
11	103	Pyruvate kinase (PKM)	P14618	7.96	57900	54%	413	Down-regulated	Kinase	Transferase
12	120	Peroxiredoxin 3 (PRDX3)	P30048	7.67	27675	25%	128	Down-regulated	Enzyme	Peroxidase

13	121	Heterogeneous nuclear ribonucleoprotein A1 (HRRNPA1)	P09651	9.17	38723	45%	195	Down-regulated	Other	Ribonucleoprotein
14	130	Galectin-3 (LGALS3)	P17931	8.57	26136	27%	235	Down-regulated	Other	IgE-binding protein
15	134	Elongation factor 1-alpha 1 (EEF1A1)	P68104	9.1	50109	33%	153	Down-regulated	Translation regulator	Elongation factor

[a]Accession numbers were derived from the Mascot database. [b]Theoretical molecular weight (MW) or isoelectric point (p*I*) from the Mascot database. [c] https://www.uniprot.org

found to be involved in the expression of genes in the bioenergetic pathways [40]. Kinase and phosphatase usually co-work together in the opposite function where kinase attaches a phosphate group to a protein and a phosphatase removes a phosphate group from protein. These two families equally contribute to balance phosphorylation levels and thus act to modulate the activities of the proteins in a cell where these family responds to external stimuli [41, 42]. In other words, kinases catalysed the posttranslational modification of protein via protein phosphorylation, and protein phosphatases reverse the effect. Protein kinases involve cell metabolism, transcription, cell division and movement, programmed cell death, and the immune response and nervous system function. Mutations and disregulation of kinase play causal roles in human disease. [43]. Translation regulator plays an important role in most processes in the cell and very essential for maintaining cell homeostasis. The protein synthesis rate depends on the concentration and translational efficiency of its mRNA, where endogenous or exogenous signals such as nutrient supply, hormones, or stress were governed by translational regulator to respond [44].

CONCLUSION

In conclusion, our experimental results show that 9-methoxycanthin-6-one compound from *E. longifolia* inhibit the proliferation of ovarian cancer cell line A2780. Using proteomic analysis after treatment with 9-methoxycanthin-6-one compound, we observed proteome changes where various protein families functioned in various molecular functions affected. These enzymes may be a part of the mode of action of 9-methoxycanthin-6-one compound in killing ovarian cancer cells A2780. These results shall be validated by using immunoassay and other cellular-based assays (s) and furthermore, biochemical pathway analysis can be done to observe the global network of the ovarian cancer cellline that was affected.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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