

Research Paper

Inhibition of MAPKs, Myc/Max, NFkB, and Hypoxia Pathways by *Phyllanthus* Prevents Proliferation, Metastasis and Angiogenesis in Human Melanoma (MeWo) Cancer Cell Line

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Abstract

Background: Melanoma is the most fatal form of skin cancer. Different signalling pathways and proteins will be differentially expressed to pace with the tumour growth. Thus, these signalling molecules and proteins are become potential targets to halt the progression of cancer. The present works were attempted to investigate the underlying molecular mechanisms of anticancer effects of *Phyllanthus* (*P.amarus*, *P.niruri*, *P.urinaria* and *P.watsonii*) on skin melanoma, MeWo cells. **Methods:** The ten cancer-related pathways reporter array was performed by transfection of plasmid construct of transcription factor-responsive reporter of each pathway in MeWo cells. The affected pathways in MeWo cells after treatment of *Phyllanthus* extracts were determined using luciferase assay. Western blot, 2D gel electrophoresis and mass spectrometry analysis were performed to identity and confirm the affected proteins and signalling molecules in treated cells. **Results:** The ten-pathway reporter array revealed five different cancer-related signalling pathways were altered by *Phyllanthus* species in MeWo cells; NFkB, Myc/Max, Hypoxia, MAPK/ERK and MAPK/JNK ($p < 0.05$). Western blot revealed that their intracellular signalling molecules including pan-Ras, c-Raf, RSK, phospho-Elk1, c-myc, Akt, HIF-1 α , Bcl-2, and VEGF were down-regulated with concurrent of up-regulation; Bax, phospho-JNK-1/2 and phospho-GSK3 β , in MeWo cells upon *Phyllanthus* treatment ($p < 0.05$). Proteomics-based approach was performed and MS/MS results revealed that 52 differential expressed proteins were identified ($p < 0.05$) and involved in tumour growth, metastasis, apoptosis, glycolysis and glycolysis, angiogenesis, protein synthesis and energy metabolism. **Conclusion:** This study provides insight into the regulation on multiple survival signalling pathways by *Phyllanthus* in melanoma and might be a therapeutic target for cancer treatment.

Key words: *Phyllanthus*, apoptosis, signaling, melanoma.

Introduction

Malignant melanoma is the most aggressive and life-threatening skin cancer with increasing incidences over the past decades. This disease is prevalent in faired-skin populations, and is easy to detect due to its abnormal colour, size and pigmentation. Despite ac-

counting for only 4% of all skin cancers, melanoma confers 80% of skin cancer induced death [1]. Although, melanoma has high recovery rates if detected early, it has a high tendency of metastasizing, dropping the 5-year survival rate less than 5% [2]. The

underlying cause of melanoma progression and metastasis is poorly understood, hence limiting the effectiveness of current treatments and contributing to increased cases of recurrence or refractory melanoma. Thus, it is vital that future anticancer agents can combat both local and metastatic melanoma.

Many vital biological processes are tightly regulated by complex signalling networks and signal transduction occurs when an external stimuli (e.g. stress, UV) initiate changes in a cell upon binding of ligand to surface receptor. This causes conformational changes to the intracellular signalling molecules, which in turn elicit cellular responses. Disruption of these pathways or intracellular communication can result in various diseases, including cancer [3, 4]. Thus, signalling molecules have become potential targets to halt cancer progression by inducing apoptosis and/or inhibiting tumour metastasis and angiogenesis.

The mitogen activated protein kinase family members (MAPKs) are highly expressed in melanoma and it is believed this pathway mediates melanoma metastasis by inducing proteolytic enzymes (e.g. MMP) activation that leads to degradation of basement membrane, and also by regulating genes involved in cell migration, cell survival and growth [5]. MAPK pathway also has been found in other cancers such as breast, colon, prostate and lung, suggesting a role for MAPK pathway in tumour progression and metastasis [6-9]. Disrupting this pathway may halt cancer progression by inhibition of tumour angiogenesis, proliferation, invasion and metastasis. In melanoma, several other pathways have also been shown to be highly expressed including PI3K/Akt and NF κ B signal transduction pathways and have been associated with tumour development and progression [10-12].

During tumour metastasis, high oxygen delivery and consumption requires activation of hypoxia-inducible factors (HIFs), which induce transcription of growth-related genes such as vascular endothelial growth factor (VEGF). The activation of VEGF can stimulate tumour angiogenesis and thereby increase the oxygen delivery [13]. These changes in hypoxic cancer cells allow them to acquire invasive and metastatic properties as well as to develop resistance to chemotherapy [14].

The pharmacological effects of *Phyllanthus*, a widely distributed medicinal plant, are many including antiviral [15], anti-bacteria [16] and anti-hepatotoxic [17] as well as with anticancer properties [18]. Previously, we reported four species of *Phyllanthus* to possess anti-proliferative effect and apoptosis inducing capabilities in melanoma cell [19]. Currently, to understand the mechanism behind the

anticancer properties of *Phyllanthus* on melanoma cells, we investigated the changes to intracellular signalling network upon treatment. At the same time, we also seek to identify potential new targets for therapeutic intervention in skin melanoma.

Materials and Methods

Preparation of *Phyllanthus* Extracts

P.amarus, *P.niruri*, *P.urinaria*, and *P.watsonii* were the four different species of *Phyllanthus* plant used in this study. Both aqueous and methanolic extracts of each *Phyllanthus* were prepared as described previously by Tang et al. [19]. Briefly, plant samples were freshly harvested, washed, and freeze dried. For aqueous extract preparation, ultra-pure water was used to soak the dried plant samples and absolute methanol was used to prepare the methanolic extract. The samples then were homogenized with extraction buffer and the supernatant was collected after three rounds of extraction. Lyophilized forms of aqueous and methanolic extracts were obtained after evaporation and stored at -20°C prior to experiments.

Chemicals and antibodies

EMEM (Eagle's minimum essential medium) was purchased from Sigma (St. Louis, MO). Foetal bovine serum (FBS) was obtained from Gibco. Antibodies; pan-Ras, c-Raf, RSK, phospho-Elk1, c-Jun, phospho-JNK-1/2, c-myc, HIF-1 α , Bcl-2, Bax, NF κ B p50 and p52, VEGF, phospho-GSK3 β , phospho-p38 MAPK and p53 were obtained from MERCK, San Diego, USA. While urea, CHAPS, DTT IPG buffer (pH 3-11NL), SDS, bromophenol blue Tris, glycine, acrylamide, bis-acrylamide were purchased from GE Healthcare.

Cell culture

Skin melanoma MeWo (HTB-65) cell line was purchased from American Type Culture Collection (Rockville, MD) and cultured with EMEM. This medium was supplemented with 10% heat-inactivated foetal bovine serum (FBS, Gibco). Cells were maintained in culture at 37°C with 5% CO₂ and 95% humidity. Cells were harvested using 0.25% trypsin (Hyclone) when they reach 70-80% confluency in culture flasks. Cells undergoing exponential growth were used throughout the experiments.

Dual luciferase pathway reporter transient transfection

Ten different cancer-related pathways analysis was performed using the Signal Finder 10-Pathway Reporter Arrays (SA Biosciences, Fredrick, MD). Optimization of the conditions, including amount and incubation time of plasmid construct of transcription

factor-responsive reporter of each pathway into the cells, was performed to ensure highly transfection efficiency and inhibit transformation. After optimization, MeWo cells were seeded into a 96-well white plate and incubated overnight at 37°C. Transient transfection was conducted by adding plasmid construct of transcription factor-responsive reporter of each pathway and controls to cells and incubated overnight in a 37°C incubator. Then, cells were treated with *Phyllanthus* extracts at their respective IC₅₀ values (Table 1) and further incubated for 48 hours. Each transfection condition was carried in triplicates. Each of the pathways/reporters consist an inducible transcription factor responsive firefly luciferase reporter and constitutively expressing *Renilla* construct. *Renilla* construct is to act as an internal control for normalizing transfection efficiencies and monitor cell viability. After 48h of *Phyllanthus* treatment, the changes in expression of each pathway in cells were determined by measuring the generated firefly and *Renilla* luminescent signals using the Dual-Glo Luciferase Assay system (Promega, Madison, WI) on the Glomax machine (Promega, USA). The relative luciferase units were determined by dividing the firefly to *Renilla* luciferase activity ratio.

Table 1. Treatment of MeWo cells at different IC₅₀ values of *Phyllanthus* extracts.

		IC ₅₀ ± SEM (µg/ml)
<i>Phyllanthus</i> species	Extracts	MeWo
<i>P.amarus</i> (PA)	Aqueous (H ₂ O)	193.3 ± 1.3
	Methanolic (MeOH)	133.3 ± 2.9
<i>P.niruri</i> (PN)	Aqueous (H ₂ O)	260.0 ± 2.4
	Methanolic (MeOH)	153.3 ± 2.6
<i>P.urinaria</i> (PU)	Aqueous (H ₂ O)	193.3 ± 1.1
	Methanolic (MeOH)	56.2 ± 3.2
<i>P.watsonii</i> (PW)	Aqueous (H ₂ O)	160.0 ± 3.2
	Methanolic (MeOH)	100.7 ± 2.0

Western Blot analysis

The intracellular signalling molecules in MAPK (pan-Ras, c-Raf, phospho-Elk1, RSK, c-Jun, phospho-JNK-1/2, Akt and phospho-p38 MAPK), Myc/Max (c-myc) and Hypoxia (c-myc, HIF-1α, VEGF and GSK3β), NFκB (p50 and p52), as well as p53 (p53, Bcl-2, Bax) pathways were chosen for western blot analysis. Beta actin was used as a loading control. Briefly, protein lysates from *Phyllanthus*-treated (Table 1) and untreated groups after 48 hours of treatment were resolved on 12% SDS-PAGE gels. After electrophoresis, proteins were transferred onto nitrocellulose membranes, followed by blocking and incubation with primary antibodies overnight at 4°C. Subsequently, the membranes were incubated

with appropriate secondary antibodies (horseradish-conjugated goat anti-mouse or anti-goat IgG) for 1h. Open-source software, ImageJ was used to measure band intensities. The percentage of protein expression was calculated by dividing the band intensity of treated group with the untreated group.

Two-Dimensional Gel Electrophoresis

A total of 500mg protein was subjected to 2D gel electrophoresis according to the manufacturer's instructions (GE Healthcare). Briefly, total protein was extracted from cells (untreated and treated groups) after 48 hours of treatment (Table 1), by incubation with cold lysis buffer on ice for 30 minutes. The protein pellets were re-solubilized in rehydration solution (8 M urea, 2% CHAPS, 40 mM DTT, 0.5% IPG buffer pH3-11NL, bromophenol blue). A total of 500mg protein rehydrated into 13cm immobilized pH gradient (IPG) strips (pH 3-11 nonlinear) (GE Healthcare) overnight. The first dimension was run on the IPGphor III machine (GE Healthcare) at 20°C with the following settings: step 1 at 500V for 1h; step 2 at 500-1000V for 1h; step 3 at 1000-8000V for 2.5h, and step 4 at 8000V for 0.5h. Upon completion of first dimensional separation, the strip was equilibrated as following; first reduction with 64.8 mM of dithiothreitol-SDS equilibration buffer (50 mM Tris-HCl [pH 8.8], 6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue) for 15 minutes, followed by alkylation with 135.2 mM of iodoacetamide-SDS equilibration buffer for another 15 minutes. The second dimension electrophoresis was performed by electrophoresing the samples in 12.5% SDS acrylamide gels by using the SE600 Ruby system (GE Healthcare) at 25°C in an electrode buffer (25 mM Tris, 192 mM glycine, and 0.1% [wt/vol] SDS) with the following settings: step 1 at 100V/gel for 45 minutes; step 2 at 300V/gel until the run is completed. After electrophoresis, the gels were fixed with destaining solution for 30 minutes, followed by staining with hot Coomassie blue for 30 minutes. Lastly, the gels were scanned using Ettan DIGE Imager (GE Healthcare). Gel images were analyzed using PDQuest 2-D Analysis Software (Bio-Rad, USA) and only protein spots which showed significant differences (more than 1.0 fold) were selected for mass spectrometry analysis.

Protein Digestion, Desalting and MALDI-TOF/TOF Analysis

The significant differential expressed protein spots were excised from the polyacrylamide gels and kept in sterile 1.5ml eppendorf tubes. Excised spots (gel plugs) were washed with destaining solution (50 mM NH₄HCO₃) until the gel plugs were clear. The gel plugs were then incubated with reducing solution

(100 mM NH_4HCO_3 containing 10 mM DTT) for 30 min at 60°C. The gel plugs were alkylated with 100 mM NH_4HCO_3 containing 55 mM of IAA for 20 min in the dark and followed with three times washed with 50% acetone in 100 mM NH_4HCO_3 for 20 min each. The gel plugs were then rehydrated with 100% ACN. In-gel digestion step, trypsin gold (Promega, Mass Spectrometry Grade) was used and added into gel plug and incubated overnight at 37°C. Proteins were extracted from gel plugs and purified by using Ziptip (Ziptip C18, Millipore, Bedford, MA, USA). The eluted proteins were mixed with MATRIX solution and spotted on MALDI plate using dry droplet method and analysed using Ab Sciex Tof/Tof™ instrument. The generated peptides were blasted with MASCOT Search Algorithm (Version 2.1.0) to identify the possible proteins.

Statistical Analysis

For all experiments, results were expressed as the mean \pm standard error (SEM) of data obtained from triplicate experiments using SPSS software. The Student's *t*-test was used where values of $p < 0.05$ were considered significant.

Results

Alteration in expression of several cancer pathways

Ten cancer-related pathways including Wnt, Notch, p53, TGF- β , cell cycle/pRB-E2F, NF κ B, Myc/Max, Hypoxia, MAPK/ERK and MAPK/JNK pathways were investigated. The differential expression of each of these pathways in treated and un-

treated MeWo cells is presented in Figure 1. From Figure 1, it was noted, that in the untreated MeWo cells, six (p53, NF κ B, Myc/Max, Hypoxia and MAPK/ERK and MAPK/JNK) investigated pathways were expressed to regulate the cells growth and survival. However, the expressions of NF κ B, Myc/Max, hypoxia and MAPK/ERK showed significant down-regulation in *Phyllanthus* treated MeWo cells ($p < 0.05$). Contrarily, the expressions of p53 and MAPK/JNK pathways showed significant up-regulation in the treated cells ($p < 0.05$). Other pathways investigated were found to be not significantly affected by *Phyllanthus* extracts ($p > 0.05$).

Induction of apoptosis through p53 protein and disruption in anti-apoptotic/pro-apoptotic balance

As shown in Figure 2, p53 protein was detected at 53 kDa and their expression was significantly up-regulated in *Phyllanthus* treated MeWo as compared to untreated MeWo cells ($p < 0.05$). In addition, other apoptosis related proteins including Bcl-2 and Bax were detected at 23 kDa and 26 kDa, respectively. The graph shows a significant increase of pro-apoptotic protein, Bax in treated MeWo cells ($p < 0.05$) with a concurrent decrease in anti-apoptotic protein, Bcl-2 ($p < 0.05$) as compared to untreated MeWo cells. Among the species of *Phyllanthus*, both aqueous and methanolic extracts of *P.watsonnii* showed the most significant changes on p53, Bax and Bcl expression, followed by *P.urinaria*, *P.niruri* and *P.amarus*.

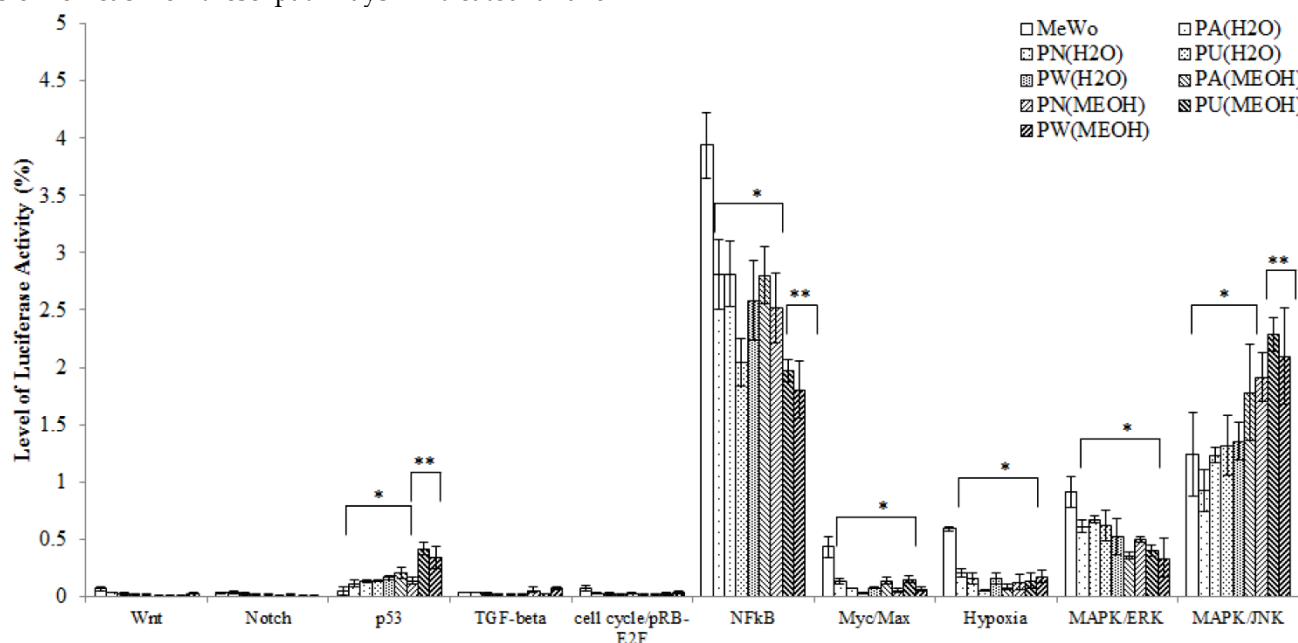


Figure 1. The expression of transcription activities in ten-cancer related pathways of untreated and treated MeWo. Four different pathways (NF κ B, Myc/Max, Hypoxia, and MAPK/ERK) were significantly down-regulated ($p < 0.05$) and two pathways (p53 and MAPK/JNK) were up-regulated ($p < 0.05$). Other pathways showed no significant change. Bars show the mean percentage \pm SEM. * $P < 0.05$ and ** $P < 0.01$ vs control.

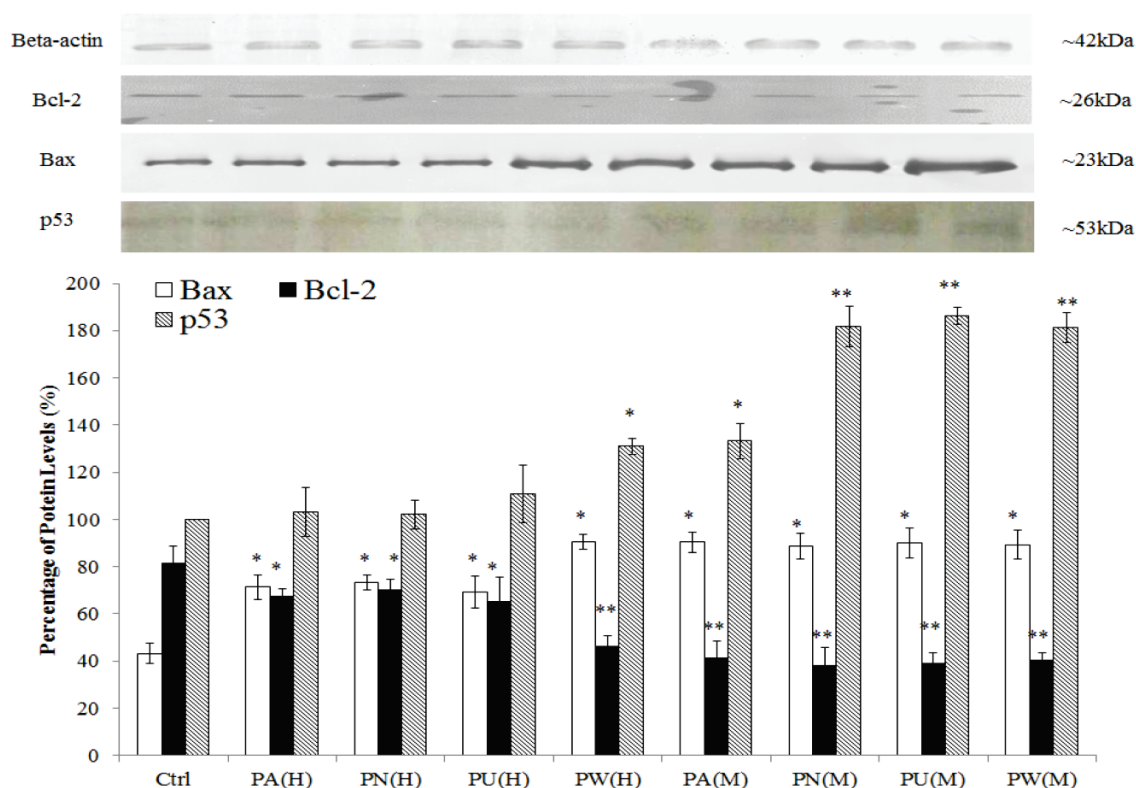


Figure 2. Induction of apoptosis through p53 pathway and disruption in anti-apoptotic/pro-apoptotic balance. The expression of pro-apoptotic (Bax) was up-regulated and anti-apoptotic (Bcl-2) protein was down-regulated in MeWo cells after treatment with *Phyllanthus* extracts. Bars show the mean percentage \pm SEM. * $P < 0.05$ and ** $P < 0.01$ vs control.

***Phyllanthus* alters activities of intracellular signalling molecules in affected pathways**

Three up-stream activator molecules in MAPK pathway; pan-Ras, c-Raf and Akt were highly expressed in untreated MeWo cells (Figure 3). The constitutive activations of these molecules can activate their downstream targets including MAPK/ERK (RSK, phospho-Elk1, c-Jun/AP-1), MAPK/JNK (phospho-JNK-1/2) and p38 (phospho-p38 MAPK). As shown in Figure 3, the expressions of all these intracellular signalling molecules were detected in untreated MeWo cells indicating their involvement in regulating MeWo cells' growth. However, all these intracellular signalling molecules had notable down-regulated expression ($p < 0.05$) except for phospho-JNK-1/2 protein, which showed a slight increase in expression in treated MeWo cells.

The expression of Myc/Max and hypoxia signalling pathway was detected at increased levels in MeWo cells (Figure 1) with a high percentage down-regulation after treatment with *Phyllanthus*. In these pathways, four intracellular signalling molecules were detected by western blot; c-myc, HIF-1 α , VEGF and phospho-GSK3 β . As shown in Figure 4, the expression of the c-myc was detected at 65 kDa and its expression was noticed significantly decreased by aqueous- ($p < 0.05$) and methanolic-*Phyllanthus*

($p < 0.01$) treated MeWo cells as compared to untreated cells. The downstream targets of c-myc; HIF-1 α and VEGF were detected at 120 kDa and 50 kDa, respectively. Their expressions were significantly down-regulated in treated MeWo cells as compared to untreated cells ($p < 0.05$). A negative regulator of c-myc, phosphorylated glycogen synthase kinase 3-beta (phospho-GSK3 β) was detected at 51 kDa and its expression was significantly up-regulated in treated cells as compared to untreated cells ($p < 0.01$). Two intracellular signalling molecules in NF κ B pathway; p50 and p52 were detected at 50 kDa and 52 kDa, respectively (Figure 5). Both NF κ B proteins were significantly down-regulated in MeWo cells after treatment with *Phyllanthus* extracts ($p < 0.05$).

Among the *Phyllanthus* species, *P.urinaria* showed strongest effect on expression of intracellular signalling molecules in affected pathways, followed by *P.watsonii*, *P.amarus* and *P.niruri* for both aqueous and methanolic extracts.

Proteomic profiling of the differentially expressed proteins in *Phyllanthus* treated MeWo cells

Differentially expressed proteins in MeWo cells were statistically defined based on two criteria: 1) degree of intensity > 1.0 fold (Protein scores greater

than 52 are significant, $p < 0.05$) and 2) reoccurrence in the three repeated experiments (Figure 6). According to these criteria, 52 proteins were identified by MS/MS and grouped in four biological processes based on their functions described in UniProtKB/Swiss-Prot protein database (Table 2).

In Group I (cell adhesion, migration, invasion and metastasis), 8 proteins were found to be differentially expressed in treated MeWo cells. Of these, type I cytoskeletal 13 keratin was found to be up-regulated ($p < 0.05$). Its expression was significantly up-regulated about 1.4-1.8 folds higher than untreated cells. The other identified proteins were down-regulated in treated cells; Ephrin-B1, alpha-centractin, bystin, superoxide dismutase, tubulin beta chain, F-actin-capping protein subunit alpha-2 and partitioning defective 3 homolog.

In Group II (proliferation, cell cycle and apoptosis), 21 proteins were significantly down-regulated in MeWo cells after treatment with *Phyllanthus* extracts. Among these down-regulated proteins, HCLS1-associated protein X-1 and casein kinase II

subunit alpha proteins showed the greatest reduction at 1.5-2.3 folds in their expression as compared to untreated cells ($p < 0.05$).

In Group III (glycogenesis and glycolysis), 5 down-regulated enzymes were detected in treated MeWo cells. These identified enzymes were glucosamine-fructose-6-phosphate aminotransferase, protein phosphatase 1 regulatory subunit 3D, alpha-enolase, pyruvate kinase isozymes M1/M2 and phosphoglycerate kinase 1.

In Group IV (protein synthesis and energy metabolisms), 15 proteins were found to be down-regulated at the range of 1.3-1.9 in *Phyllanthus* treated MeWo cells as compared to untreated cells. Eight of these proteins; E3 ubiquitin-protein ligase ARIH2 and RNF115, aspartate aminotransferase, 26S protease regulatory subunit 8, putative elongation factor 1-alpha-like 3, mitochondrial inner membrane organizing system protein 1 (MINOS), zinc finger protein, Sec1 and trimethyllysine dioxygenase, have been known to be involved in regulation of mitochondrial integrity and protein ubiquitination.

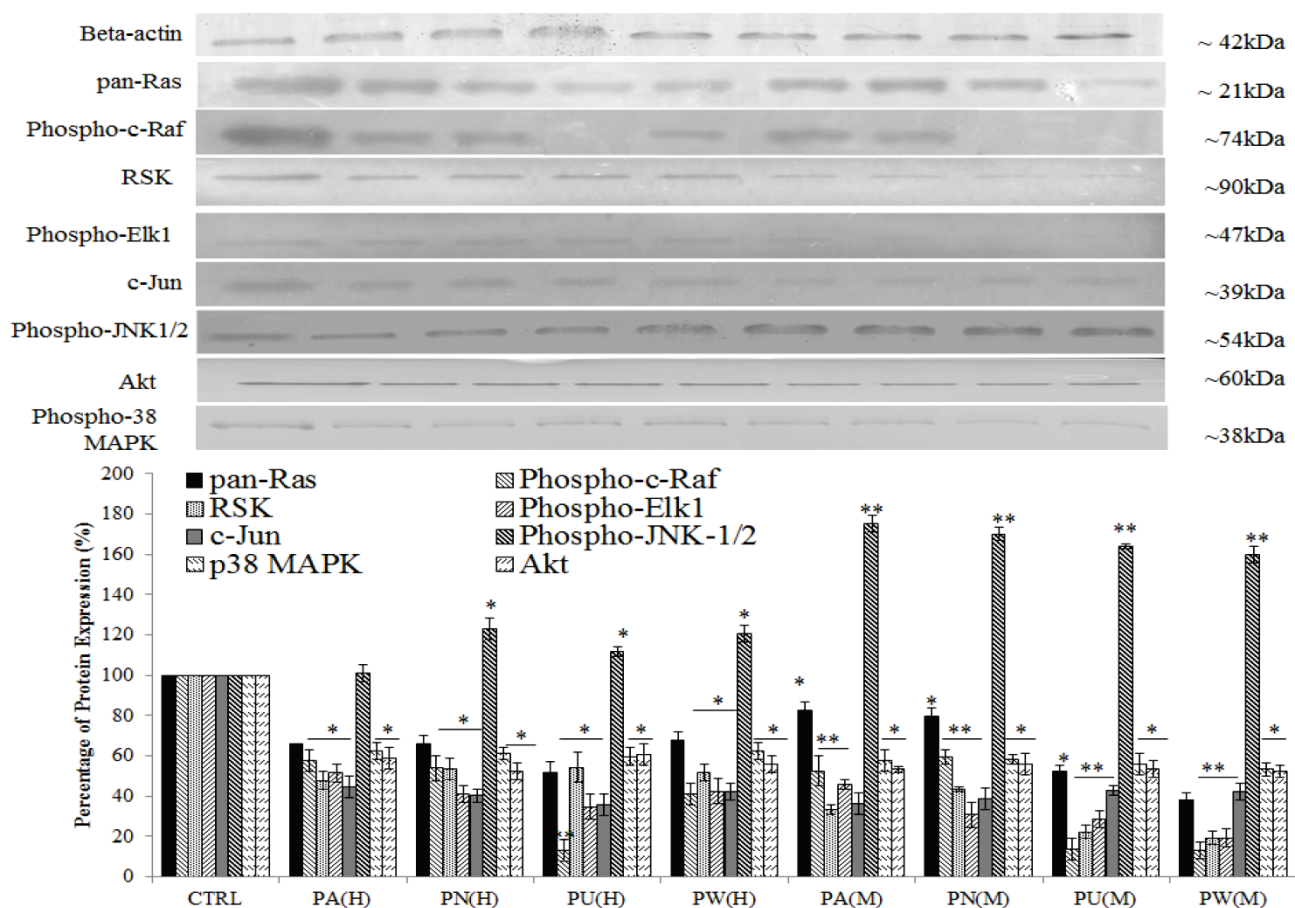


Figure 3. Inhibition of MAPK/ERK and p38 MAPK but activation of MAPK/JNK pathways in MeWo cells. The expressions of Ras, Raf, phospho-Elk1, RSK, phospho-JNK-1/2, c-Jun, phospho-p38 MAPK and Akt in treated MeWo were significantly down-regulated. Bars show the mean percentage \pm SEM. * $P < 0.05$ and ** $P < 0.01$ vs control.

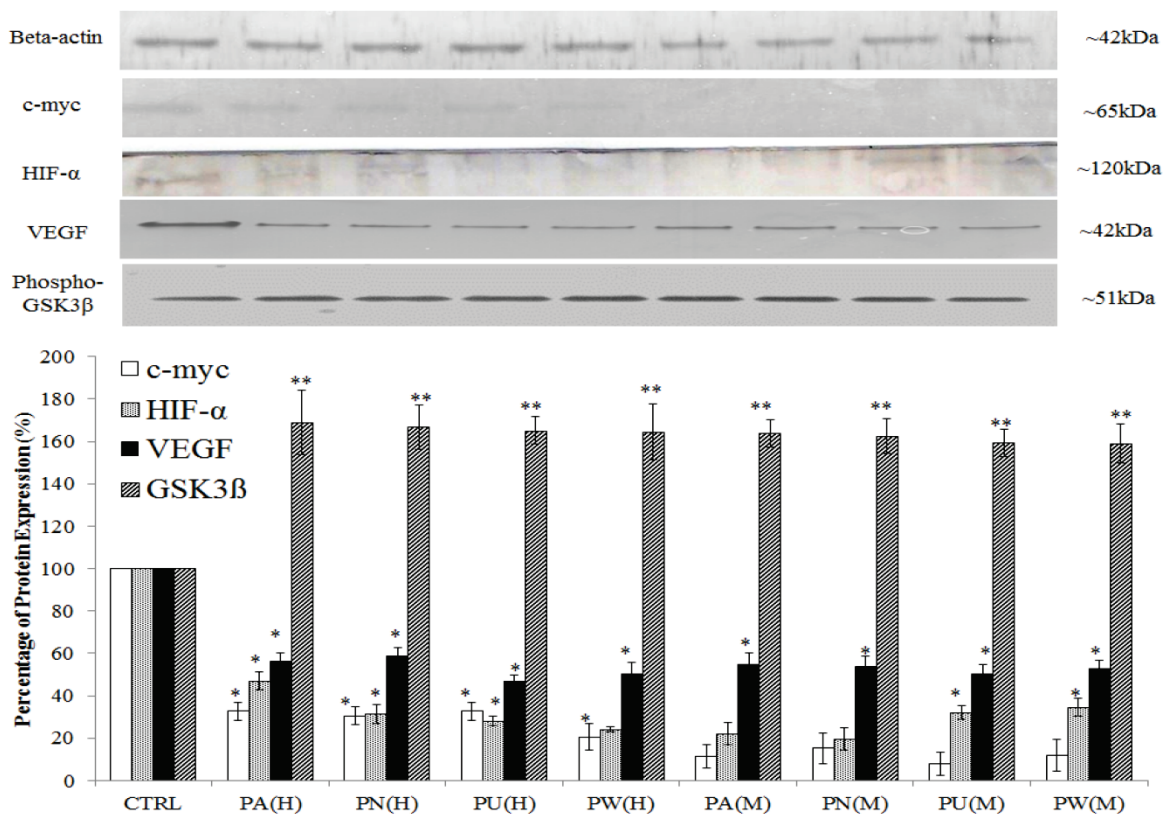


Figure 4. *Phyllanthus* alters expression in c-Myc and Hypoxia pathways of MeWo cells. The expressions of c-Myc, HIF-1 α and VEGF were significantly down-regulated in conjunction with up-regulation of phospho-GSK3 β . Bars show the mean percentage \pm SEM. *P<0.05 and **P<0.01 vs control.

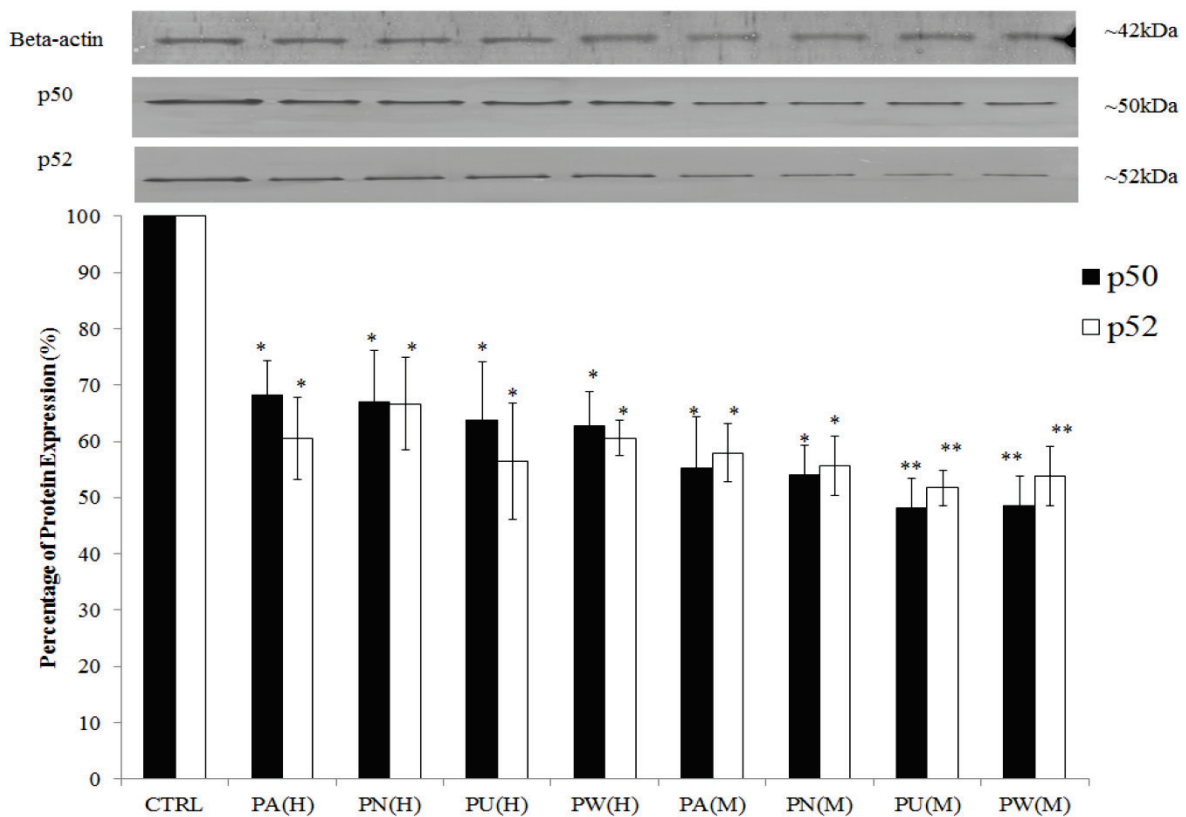


Figure 5. Inhibition of NF κ B pathway in MeWo cells by *Phyllanthus* extracts. The expressions of p50 and p52 proteins were significantly down-regulated. Bars show the mean percentage \pm SEM. *P<0.05 and **P<0.01 vs control.

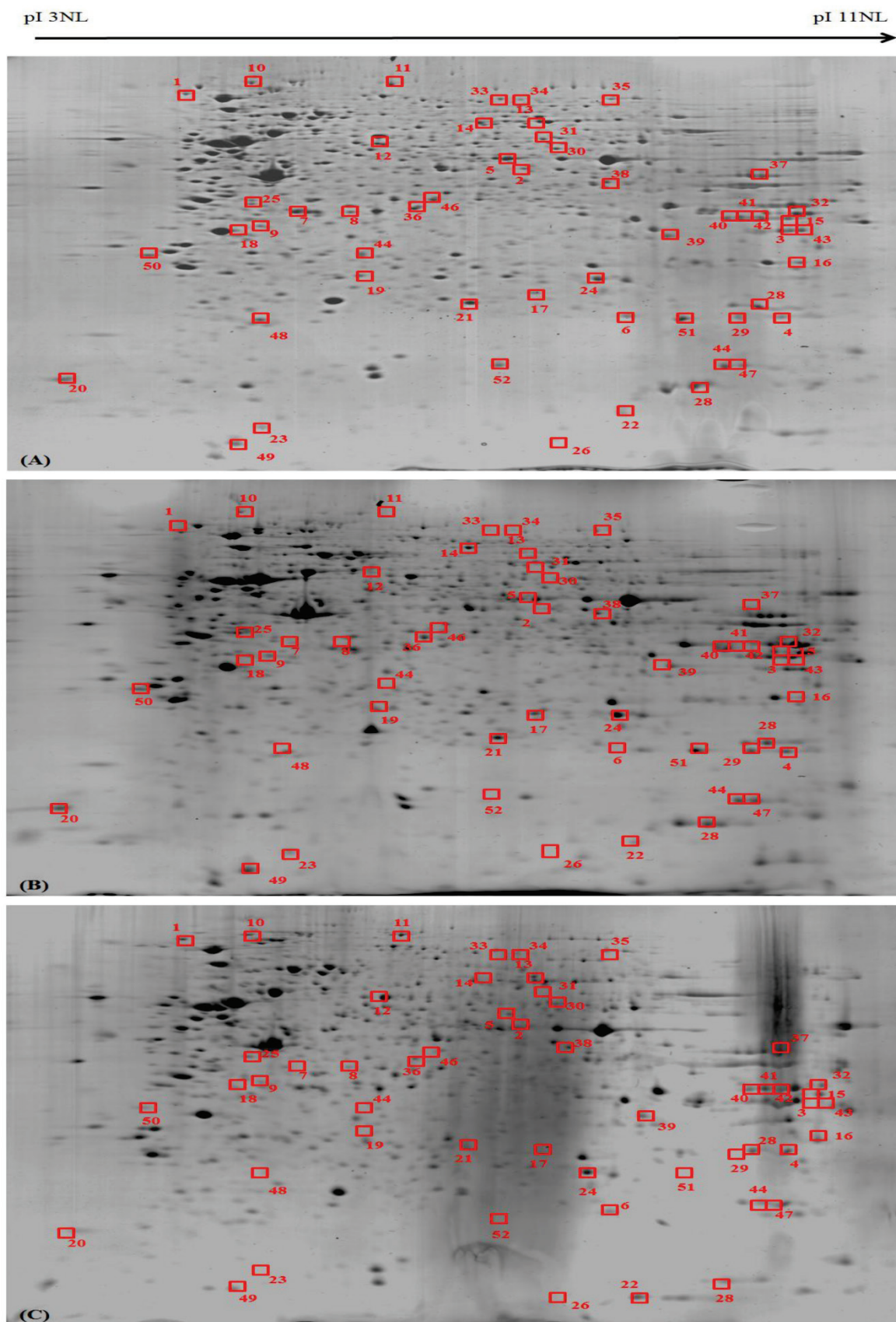


Figure 6. Proteomic profiling of the differentially expressed proteins in *Phyllanthus* extracts-treated MeWo cells. The differentially expressed proteins in (A) untreated, (B) aqueous- and (C) methanolic-treated of MeWo cells.

Table 2: Fold changes of differential expressed proteins in aqueous- and methanolic-treated MeWo cells. Symbols“+” indicate up-regulation, and “-” indicate down-regulation. PA: *P.amarus*; PN: *P.niruri*; PU: *P.urinaria* and PW: *P.watsonii*.

No	UniProtKB/Swiss-Prot (Accession number)	Protein	<i>Phyllanthus</i>							
			PA(H)	PN(H)	PU(H)	PW(H)	PA(M)	PN(M)	PU(M)	PW(M)
I Cell Adhesion, Migration, Invasion and Metastasis and Angiogenesis										
1	P98172	Ephrin-B1	-1.46	-1.47	-1.53	-1.48	-1.59	-1.58	-1.57	-1.65
2	P61163	Alpha-centractin	-1.42	-1.33	-1.57	-1.45	-1.58	-1.75	-1.58	-1.74
3	Q13895	Bystin	-1.43	-1.57	-1.34	-1.45	-1.46	-1.59	-1.48	-1.65
4	P13646	Keratin, type I cytoskeletal 13	1.42	1.47	1.46	1.57	1.74	1.58	1.57	1.64
5	P08670	Vimentin	-1.73	-1.92	-1.72	-2.01	-1.85	-1.73	-1.72	-1.92
6	P04179	Superoxide dismutase [Mn], mitochondrial	-1.29	-1.54	-1.57	-1.39	-1.58	-1.85	-1.65	-1.48
7	P07437	Tubulin beta chain	-1.42	-1.48	-1.67	-1.97	-1.57	-1.58	-1.59	-1.54
8	P47755	F-actin-capping protein subunit alpha-2	-1.34	-1.33	-1.63	-1.58	-1.68	-1.58	-1.53	-1.63
9	Q8TEW0	Partitioning defective 3 homolog	-1.32	-1.49	-1.32	-1.68	-1.50	-1.56	-1.68	-1.58
II Proliferation, Cell Cycle, and Apoptosis										
10	P68400	Casein kinase II subunit alpha	-2.28	-1.97	-1.83	-2.08	-2.18	-2.08	-1.95	-2.07
11	P50583	Bis(5'-nucleosyl)-tetrphosphatase [asymmetrical]	-1.47	-1.53	-1.48	-1.37	-1.58	-1.74	-1.63	-1.75
12	O60220	Mitochondrial import inner membrane translocase subunit Tim8	-1.56	-1.35	-1.64	-1.62	-1.63	-1.65	-1.68	-1.58
13	Q9BQ83	Structure-specific endonuclease subunit SLX1	-1.37	-1.49	-1.57	-1.48	-1.93	-1.57	-1.74	-1.63
14	Q6Q0C0	E3 ubiquitin-protein ligase TRAF7	1.48	1.57	1.65	1.57	1.45	1.78	-1.69	-1.549
15	P0CJ76	Humanin-like protein 9	-1.56	-1.68	-1.58	-1.58	-1.62	1.67	1.73	1.78
16	P17931	Galectin-3	-1.58	-1.75	-1.63	-1.69	-1.68	-1.68	-1.64	-1.78
17	P31943	Heterogeneous nuclear ribonucleoprotein H	-1.49	-1.57	-1.34	-1.63	-1.56	-1.68	-1.58	-1.84
18	P08758	Annexin A5	-1.42	-1.57	-1.73	-1.57	-1.57	-1.47	-1.59	-1.47
19	Q969E4	Transcription elongation factor A protein-like 3	-1.32	-1.47	-1.57	-1.74	-1.62	-1.48	-1.68	-1.57
20	Q9H930	Nuclear body protein SP140-like protein	-1.32	-1.38	-1.55	-1.57	-1.58	-1.58	-1.57	-1.58
21	P27348	14-3-3 protein theta	-1.42	-1.47	-1.29	-1.58	-1.57	-1.67	-1.45	-1.34
22	P62937	Peptidyl-prolyl cis-trans isomerase A	-1.54	-1.23	-1.47	-1.54	-1.58	-1.62	-1.57	-1.46
23	Q14929	Zinc finger protein 169	-1.69	-1.57	-1.33	-1.84	-1.75	-1.55	-1.47	-1.56
24	O75791	GRB2-related adapter protein	-1.59	-1.43	-1.4	-1.83	-1.56	-1.48	-1.53	-1.28
25	O00165	HCLS1-associated protein X-1	-1.69	-1.72	-1.97	-1.66	-1.84	-1.86	-1.67	-1.78
26	O95243	Methyl-CpG-binding domain protein 4	-1.49	-1.63	-1.56	-1.57	-1.66	-1.54	-1.63	-1.64
27	P78417	Glutathione transferase omega - 1	-1.45	-1.53	-1.46	-1.57	-1.64	-1.53	-1.48	-1.58
28	Q06830	Peroxiredoxin-1	-1.45	-1.42	-1.46	-1.47	-1.64	-1.74	-1.53	-1.48
29	P50453	Serpin B9	-1.32	-1.31	-1.53	-1.56	-1.57	-1.45	-1.55	-1.63
30	Q8ND25	E3 ubiquitin-protein ligase ZNRF1	-1.48	-1.64	-1.67	-1.57	-1.56	-1.46	-1.53	-1.58
31	Q06210	E3 ubiquitin-protein ligase ARIH2	-1.48	-1.58	-1.64	-1.6	-1.57	-1.65	-1.45	-1.64
32	Q9Y4L5	E3 ubiquitin-protein ligase RNF115	-1.56	-1.43	-1.66	-1.73	-1.69	-1.58	-1.58	-1.56
III Glycogenesis and glycolysis										
33	Q969E3	Glucosamine--fructose-6-phosphate aminotransferase [isomerizing] 2	-1.57	-1.58	-1.58	-1.53	-1.65	-1.73	1.67	-1.48
34	P00558	Protein phosphatase 1 regulatory subunit 3D	-1.33	-1.58	-1.53	-1.64	-1.56	-1.37	-1.57	-1.74
35	P06733	Alpha-enolase	-1.42	-1.57	-1.74	-1.73	-1.58	-1.57	-1.65	-1.69
36	P04406	Pyruvate kinase isozymes M1/M2	-1.45	-1.67	-1.47	-1.63	-1.78	-1.36	-1.36	-1.75
37	P04075	Phosphoglycerate kinase 1	-1.57	-1.58	-1.61	-1.64	-1.48	-1.53	-1.52	-1.58
IV Protein Synthesis and Energy Metabolism										
38	P17174	Aspartate aminotransferase, cytoplasmic	-1.26	-1.67	-1.68	-1.56	-1.57	-1.58	-1.53	-1.58
39	Q9UBC9	Small proline-rich protein 3	-1.63	-1.75	-1.74	-1.56	-1.67	-1.68	-1.73	-1.57
40	P41247	Patatin-like phospholipase domain-containing protein 4	-1.63	-1.32	-1.49	-1.66	-1.53	-1.53	-1.58	-1.64
41	Q9HBU6	Ethanolamine kinase 1	-1.39	-1.75	-1.57	-1.83	-1.48	-1.75	-1.68	-1.74
42	P62195	26S protease regulatory subunit 8	-1.53	-1.64	-1.57	-1.74	-1.75	-1.56	-1.64	-1.54
43	Q5VTE0	Putative elongation factor	-1.48	-1.64	-1.64	-1.57	-1.57	-1.64	-1.58	-1.85

No	UniProtKB/Swiss-Prot (Accession number)	Protein	<i>Phyllanthus</i>							
			PA(H)	PN(H)	PU(H)	PW(H)	PA(M)	PN(M)	PU(M)	PW(M)
		1-alpha-like 3								
44	Q5TGZ0	Mitochondrial inner membrane organizing system protein 1	-1.53	-1.63	-1.74	-1.56	-1.67	-1.68	-1.64	-1.75
45	P07339	Cathepsin D	-1.54	-1.54	-1.65	-1.46	-1.68	-1.68	-1.47	-1.58
46	Q9Y587	AP-4 complex subunit sigma-1	-1.33	-1.57	-1.56	-1.36	-1.68	-1.74	-1.58	-1.67
47	P00568	Adenylate kinase	-1.58	-1.39	-1.53	-1.57	-1.56	-1.63	-1.68	-1.58
48	P80294	Metallothionein-1H	-1.29	-1.74	-1.73	-1.45	-1.59	-1.58	-1.76	-1.68
49	Q76KX8	Zinc finger protein 534	-1.59	-1.46	-1.57	-1.64	-1.54	-1.63	-1.58	-1.58
50	Q9Y6H3	Mitochondrial inner membrane protease ATP23 homolog	-1.59	-1.56	-1.57	-1.67	-1.68	-1.67	-1.68	-1.63
51	Q8WVM8	Sec1 family domain-containing protein 1	-1.44	-1.56	-1.64	-1.54	-1.76	-1.65	-1.75	-1.64
52	Q9NVH6	Trimethyllysine dioxygenase, mitochondrial	-1.58	-1.48	-1.58	-1.54	-1.79	-1.68	-1.53	-1.66

Discussion

Understanding cell signalling in cancer is of paramount importance and may provide potential targets for anticancer agents to induce apoptosis and/or inhibitions tumour metastasis and angiogenesis. Previously, we showed that *Phyllanthus* was capable of exhibiting selective cytotoxicity on melanoma cancer cells and apoptosis induction [19], was most likely attributed to the mixture bioactive compounds within the plant.

Bcl-2-family proteins play an important role in cell death regulation including apoptosis, necrosis and autophagy [26]. Overexpression of the anti-apoptotic proteins (e.g. Bcl-2) and suppression of pro-apoptotic proteins (e.g. Bax) have been demonstrated to inhibit cell death leading to formation of cancer [26]. In our study, the expression of Bax proteins was greatly up-regulated accompanied by down-regulation of Bcl-2 expression in *Phyllanthus*-treated MeWo cells (Figure 2). The highly expressed Bax protein could induce changes in mitochondrial outer membrane permeabilization (MOMP), causing the release of cytochrome *c*. This release will then induce caspase activation [27, 28] as detected in our previous report [19] and also inhibit the inhibitors of apoptosis (IAP) (e.g. SMAC and OMI/Htra2) [28, 29]. Finally, this will leads to apoptosis induction in MeWo cells. In addition, another apoptotic protein, p53 was also found to be up-regulated in treated MeWo cells which could induce apoptosis through its transcription activity to produce pro-apoptotic proteins (e.g. Bid, Bax, Nova and PUMA) which in turn leads to cytochrome *c* release and activation of caspases [30, 31].

Ras gene is frequently found high expressed in human cancers [32] whereby it triggers various other intracellular signalling cascades to regulate genes-driven malignancy of cancer including prolif-

eration, evasion of apoptosis, metastasis and angiogenesis [33]. When MeWo cells were treated with *Phyllanthus*, a down-regulation of Ras proteins was observed and this will eventually lead to suppression of its downstream targets; Raf and Akt as observed (Figure 3).

In melanoma, active ERK1/2 molecule has been shown to activate RSK and Elk molecules; subsequently activating c-Jun and c-Fos proteins. The combination of c-Jun and c-Fos will form activator protein 1 (AP-1), a transcription factor that translocates into nucleus and regulates cell survival genes [33]. In addition, p38 MAPK also enhances AP-1 formation by producing c-Fos through activation on Elk protein [5]. However, in *Phyllanthus*-treated MeWo cells, all the above-mentioned MAPK intracellular signalling molecules were noted to be down-regulated (Figure 3).

Tumour necrosis factor-alpha (TNF- α) is a cytokine capable of activating multiple downstream signalling pathways, including caspases, I κ B kinase (IKK) and JNK [34, 35]. TNF- α does not usually induce apoptosis unless NF κ B has been inactivated [35]. During TNF- α induced apoptosis, caspases will be activated, and IKK will be inactivated causing NF κ B to bind with its inhibitor; subsequently inhibiting the ability to regulate genes including inhibitors of apoptosis (IAPs) family.

The inhibition of NF κ B was observed in *Phyllanthus*-treated MeWo cells by measurements of NF κ B-1 (p50) and NF κ B -2 (p52). Furthermore, we believed that the down-regulation of NF κ B pathway in treated MeWo cells can inhibit proliferation, metastasis and angiogenesis by suppressing the MMP, VEGF, and IL-8, and anti-apoptotic proteins (bcl-xl, cIAP) as well as inducing programmed cell death in MeWo cells [37, 42].

The PI3K/Akt pathway is found highly expressed in advanced melanoma and inhibits apoptosis

[43-45]. However, the suppression of Akt protein in treated MeWo could induce apoptosis by the activation pro-apoptotic factors (Bad, GSK3 β , procaspase-9 and TRAIL/APO-2L (TNF-Related Apoptosis-Inducing Ligand) [46, 47] and inhibition of anti-apoptotic factors (cyclic AMP response element-binding protein (CREB) and the I κ B kinase (IKK) [47]. The up-regulation of phosphorylated GSK3 β was detected in treated MeWo cells can further degrade c-myc as was observed; possibly halting tumour growth with induction of S-phase arrest as we report previously [19]. In addition, the cell cycle arrest at S-phase could be due to the activation of p53 as p21 is a major target for transactivation by p53 [51] which will then induce cell cycle arrest at S-phase through two mechanisms; (1) interaction of p21 with the proliferating cell nuclear antigen (PCNA) [52] and/or (2) p21 directly inhibiting the activity of cyclin E/CDK2 complexes [53].

According to Warburg effect, the microenvironment surrounding a solid tumour is an anaerobic/hypoxia state. This will lead cancer cells to up-regulate the expression of intracellular signalling molecules and enzymes that involved anaerobic glycolytic pathway [54-56]. Hypoxia-inducible factor (HIF) is an important protein activated upon hypoxic condition or low oxygen level [13]. The target genes of HIF are involved in angiogenesis (e.e VEGF), cell survival (e.g. IGF-1) and metastasis (e.g. LOX, PAI-1) and all these process can drive tumour progression [14]. In our study, *Phyllanthus* extracts were noted to inhibit the glycolytic pathway and ATP production in melanoma cells by down-regulating the HIF-1 α protein and vascular endothelial growth factor (VEGF) (Figure 5). In addition, several of glycolytic enzymes were found to be down-regulated in MeWo cells after treatment with *Phyllanthus* extracts such as glucosamine-fructose-6-phosphate aminotransferase, protein phosphatase 1 regulatory subunit 3D, alpha-enolase, pyruvate kinase isozymes M1/M2 and phosphoglycerate kinase 1. The down-regulation in PI3K/Akt and Ras/MAPK pathways in treated MeWo cells are believed to be involved in the inhibition of hypoxia pathway [14].

The epithelial-mesenchymal transition (EMT) is a cellular process that allows immotile epithelial cells to become motile mesenchymal cells, promoting carcinoma invasion and metastasis as well as resistance to apoptosis [57]. Several signalling networks including Ras/MAPK-, PI3K/Akt-, Wnt- and NF κ B-dependent pathways in EMT were found to be down regulated in treated MeWo cells, and thus were believed to inhibit metastasis of melanoma [58]. The inhibition on melanoma metastasis was further imposed with the up-regulation of type I cytoskeletal 13

keratin and concurrent down-regulation of vimentin in MeWo cells after treatment with *Phyllanthus*. Despite the exact mechanism of up-regulation of keratin during EMT being unclear but its down-regulation in treated cells can restrict the motility of cells, thus halting metastasis [59, 60]. Vimentin also plays an important role in cell survival by stabilizing the ERK protein and governing its translocation into the nucleus [61] and by preventing assembly of Raf-14-3-3 complex enabling Ras protein to be continuously expressed [62]. Therefore, the down regulation of vimentin could induce apoptosis via suppression on ERK- and Ras-dependent survival pathways in MeWo cells.

Following the treatment of *Phyllanthus*, many tightly regulated proteins were found to be altered in MeWo cells. Of these, two proteins, HCLS1-associated protein X-1 (HAX1) and casein kinase II subunit alpha. HAX-1 interacts with HS1 and α -subunit of G α 13 heterotrimeric G protein for cell migration [63, 64] and is highly expressed in hypoxic tumor progression [65, 66]. Protein kinase casein kinase II (CK2) is a highly conserved protein serine/threonine kinase and is found highly expressed in cancers [67-70]. CK2 can be anti-apoptotic via (1) inhibition on Max, a transcriptional partner of the c-myc, from caspase-mediated degradation [71], (2) activation of Bid, a pro-apoptotic protein [72], and (3) confer protection from Fas- and drug-triggered apoptosis [72, 73]. Therefore, down-regulation of CK2 in treated MeWo cells could elicit apoptosis induction and could increase the susceptibility of MeWo cells to apoptotic inducer chemotherapeutic agents [74-76].

Mitochondrion is involved in protein synthesis and energy metabolism for cancer cell survival, transformation, invasion, and metastasis. The alteration in mitochondrial integrity and ubiquitination components such as E3 ubiquitin-protein ligase ARIH2 and RNF115, aspartate aminotransferase, 26S protease regulatory subunit 8, putative elongation factor 1-alpha-like 3, mitochondrial inner membrane organizing system protein 1 (MINOS), zinc finger protein, Sec1 and trimethyllysine dioxygenase, by *Phyllanthus* in MeWo cells could inhibit ATP production and stability of proteins, therefore halting cancer growth. In addition, disruption on mitochondria stability could elicit apoptosis in MeWo by releasing its components, cytochrome c [36].

Based on the findings, we proposed a schematic presentation of the possible mechanism behind the anti-cancer activity of *Phyllanthus* extracts in MeWo cells (Figure 7). In conclusion, this study revealed a comprehensive perspective of the possible mechanism behind the anti-cancer activity of *Phyllanthus* extracts by inspection of their regulation in multiple

signalling pathways and protein-protein interaction in melanoma MeWo cells. In addition, the identified

differentially expressed proteins could become potential targets for new anticancer agent development.

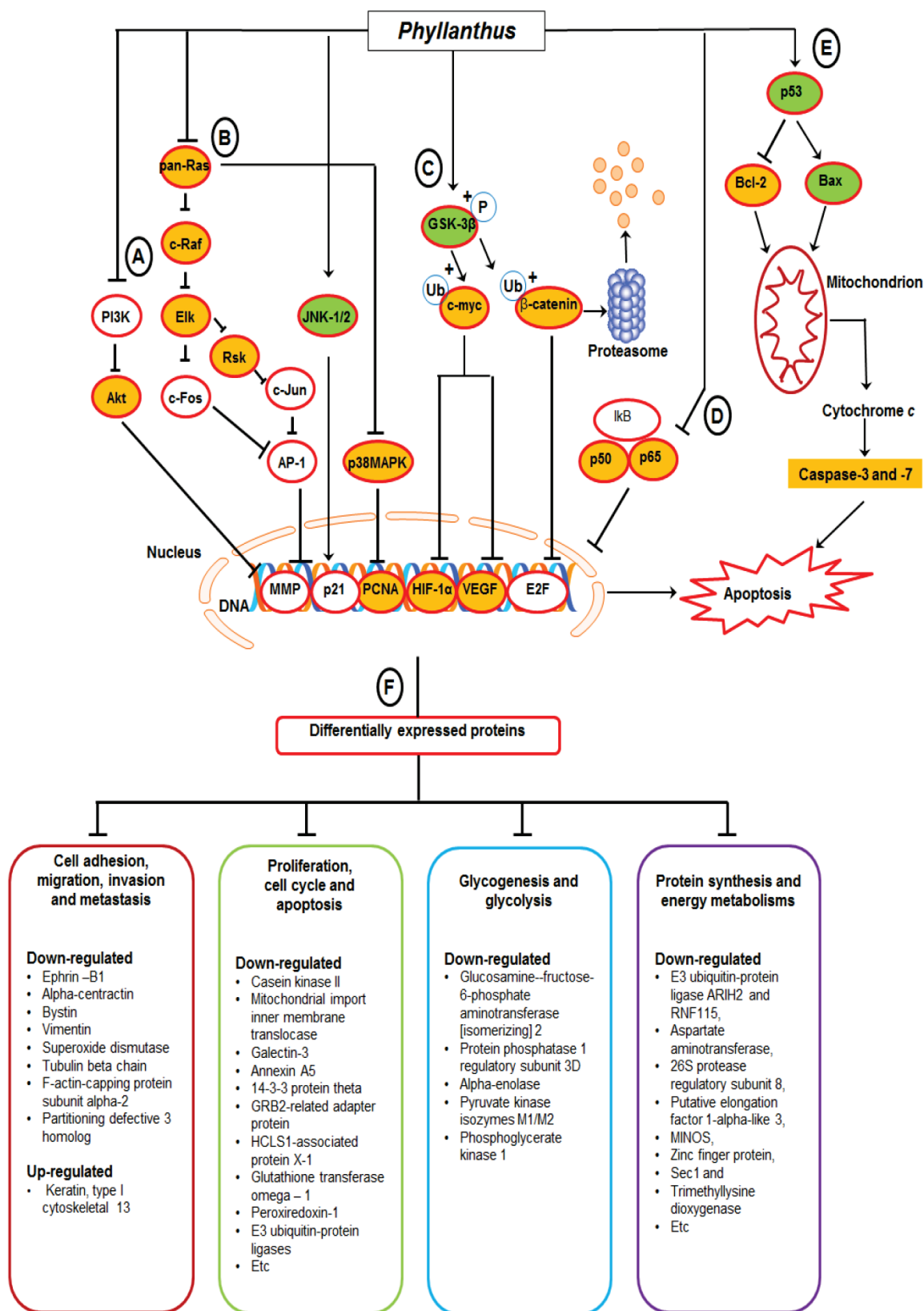


Figure 7. A proposed schematic diagram of (A) PI3K/Akt, (B) MAPKs, (C) Myc/Max and Hypoxia, (D) NFκB, and (E) p53 apoptosis signalling pathways as well as (F) multiple proteins regulations in *Phyllanthus*-treated MeWo cells. See the text for discussion.

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Abbreviations

NF κ B, Nuclear Factor-kappa B; MAPK, Mitogen-Activated Protein Kinases; ERK, Extracellular signal-regulated kinases; JNK, c-Jun N-terminal kinase; RSK, Ribosomal s6 kinase; Elk1, E twenty-six (ETS)-like transcription factor 1; HIF-1 α , Hypoxia-inducible factor 1alpha; VEGF, vascular endothelial growth factor; GSK3 β , Glycogen synthase kinase-3 beta; MMP, matrix metalloproteinase; PI3K, Phosphatidylinositol 3-kinase.

Competing Interests

The authors have declared that no competing interest exists.

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