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Original Article

Suppression of ERK1/2 and hypoxia pathways by four *Phyllanthus* species inhibits metastasis of human breast cancer cells

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ABSTRACT

Chemotherapies remain far from ideal due to drug resistance; therefore, novel chemotherapeutic agents with higher effectiveness are crucial. The extracts of four *Phyllanthus* species, namely *Phyllanthus niruri, Phyllanthus urinaria, Phyllanthus watsonii,* and *Phyllanthus amarus*, were shown to induce apoptosis and inhibit metastasis of breast carcinoma cells (MCF-7). The main objective of this study was to determine the pathways utilized by these four *Phyllanthus* species to exert anti-metastatic activities. A cancer 10-pathway reporter was used to investigate the pathways affected by the four *Phyllanthus* species. Results indicated that these *Phyllanthus* species suppressed breast carcinoma metastasis and proliferation by suppressing matrix metalloprotein 2 and 9 expression via inhibition of the extracellular signal-related kinase (ERK) pathway. Additionally, inhibition of hypoxia-inducible factor 1-α in the hypoxia pathway caused reduced vascular endothelial growth factor and inducible nitric oxide synthase expression, resulting in anti-angiogenic effects and eventually anti-metastasis. Two-dimensional gel electrophoresis identified numerous proteins suppressed by these *Phyllanthus* species, including invasion proteins, anti-apoptotic protein, protein-synthesis proteins, angiogenic and mobility proteins, and various glycolytic enzymes. Our results indicated that ERK and hypoxia pathways are the most likely targets of the four *Phyllanthus* species for the inhibition of MCF-7 metastasis.

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1. Introduction

Breast cancer is the most commonly diagnosed malignancy in women of all ages, with >1 million cancer incidences each year [1–3]. Early-stage breast cancers become life threatening when they begin to metastasize [4], resulting in >400,000 deaths every year and constituting the leading cause of cancer-related deaths in women [3]. Current chemotherapies are still far from ideal due to drug-resistance development following prolonged exposure and leading to a more aggressive metastatic phenotype [3,5]. Therefore, the search for novel chemotherapeutic agents with higher effectiveness surpassing drug resistance and metastasis is crucial to improve the survival of patients with advanced or recurrent breast cancer. For this reason, natural-product-derived drugs have increased in popularity as preventive medication or for health management [6]. The genus *Phyllanthus* is commonly found throughout the tropical and subtropical regions and having various reported therapeutic properties [7–10]. Interestingly, the four species of *Phyllanthus* (*Phyllanthus niruri, Phyllanthus urinaria, Phyllanthus watsonii,* and *Phyllanthus amarus*) exhibit anti-metastatic and anti-proliferative activities against cancer cell lines, including lung (A549) and breast (MCF-7) carcinomas [7].

However, the exact mechanisms that confer the anticancer activities of the four *Phyllanthus* species remains uncertain. Cell transformation is normally due to the activation of signaling pathways through protein kinases that constitute a large family of >500 regulatory enzymes [2]. The mitogen-activated protein kinase (MAPK) family consists of three main subgroups, including extracellular signal-related kinase 1/2 (ERK1/2), Jun amino-terminal kinase (JNK), and p38 MAPK. These pathways respond to various extracellular stimuli to regulate cell survival and proliferation, differentiation, cell death, as well as metastasis [1,3]. Regulation of metastasis by MAPK occurs mainly through control of cell expression of matric metalloproteins (MMPs) or urinary-type plasminogen activator, which are capable of degrading the extracellular matrix to allow access to the vasculature [11,12]. Additionally, hypoxia-inducible factor-1 (HIF-1) is specifically activated and becomes abundant during hypoxia due to the stable accumulation of HIF-1α, which is usually overexpressed in most solid tumors, including those associated with breast cancer [4]. Elevation of HIF-1α expression is intimately linked with the upregulation of over 70 target genes, including vascular endothelial growth factor (VEGF) and nitric oxide synthase (NOS), that favor tumor growth and metastasis [4,13], as well as genes that regulate major cellular processes, including energy production, proliferation, vascular development, and remodeling, during angiogenesis [14,15].

P. niruri, P. urinaria, P. watsonii, and *P. amarus* inhibit MCF-7 (breast carcinoma) cell growth [7]. Additionally, extracts reduced invasion, migration, and adhesion of MCF-7 cells in a dose-dependent manner and were shown to induce apoptosis in conjunction with its anti-metastatic activity [7]. Therefore, this study aimed to determine the signaling pathways activated by extracts from the four *Phyllanthus* species to exert their anti-proliferative and anti-metastatic effects in breast cancer MCF-7 cells. In order to investigate this, we screened and investigated protein-expression profiles in MCF-7 following extract treatment.

2. Materials and methods

2.1. *Cell line and* Phyllanthus *species extracts*

Human breast carcinoma cell line MCF-7 was obtained from American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Waltham, MA, USA) and kept in a humidified atmosphere with 5% CO_2 at 37°C. Whole-plant crude extracts [aqueous (Aq) and methanolic (MeOH)] from each *Phyllanthus* species, namely *P. niruri* (PN), *P. urinaria* (PU), *P. watsonii* (PW), and *P. amarus* (PA), were obtained from Malaysian Agricultural Research and Development Institute, Malaysia. A single batch of the Aq (10 mg/mL in phosphate-buffered saline) and MeOH *Phyllanthus* species extracts (40 mg/mL in dimethyl sulfoxide) for the four species were stored at −20°C until use. The bioactive components present in the extracts were described previously [7] and are shown in Table 1. Generally, Aq extracts of the four *Phyllanthus* species contained higher concentrations of bioactive components as compared with the MeOH extracts, consistent with another study carried out by Lou et al [16]. Here, IC_{50} concentrations for the individual extracts (Aq PN: 179.7 μg/mL; Aq PU: 139.3 μg/mL; Aq PW: 104 μg/mL; Aq PA: 156.7 μg/mL; MeOH PN: 62.3 μg/mL; MeOH PU: 48.7 μg/mL; MeOH PW: 49 μg/mL; MeOH PA: 56.3 μg/mL) were used as treatment conditions in MCF-7 cells that showed minimal effects on a normal breast epithelial cell line (184B5).

Table 1 – Bioactive components detected in each *Phyllanthus* species.

Bioactive components	*Phyllanthus* species
Aqueous extracts	
Gallic acid	*P. niruri, P. urinaria, P. watsonii, P. amarus*
Galloylglucopyranoside	*P. niruri, P. urinaria, P. watsonii, P. amarus*
Corilagen	*P. niruri, P. urinaria, P. watsonii, P. amarus*
Geraniin	*P. niruri, P. urinaria, P. watsonii, P. amarus*
Rutin	*P. niruri, P. urinaria, P. watsonii, P. amarus*
Quercetin glucoside	*P. niruri, P. urinaria, P. watsonii, P. amarus*
Caffeolquinic acid	*P. niruri, P. urinaria, P. watsonii, P. amarus*
Digalloylglucopyranoside	*P. niruri, P. watsonii, P. amarus*
Quercetin rhamnoside	*P. urinaria, P. watsonii*
Trigalloylglucopyranoside	*P. urinaria*
Methanolic extracts	
Geraniin	*P. niruri, P. urinaria, P. watsonii, P. amarus*
Quercetin diglucoside	*P. niruri*
Trigalloylglucopyranoside	*P. urinaria*
Tetragalloylglucopyranoside	*P. urinaria*

2.2. Transient transfection and cancer 10-pathway-reporter array

Analysis of various key signaling pathways implicated in human tumorigenesis was performed using Cignal Finder Cancer 10-pathway Reporter Array kit (SABiosciences: Qiagen, Hilden, Germany). Cellular transfection was performed with TransIT-LT1 (MirusBio, Madison, WI, USA). Plasmid DNA for each signaling pathway provided in the kit (Cat. #: CCA-001L; Invitrogen, Carlsbad, CA, USA) was mixed with TransIT-LT1 to form TransIT-LT1/DNA complexes before being added to the cell suspension and incubated at 37°C overnight. The transfected cells were then incubated with extracts of the four *Phyllanthus* species for another 24 h. Dual-Glo luciferase reagent was added to each well and incubated at room temperature for 10 min to obtain firefly luminescence readings using the GloMax Multi-detection System (Promega, Durham, NC, USA). This was followed by addition of Dual-Glo Stop & Glo reagent to obtain renilla-luminescence readings. Firefly constructs visualized modulation of key transcription-factor activity, usually representing a downstream target of a particular signaling pathway. The renilla construct functions as an internal control to normalize transfection efficiencies and to monitor cell viability. Luminescence for each sample was calculated based on the firefly-to-renilla luminescence ratio.

2.3. Cell-cycle analysis

Cell-cycle analysis was performed according to a previously published method [17]. Data acquisition was performed with a Becton Dickinson FACSCalibur flow cytometer and CellQuest software (BD, Franklin Lakes, NJ, USA). Subsequent analysis was performed using WinMDI 2.9 software (http://www.cyto.purdue.edu/flowcyt/software/Winmdi.htm).

2.4. Western blot

Protein lysates were prepared for the four *Phyllanthus* species-treated and -untreated samples. Protein concentrations were determined using a 2D Quant kit from GE Healthcare (Little Chalfont, UK). Briefly, 150 μg of each sample was either resolved by 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) or by 2D-gel electrophoresis with 7-cm immobilized pH gradient (IPG) gel strips (pH 3–11 NL; GE Healthcare). The proteins were transferred onto a membrane at 250 mA for 1 h, blocked, and incubated with various primary and secondary antibodies. The target proteins were detected using a colorimetric-based 3,3′-diaminobenzidine substrate. Anti-pan-Ras, anti-Bcl-2, anti-HIF-1α, anti-c-Raf, anti-c-Myc, anti-p53, anti-Elk1, anti-c-Jun/activator protein (AP)-1, anti-JNK1/2, anti-VEGF, goat anti-mouse, and goat anti-rabbit IgG peroxidase-treated antibodies were obtained from Merck Millipore (Darmstadt, Germany), while the anti-RSK antibody was obtained from Thermo Fischer Scientific. These proteins play a crucial role in the pathways affected by the four *Phyllanthus* species as confirmed using the pathway-reporter array.

2.5. Zymography assay

This assay was performed according to a method described previously [18] to determine the presence of MMPs, one of the major proteolytic enzymes implicated in breast-cancer metastasis due to their ability to hydrolyze the extracellular matrix [19].

2.6. Human total inducible NOS (iNOS) and glyceraldehyde 3-phosphate dehydrogenase (GADPH) immunoassay

Total iNOS in the four *Phyllanthus* species-treated cells was measured using a human total-iNOS immunoassay enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA) according to manufacturer instructions. The GAPDH antibody supplied in this kit detects the housekeeping GAPDH protein for normalization.

2.7. Two-dimensional gel electrophoresis

Proteins (500 μg) for both treated and untreated MCF-7 samples were rehydrated on 13-cm IPG gel strips (pH 3–11 NL; GE Healthcare), and isoelectric focusing was performed with an Ettan IPGphor Isoelectric Focusing unit (GE Healthcare). After a two-step sequential equilibration with 2% (w/v) dithiothreitol and 2.5% (w/v) iodoacetamide, the proteins were resolved by 12.5% SDS-PAGE using an Ettan Dalt*twelve* Separation Unit (GE Healthcare). The Coomassie-stained gels were then imaged using an Ettan DIGE Imager (GE Healthcare). Experiments were performed in triplicate for each treatment group ($n = 3$), and analysis was performed using PDQuest 2D Analysis Software (Bio-Rad). Protein spots indicating >2-fold differential expression were considered significant ($p < 0.05$) and were excised for mass spectrometry analysis.

2.8. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF/TOF) mass spectrometry and database search

Excised protein spots were digested with 6 ng/μL trypsin and subjected to MALDI-TOF/TOF analysis using ABSCIEX 4800 MALDI-TOF/TOF (Ab Sciex, Framingham, MA, USA). Data collected were analyzed against the SwissProt database (http://web.expasy.org/docs/swiss-prot_guideline.html) using the MASCOT search algorithm (v2.1.0; Matrix Science, London, UK) with typical search parameters. Protein scores >55 were deemed significant ($p < 0.05$). The protein with the highest number of peptides was considered to correspond to the spot if multiple proteins were identified in a single spot. The proteins identified were then compared against the Uniprot KB/Swiss-Prot database (http://web.expasy.org/docs/swiss-prot_guideline.html) and grouped based on the Eukaryotic Orthologous Group of Classifications (COGs).

2.9. Data analysis

Results are presented as mean ± standard error of the mean of data acquired from three replicates. All data were analyzed with one-way analysis of variance and Dunnett's test for

pairwise comparison. A $p < 0.05$ was deemed statistically significant for all assays.

3. Results

3.1. *Signaling pathways affected by* Phyllanthus *species*

A Cignal Finder cancer 10-pathway reporter array was utilized, because it permits the concurrent screening of 10 major cellular pathways [p53/DNA damage, hypoxia, tumor growth factor-beta (TGFβ), Wnt, Notch, cell cycle/pRb-E2F, Myc/Max, nuclear factor (NF)-κB, MAPK/ERK, and MAPK/JNK). Figures 1A and 1B show stable expression of a green fluorescence protein (GFP) construct in both the extract-treated and -untreated MCF-7 cells. Therefore, results obtained from this assay were deemed valid. Among the 10 pathways, the hypoxia, Myc/Max, MAPK/JNK, and MAPK/ERK pathways were highly expressed in untreated MCF-7 cells as compared with the other pathways; however, treatment with various Aq and MeOH extracts of the four *Phyllanthus* species reduced the expression of these pathways significantly ($p < 0.05$). Comparing the effect of both extracts, MeOH extracts from the four *Phyllanthus* species demonstrated slightly better inhibitory activity on these pathways as compared with the Aq extracts. Additionally, no significant differences ($p > 0.05$) were observed between the four *Phyllanthus* species, although *P. urinaria* appeared slightly more effective at inhibiting the named pathways.

NF-κB is likely utilized by MCF-7 to ensure cell growth based on its high levels of expression in untreated cells. Our findings suggested that extracts from the four *Phyllanthus* species did not modulate this pathway to exert antiproliferative effects, except for MeOH *P. niruri* and *P. watsonii* extracts, which showed significant differences in cell proliferation as compared with that observed in untreated cells. Involvement of the cell cycle/pRb-E2F pathway in suppressing cell growth and survival was inconclusive, given that their expression percentages were lower than that measured for GFP expression. Subsequent cell-cycle analysis showed that extracts from the four *Phyllanthus* species did not result in cell-cycle arrest in the extract-treated MCF-7 cells (Figures 1C–1E) due to insignificant changes ($p > 0.05$) in the cell distribution at each phase. However, the number of cells in G_2/M phase increased ion MCF-7 cells treated with cisplatin and doxorubicin. Other pathways, including Wnt, Notch, p53/DNA damage, and TGFβ, may play insignificant roles in MCF-7 viability, given that their expression in both untreated and extract-treated cells was low.

3.2. *Western blot analysis of signaling pathways affected by* Phyllanthus *species*

Extracts from the four *Phyllanthus* species demonstrated repression of MCF-7 cell growth, survival, and metastasis

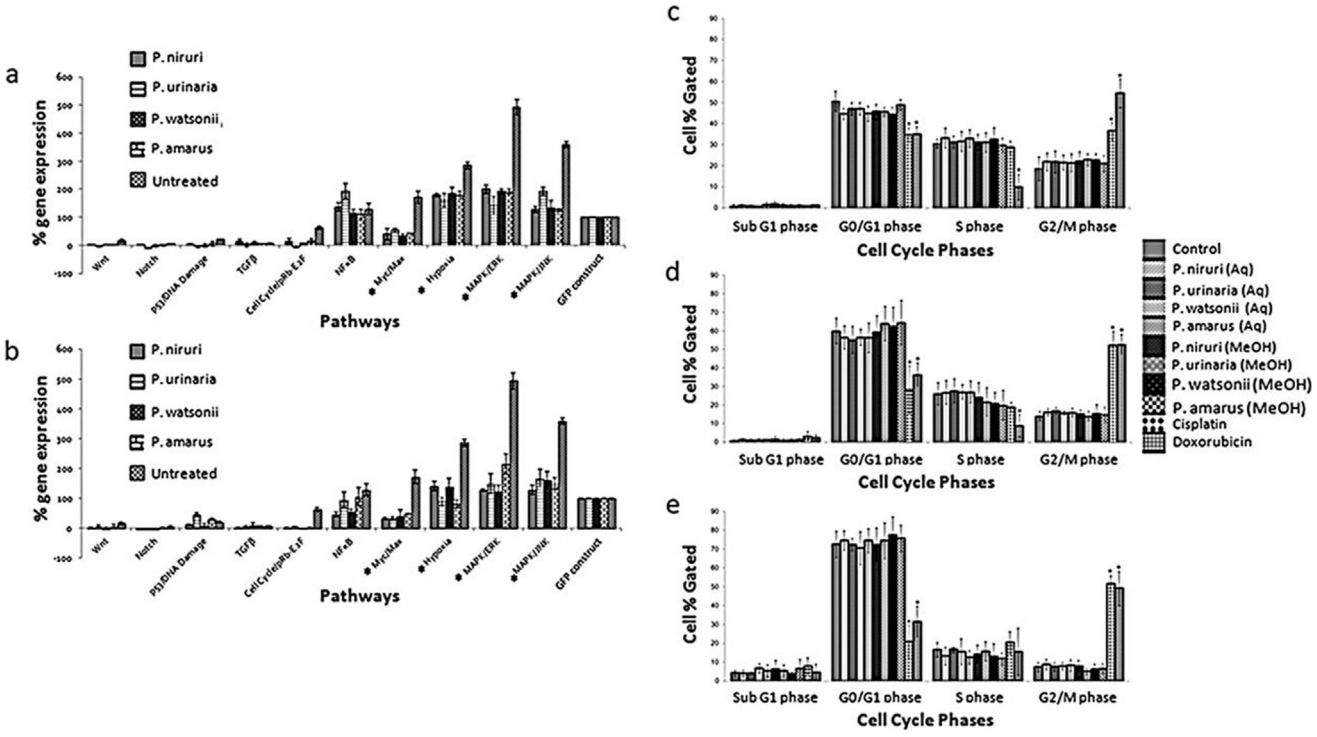

Figure 1 – Expression levels of 10 major cell-signaling pathways in cells treated with (A) aqueous and (B) methanolic extracts from the four *Phyllanthus* species. (C–E) Cell cycle phase-distribution percentage for cells treated with extracts from the four *Phyllanthus* species at their IC_{50} (μg/mL) concentrations for 24 h, 48 h, and 72 h, respectively. Error bars indicates the standard error of the mean of three independent experiments. * $p < 0.05$ versus untreated controls. Aq = aqueous; MeOH = methanolic.

primarily through MAPK and hypoxia pathways. Two-dimensional gel electrophoresis-based western blot analysis was performed to observe the individual expression of various proteins in these pathways to elucidate extract targets during early and late stages of the pathways. Similar to standard two-dimensional gel electrophoresis, isomers and subunits of each protein are fully separated based on their isoelectric value and molecular weight before transfer onto a membrane. This

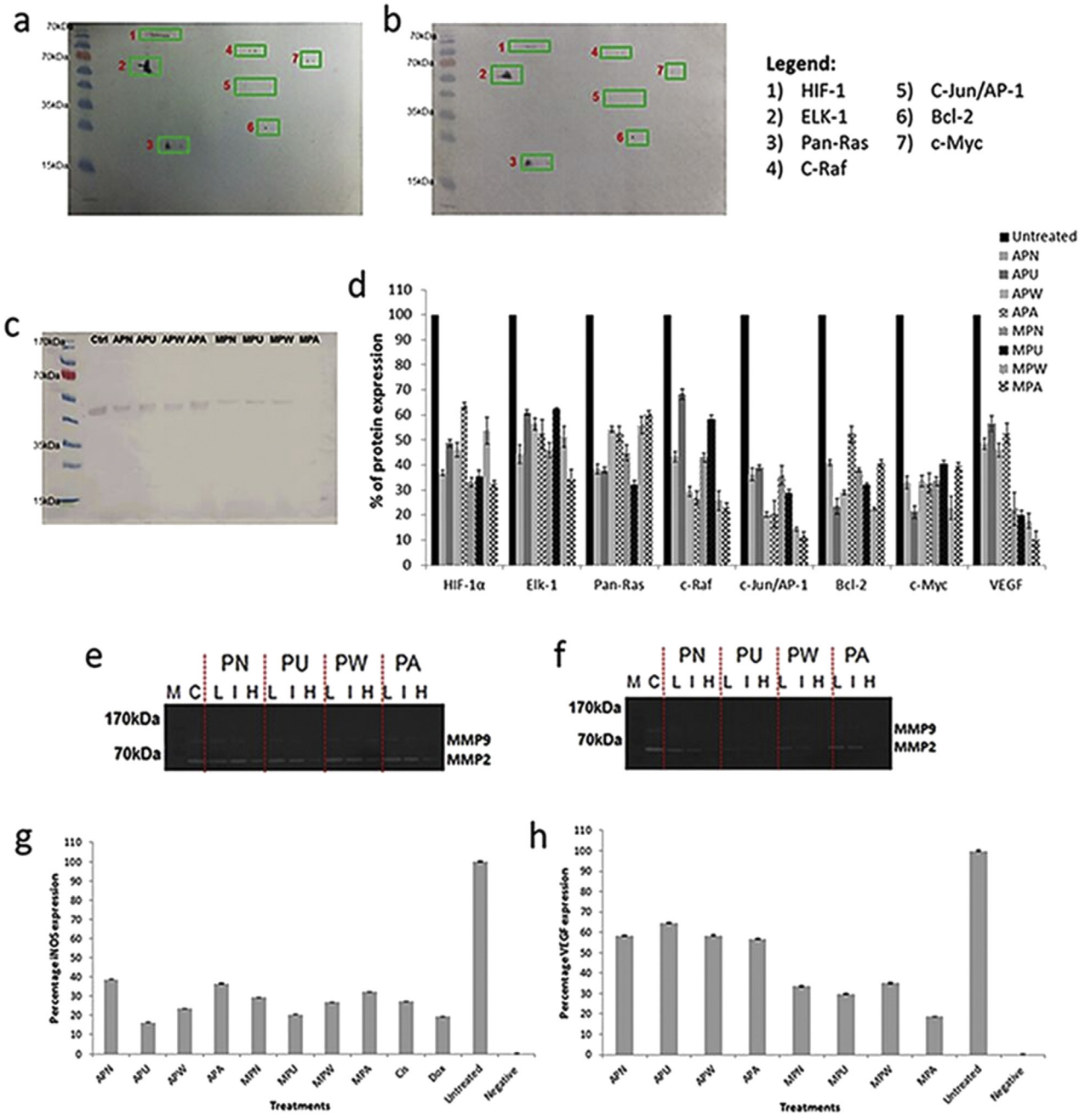

Figure 2 – Protein-expression levels in (A) untreated cells and (B) representative images of cells treated with aqueous *Phyllanthus watsonii*. (C) Western blot showing VEGF expression in untreated cells and cells treated with extracts from the four *Phyllanthus* species. (d) Percentage of individual protein expression. (E, F) MMP expression in cells after treatment with aqueous and methanolic extracts from four *Phyllanthus* species, respectively. ELISA-based detection of (G) iNOS and (H) VEGF expression in untreated cells treated with extracts from the four *Phyllanthus* species. Error bars indicate the standard error of the mean of three independent experiments. $p < 0.05$ for each *Phyllanthus* treatment as compared with the untreated group. APN = aqueous *P. niruri*; APU = aqueous *P. urinaria;* APW = aqueous *P. watsonii;* APA = aqueous *P. amarus;* C = untreated control; Cis = cisplatin; Dox = doxorubicin; ELISA = enzyme-linked immunosorbent assay; H = 500 μg/mL; I = IC_{50} dosage; iNOS = inducible nitric oxide synthase; L = 50 μg/mL; M = DNA marker; MMP = matrix metalloproteinase; MPA = methanolic *P. amarus;* MPN = methanolic *P. niruri;* MPU = methanolic *P. urinaria;* MPW = methanolic *P. watsonii;* VEGF = vascular endothelial growth factor.

assay allows a more rapid, simultaneous detection of various pathway-specific regulatory proteins in a single sample, thereby allowing for a more reliable comparison of their expression in the treated versus the untreated sample.

Figure 2A shows the blot for untreated cells, while Figure 2B shows the blot for cells treated with Aq *P. watsonii* extract. The expression level of each protein associated with the individual extracts is presented in Figure 2D. Proteins detected in untreated MCF-7 cells were c-Jun/AP-1, Pan-Ras, c-Raf, c-Myc, Elk-1, and HIF-1α, suggesting roles of the MAPK/ERK and hypoxia pathways in controlling cell growth and survival. Bcl-2 was also present in untreated MCF-7 cells, indicating its function as an anti-apoptotic agent to promote cell survival [20]. As predicted, expression of these proteins decreased following MCF-7 treatment with extracts from the four *Phyllanthus* species. Generally, MeOH extracts from *P. watsonii* and *P. amarus* exhibited better suppression of these proteins as compared with that observed following treatment with the Aq extracts. Among the Aq extracts, these two species also showed slightly better inhibitory activity relative to that observed from Aq extracts from *P. urinaria* and *P. niruri*. Furthermore, p53 expression remained undetected in both treated and untreated MCF-7 cells, confirming results from the cancer-reporter array demonstrating low levels of p53 expression in MCF-7 cells.

3.3. *Inhibition of MMP expression by* Phyllanthus *species*

MMPs play a crucial role in cancer metastasis, and their expression is often associated with tumor invasiveness due to their capability to degrade almost all types of extracellular components [18,21]. Our findings showed that MMP2 expression in MCF-7 cells was higher relative to that of MMP-9, suggesting a more prominent role for MMP-2 in MCF-7 metastasis. Comparison of the untreated- and treated-band intensity in Figures 2E and 2F demonstrated that the expression of both MMP2 and MMP9 was reduced in a dose-dependent manner. MeOH extracts from the four *Phyllanthus* species exerted greater inhibitory effects on MMP expression as compared with that observed from the Aq extracts. Among the four *Phyllanthus* species, *P. urinaria* showed the greatest inhibitory activity, followed closely by *P. watsonii*, *P. niruri*, and *P. amarus*.

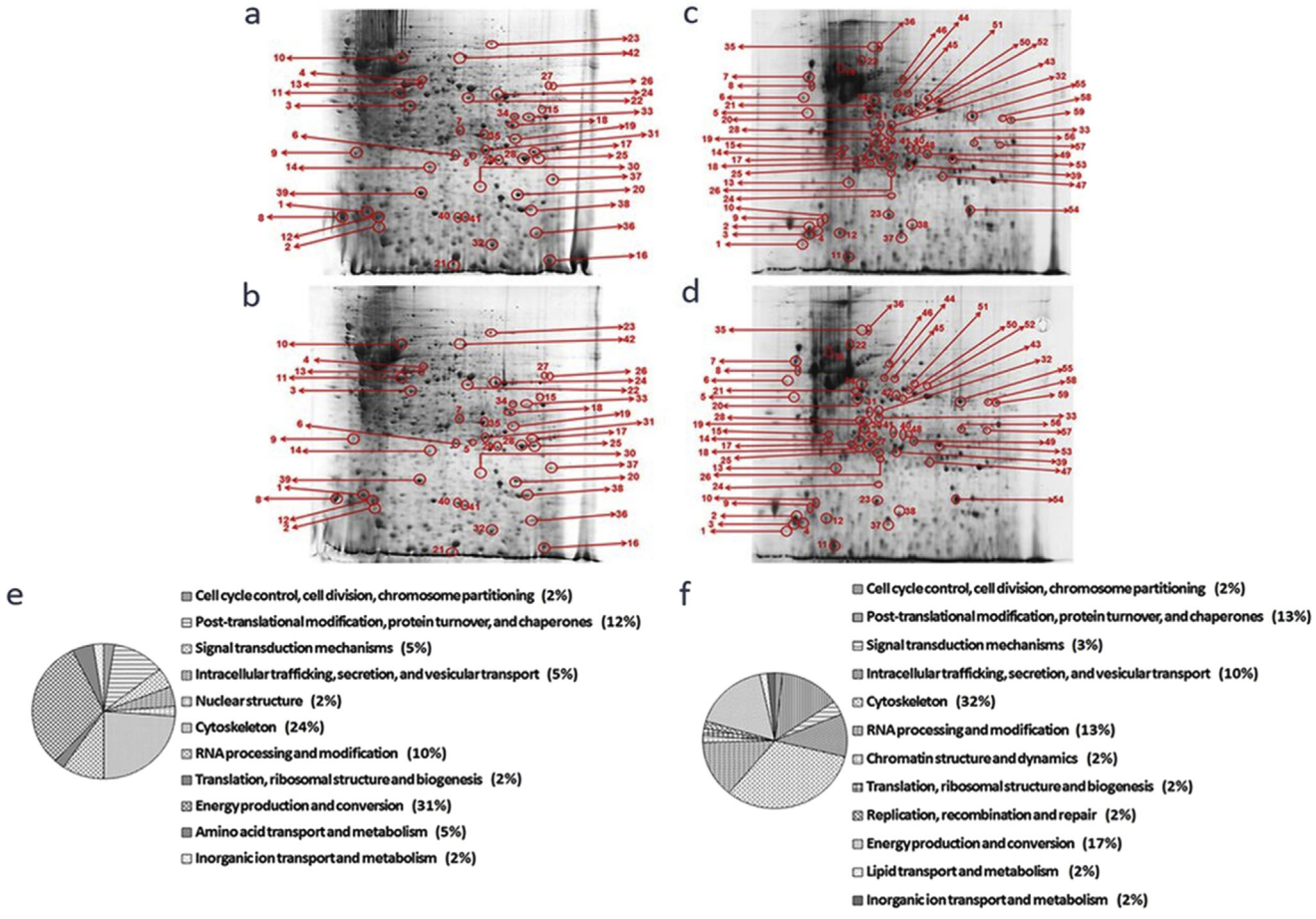

Figure 3 – Representative two-dimensional PAGE gels for (A, C) untreated cells, (B) aqueous *Phyllanthus watsonii*-treated cells, and (D) methanolic *P. watsonii*-treated cells. (E, F) COG classification of downregulated proteins in MCF-7 cells after treatment with aqueous and methanolic extracts from four *Phyllanthus* species, respectively. COG = clusters of orthologous groups; PAGE = polyacrylamide gel electrophoresis.

3.4. *Inhibition of VEGF and iNOS expression by* Phyllanthus *species*

HIF-1 primarily regulates hypoxia through activation of various genes, including VEGF and iNOS [22]. Our ELISA results detected high levels of VEGF and iNOS expression in untreated MCF-7 cells (Figures 2G and 2H); however, these levels decreased significantly to a range of 15% to 40% for iNOS and 20% to 65% for VEGF following treatment of MCF-7 cells with extracts from the four *Phyllanthus* species. Among these, Aq and MeOH *P. urinaria* showed enhanced inhibition of iNOS expression, while MeOH *P. amarus* was more effective at suppressing VEGF expression. VEGF expression was confirmed by western blot, which revealed a decrease of 40% to 90% following treatment with Aq and MeOH extracts from the four *Phyllanthus* species (Figures 2C and 2D). MeOH extracts showed greater inhibitory effects on both VEGF and iNOS expressions relative to those observed from Aq extracts.

3.5. *Differentially expressed proteins in* Phyllanthus*-treated MCF-7cells*

Two-dimensional gel electrophoresis proteomic analysis identified 62 and 61 protein spots differentially expressed in MCF-7 cells treated with Aq or MeOH extracts, respectively. Figures 3A–3D shows two-dimensional PAGE gels for untreated and Aq- and MeOH *P. watsonii*-treated MCF-7 samples. Subsequent mass spectrometry analysis and database examination by MASCOT identified 42 protein spots that were significantly downregulated by treatment with Aq extracts from the four *Phyllanthus* species, and the essential proteins classified according to COG are shown in Table 2 and Figure 3E. The MeOH extracts from the four *Phyllanthus* species resulted in 59 protein spots that were designated as being significantly inhibited (Table 3 and Figure 3F).

4. Discussion

Successful cancer treatment requires minimal drug toxicity to healthy tissues. Therefore, sourcing herbal-based anti-metastatic agents is of particular interest, given that they are repeatedly reported as being non-toxic [7,23]. Our previous work reported the toxicity of four *Phyllanthus* species on a breast carcinoma (MCF-7) cell line, wherein the migratory and invasive ability of cells was suppressed with having minimal toxicity to a normal breast epithelial (184B5) cell line. Apoptosis was induced in the MCF-7 cells following treatment with extracts from the four *Phyllanthus* species [7]; however, the underlying mechanisms conferring anti-metastatic capability remained unclear.

Fundamental regulators of metastasis include MMPs capable of hydrolyzing the extracellular matrix [24], making them targets for metastatic cancer treatment [19]. Among these MMPs, the activities of MMP2 and MMP9 are well-documented [5,24]. MMP2 is constitutively expressed as a

Table 2 – Downregulated proteins in MCF-7 following treatment with aqueous extracts from four *Phyllanthus* species.

Spots ID	Proteins	Fold change			
		APN	APU	APW	APA
Energy production and conversion					
1, 22	Pyruvate kinase isozymes M1/M2	−0.96	−0.42	−0.70	−0.67
3	Alpha-enolase	−0.41	−0.64	−0.47	−0.34
6	ATP synthase subunit alpha, mitochondrial precursor	−0.32	−0.47	−0.10	−0.53
24, 27, 34, 40	Glyceraldehyde-3-phosphate dehydrogenase	−0.72	−0.75	−0.20	−0.57
35	Triosephosphate isomerase	−0.98	−0.83	−0.74	−0.76
41	L-lactate dehydrogenase A chain	−1.00	−1.00	−1.00	−1.00
42	Beta-Enolase	−1.00	−1.00	−1.00	−1.00
Cytoskeleton					
4, 11	Tubulin beta-5 chain	−0.11	−0.84	−1.00	−0.34
10	Actin, cytoplasmic 2	−1.00	−0.55	−0.88	−1.00
12	Actin, alpha cardiac muscle 1	−0.62	−0.12	N/A	−0.28
20, 36	Cofilin-1	−0.59	N/A	−0.15	−0.21
39	Stathmin	−1.00	−1.00	−1.00	−1.00
Post-translational modification, protein turnover, and chaperones					
14	Heat-shock protein HSP 90-beta	−0.39	−0.55	−0.30	−0.37
16	10-kDa heat-shock protein, mitochondrial	−0.57	−1.00	−0.51	−1.00
18	Heat-shock cognate 71-kDa protein	−0.49	−0.57	−0.30	N/A
RNA processing and modification					
15, 30	Heterogeneous nuclear ribonucleoprotein A1	−0.40	−0.45	−0.39	N/A
19, 26	Heterogeneous nuclear ribonucleoproteins A2/B1	−0.45	−0.24	−0.19	−0.10
Drug detoxification					
2, 21	Annexin A2	−1.00	−1.00	−1.00	−1.00
Survival					
17, 29	Peroxiredoxin-1	−0.50	−0.25	−0.37	−0.26
33	Galectin-3	−0.66	−0.58	−0.46	−0.53

APA = aqueous *P. amarus*; APN = aqueous *P. niruri*; APU = aqueous *P. urinaria*; APW = aqueous *P. watsonii*; ATP = adenosine triphosphate; ID = identification; N/A = not affected.

Table 3 – Downregulated proteins in MCF-7 after treatment with methanolic extracts from four *Phyllanthus* species.

Spots ID	Proteins	Fold change			
		MPN	MPU	MPW	MPA
Cytoskeleton					
9, 10, 34, 44	Tubulin alpha-ubiquitous chain	−0.43	N/A	−0.30	−0.78
12, 19, 22	Actin, alpha cardiac muscle 1 precursor	−0.38	N/A	−0.13	−0.45
16, 20, 21	Tubulin beta-5 chain	−0.96	−0.85	−0.95	−0.62
23	Stathmin	−0.56	N/A	−0.38	−0.20
49	Filamin-A	−0.53	−0.61	−0.54	−0.86
Energy production and conversion					
18, 52	Phosphoglycerate kinase 1	−0.25	N/A	−0.28	−0.41
31	Alpha-Enolase	−0.70	−0.09	−0.58	−0.68
42	Aldose reductase	−1.00	−0.82	−0.77	−0.96
43, 46	Pyruvate kinase isozymes M1/M2	−0.50	−0.09	−0.24	N/A
47	Phosphoglycerate mutase 1	−0.29	−0.09	−0.21	−0.24
53	Triosephosphate isomerase	−0.26	N/A	−0.12	−0.34
54, 55, 56	Glyceraldehyde-3-phosphate dehydrogenase	−0.48	−0.69	−0.65	−0.78
Post-translational modification, protein turnover, and chaperones					
27	Heat-shock protein beta-1	−0.12	N/A	−0.29	−0.21
35, 36	Stress-70 protein, mitochondrial precursor	−0.53	N/A	−0.54	−0.47
57	60-kDa heat shock protein, mitochondrial precursor	−0.09	N/A	−0.14	−0.21
RNA processing and modification					
24	Splicing factor, arginine-/serine-rich 3	−0.38	N/A	−0.46	−0.50
26, 30	Heterogeneous nuclear ribonucleoprotein H	N/A	N/A	−0.10	−0.43
48	Heterogeneous nuclear ribonucleoprotein H3	−1.00	N/A	−0.39	−1.00
50	Elongation factor 2	−0.36	N/A	−0.31	−0.21
58, 59	Heterogeneous nuclear ribonucleoproteins A2/B1	−0.81	−0.83	−0.81	−0.57
Survival					
11	Thioredoxin	−0.43	−0.26	−0.39	−0.41
38	Peroxiredoxin-5, mitochondrial precursor	−0.18	N/A	−0.46	−0.66
Drug detoxification					
13, 17, 25, 29	Annexin A2	−0.70	−0.44	−0.33	−0.40
28	Annexin A8	−0.21	N/A	−0.29	−0.14

ID = identification; MPA = methanolic *P. amarus*; MPN = methanolic *P. niruri*; MPU = methanolic *P. urinaria*; MPW = methanolic *P. watsonii*; N/A = not affected.

latent proenzyme [21], while MMP9 is synthesized by various stimuli, which lead to aggravation of tumor invasiveness [25]. Here, treatment with extracts from the four *Phyllanthus* species inhibited expression of both MMPs in a dose-dependent manner, thereby preventing MCF-7 metastasis [7]. *P. urinaria* displayed higher effectiveness as compared with the other species concerning the inhibition of MCF-7 proliferation and metastasis, likely due to the presence of nine out of 10 polyphenols in the Aq extract and three out of four polyphenols in the MeOH extract [7].

Among the three human MAPK pathways, ERK1/2 is the most relevant MMP regulator in breast cancer [23]. Consistent with previous reports [18,23], our data demonstrated involvement of Ras-Raf-MEK-ERK in controlling the expression of MMP2 and MMP9. As illustrated in Figure 4A, extracts from the four *Phyllanthus* species targeted the Ras protein for inhibition, resulting in suppression of other protein expression downstream of the ERK1/2 pathway (Figure 4B) and leading to anti-proliferative and anti-metastatic activities. This result was confirmed by proteomic analysis showing downregulation of Ras-related protein Rab-11B following treatment with extracts from the four *Phyllanthus* species. Similar to our previously findings in an A549 lung-cancer cell line [26], extracts from the four *Phyllanthus* species appeared to suppress MAPK/ERK signaling, resulting in anti-proliferative and anti-metastatic effects. Contrary to results observed in MCF-7 cells, the primary target in A549 cells for suppression of the ERK pathway was the Raf protein rather than Ras. One possible explanation for this phenomenon is the presence of a constitutive-activating Ras mutation, which resulted in the A549 cells exhibiting higher motility levels [27]. Therefore, the Raf protein would represent an easier target in the pathway.

Among the DNA-bound transcription factors (Figure 4C), AP-1 and NF-κB are mainly activated by ERK1/2 in breast cancer to regulate a number of genes involved in apoptosis, cell proliferation, and MMP production [24]. Our data suggested that AP-1, but not NF-κB, was primarily affected by treatment with extracts from the four *Phyllanthus* species to inhibit MCF-7 metastasis through inhibition of c-Jun-protein induction. Another downstream target of ERK1/2 that was suppressed by extract treatment was the *c-myc* oncogene, resulting in apoptosis and/or cell-cycle arrest [28]. Therefore, in addition to regulating MMP expression, the ERK1/2 pathway also governs a number of other biological activities, including cell cycle, apoptosis, and cell proliferation, due to the diverse target genes associated with both AP-1 and c-Myc proteins. Our findings showed that treatment with extracts from all four *Phyllanthus* species did not exert cell-cycle arrest on MCF-

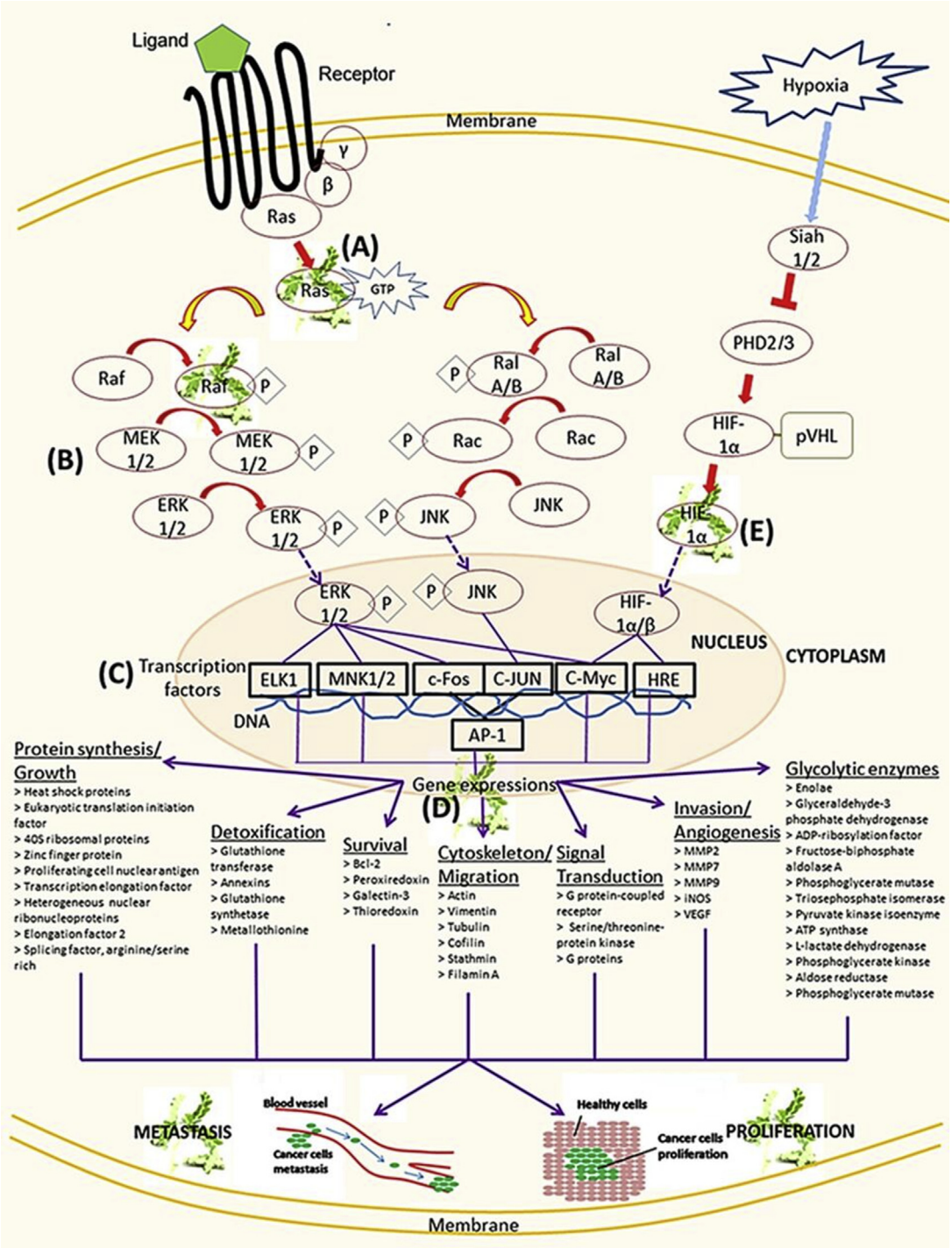

Figure 4 – Schematic diagram for anti-proliferative and anti-metastatic mechanisms associated with extracts from the four *Phyllanthus* species in MCF-7 cells. (A) GTP-binding protein Ras, (B) ERK1/2 pathway, (C) DNA-bound transcription factors, (D) various gene expressions, and (E) hypoxia pathway. ERK = extracellular Signal-related kinase; GTP = guanosine triphosphate.

7 cells. Accordingly, the only approach to inhibiting MCF-7 growth was through induction of apoptosis [7]. Preliminary data suggested that treatment with extracts from the four *Phyllanthus* species initiated the intrinsic apoptotic pathway via downregulation of anti-apoptotic Bcl-2 expression, which is the main regulator of mitochondrial outer-membrane permeabilization, resulting in cytochrome C release [29].

Regulation of MCF-7 proliferation via the ERK1/2 pathway following treatment with extracts from the four *Phyllanthus* species is primarily due to inhibition of numerous protein-

synthesizing proteins that are crucial during cell division [30], such as heterogeneous nuclear ribonucleoproteins that facilitate RNA processing and modification, as well as promote reactivation of telomerases important during carcinogenesis [31]. Other suppressed proteins are those of the arginine-/serine-rich family of proteins that include essential pre-mRNA-splicing factors required for post-transcriptional gene expression [32]. Inhibition of elongation factor 2 protein by extract treatment also prevented translocation of peptidyl tRNA from the A site to the P site in the ribosome [33]. Additionally, extract treatment downregulated the expression of a number of chaperone proteins, including Hsp90, stress-70 proteins, and Hsp60, leading to incorrect protein folding and unfolding required for normal cellular function [34]. Downregulation of these essential proteins by treatment with extracts from the four *Phyllanthus* species prohibited MCF-7 proliferation and metastasis.

During tumor hypoxia, the transcription factor HIF-1α is normally upregulated [35], which agreed with our findings in untreated MCF-7 cells. Among HIF-1α-target genes, iNOS plays a role in vascular permeability to induce degradation of the extracellular matrix, stimulate VEGF production, and induce endothelial proliferation and migration during angiogenesis [22]. Similarly, VEGF is often overexpressed in response to hypoxia [18] to promote blood-vasculature formation or remodeling. Blockage of HIF-1α expression and its downstream targets iNOS and VEGF following treatment with extracts from the four *Phyllanthus* species inhibited angiogenesis, resulting in tumor-cell death via hypoxia. In addition to HIF-1α, VEGF transcription is also controlled by AP-1 [13], suggesting a possible contribution of its upstream modulator ERK1/2 in suppressing angiogenesis. This was supported by repression of various cytoskeletal proteins (Tables 1 and 2) involved in cell mobility during vascular remodeling [36]. Perturbation of microfilament and microtubule expression by treatment with extracts from the four *Phyllanthus* species prevented MCF-7 survival and metastasis. Additionally, HIF-1α expression upregulated glucose uptake and expression of glycolytic enzymes to increase energy production during hypoxia [35]. Decreased expression of these energy-producing enzymes by treatment with extracts from the four *Phyllanthus* species could possibly deprive tumor cells of energy, resulting in their death [37].

Treatment with extracts from the four *Phyllanthus* species also decreased peroxiredoxin and thioredoxin expression, thereby exposing cancer cells to oxidative stress and death [10,38,39]. Suppression of drug detoxification (annexins) and survival (galectin-3) proteins by extract treatment also induced apoptosis in MCF-7 cells [31,38]. Interestingly, p53 was not affected by extract treatment, given that MCF-7 contains wild-type forms of p53. A possible explanation could be the suppression of Hdm2-regulated p53 degradation or defective p53 proteins.

In conclusion, the anti-proliferative and anti-metastatic activities observed following treatment with extracts from the four *Phyllanthus* species in MCF-7 cells primarily resulted from suppression of ERK1/2 and hypoxia-related pathways, which inhibited their downstream targets and numerous transcription factors. Therefore, the genus *Phyllanthus*, specifically *P. urinaria* and *P. watsonii*, could consist of potential candidates for use in cancer treatment.

Conflicts of interest

The authors have declared that no competing interest exists.

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