# PHYLA NODIFLORA MODULATES APOPTOSIS AND CELL CYCLE ARREST IN BREAST CANCER CELL LINES, MCF-7



# BIOTECHNOLOGY RESEARCH INSTITUTE UNIVERSITI MALAYSIA SABAH 2016

# PHYLA NODIFLORA MODULATES APOPTOSIS AND CELL CYCLE ARREST IN BREAST CANCER CELL LINES, MCF-7

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# THESIS SUBMITTED IN PARTIAL FULFILLMENT FOR THE DEGREE OF MASTER OF SCIENCE

# BIOTECHNOLOGY RESEARCH INSTITUTE UNIVERSITI MALAYSIA SABAH 2016

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Monica Liau 21 September 2016

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### ABSTRACT

Phyla nodiflora belongs to Verbenaceae family. It has been widely used as medicinal remedies in curing various types of diseases as it contains several of constituents. However, the precise cytotoxicity of Phyla nodiflora in relation to the apoptosis and cell cycle in breast cancer cell line, MCF-7 remains unclear. In this study, the ability of Phyla nodiflora to act as anticancer agents by modulating apoptotic pathways and cell cycle arrest was studied. Three of the extracts which consists of EA leaf, EA stem and Met stem shows inhibition of MCF-7 with  $IC_{50}$  of  $44.02\pm0.42 \text{ }\mu\text{a}/\text{m}$ ,  $57.27\pm0.11 \text{ }\mu\text{a}/\text{m}$  and  $96.26\pm0.43 \text{ }\mu\text{a}/\text{m}$  respectively. While for MDA-MB-231 cells, only cells treated with EA stem (79.36±0.58 µg/ml) and Met stem (93.48 $\pm$ 0.91 µg/ml) showed IC<sub>50</sub> value but not in EA leaf. However, there was no or less inhibition of MCF-10A was observed after treatments. To further understand Phyla nodiflora extracts in modulating apoptosis, MCF-7 was used. Apoptotic morphological changes were also seen in both methylene blue and DAPI staining in treated cells. JC-1 analysis shows that EA leaf extract (47.81±4.06%) has more cells undergone apoptosis through disruption of mitochondrial membrane potential followed by EA stem extracts (31.31±2.59%) and Met stem extracts (13.64±1.80%). In AnnexinV/PI analysis, more cells were undergoing late apoptosis after treated with Met stem (39.09±3.87%) followed by EA leaf (33.93±4.78%) and EA stem extracts (21.35±4.08%). Concurrent with this, cells treated with three different extracts showed different expression of Bcl-2, Bax, caspase 8 and caspase 9 proteins. Total 12 related apoptotic genes such as AIFM1, BAD, BCL-2, BIK, BIRC5, BAX, DFFA, CASP14, CASP2, CASP8, CASP9 and TP53 were selected for analysis and different fold change was seen in each of these gene expressions after treated with different type of extracts. Taken together, these results demonstrated that Phyla nodiflora induces the apoptosis pathway with different magnitude. To investigate the effect of plant extracts on cell cycle progression, cells were subjected to PI staining. For all treatments, S phase arrest was observed in MCF-7 cells. Altered expression of cell cycle regulatory proteins such as CDK6, CDK2, cyclin E1 and cyclin A2 suggested the perturbation of cell cycle regulation. In conclusion, these findings suggest that Phyla nodiflora has potential to be developed into anticancer agent(s).

#### ABSTRAK

### PHYLA NODIFLORA MODULASI APOPTOTIK DAN PERENCATAN KITARAN SEL PADA SEL PENGALAS KANSER PAYUDARA, MCF-7

Phyla nodiflora berasal daripada keluarga Verbenaceae. Ia telah digunakan secara meluas sebagai pengubat pelbagai jenis penyakit atas kehadiran pelbagai juzuk. Walaubagaimanapun, ketepatan sitotoksisiti Phyla nodiflora berhubung dengan apoptotik dan kitaran sel terhadap sel pengalas payudara, MCF-7 masih tidak jelas. Dalam kajian ini, kebolehan Phyla nodiflora sebagai ejen antikanser melalui modulasi laluan apoptotik dan perencatan kitaran sel telah dikaji. Ketiga-tiga ekstrak seperti EA daun, EA batang and Met batang telah menunjukan perencatan pada sel-sel MCF-7 dengan nilai with  $IC_{50}$  of  $44.02\pm0.42 \mu q/ml$ ,  $57.27\pm0.11 \mu q/ml$ dan 96.26±0.43 µg/ml masing-masing. Sementara bagi rawatan pada sel MDA-MB-231, hanya rawatan EA daun (79.36±0.58 µg/ml) and Met batang (93.48±0.91 µg/ml) mencapai nilai IC<sub>50</sub>. Walau bagaimanapun, tiada atau kurang perencatan berlaku pada sel payudara normal, MCF-10A selepas rawatan. Bagi memahami secara lanjut modulasi apoptosis oleh ekstrak Phyla nodiflora, MCF-7 telah diguna. Perubahan apoptotic morpologi turut dapat dilihat pada pewarnaan Methylene blue dan DAPI. Analisis JC-1 menunjukan bahawa EA daun (47.81±4.06%) mempunyai bilangan sel yang banyak melalui gangguan membran mitokondria diikuti dengan ekstrak EA batang (31.31±2.59%) dan Met batang (13.64±1.80%). Bagi analisis AnnexinV/PI, kebanyakan sel menjalani apoptosis lewat selepas rawatan dengan Met stem  $(39.09\pm3.87\%)$  diikuti dengan rawatan EA daun  $(33.93\pm4.78\%)$  and EA batang (21.35±4.08%). Sehubung dengan itu, sel-sel yang dirawat dengan ketigatiga ekstrak menunjukkan perbezaan ekpresi protein Bcl-2, Bax, Caspase 8 dan Caspase 9. Keseluruhan 12 gen berkait rapat dengan apoptotic seperti AIFM1, BAD, BCL-2, BIK, BIRC5, BAX, DFFA, CASP14, CASP2, CASP8, CASP9 dan TP53 telah dipilih bagi tujuan analisa dan terdapat perbezaan kali ganda pada setiap gen pada sel yang terawat. Keseluruhannya, keputusan ini menunjukkan bahawa Phyla nodiflora mendorong laluan apoptosis dengan magnitud yang berbeza. Untuk mengkaji kesan ekstrak tumbuhan pada perkembangan kitaran sel, sel-sel telah tertakluk kepada PI pewarnaan. Keseluruhan rawatan, perencatan fasa S telah diperhatikan pada sel-sel MCF-7. Gangguan pada ekspresi kawalan kitaran sel protein seperti CDK6, CDK2, siklin E1 dan siklin A2 mencadangkan gangguan terhadap kawalan kitaran sel. Kesimpulannya, hasil kajian ini menunjukkan bahawa Phyla nodiflora mempunyai potensi untuk dibangunkan sebagai ejen antikanser.

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#### LIST OF SYMBOLS

°C	-	Degree	Celsius
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- g Gram
- % Percentage
- xg Times the force of gravity
- μl Microlitre
- ml Mililitre
- **rpm** Revolutions per minute
- s Second
- IC<sub>50</sub> 50 Percent Inhibition Concentration
- C<sub>T</sub> Threshold cycle
- µg/ml Microgram per mililitre

Beta

β

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### LIST OF ABBREVIATIONS

ABL1	*	C-abl oncogene 1, non-receptor tyrosine kinase
AIFM1	-	Apoptosis-inducing factor, mitochondrion-associated, 1
AKT1	•	V-akt murine thymoma viral oncogene homolog 1
APAF1	-	Apoptotic peptidase activating factor 1
BAD		BCL2-associated agoinst of cell death
BAG1	-	BCL2-associated athanogene
BAG3	-	BCL2-associated athanogene 3
BAK1	-	BCL2-associated antagonist/killer 1
BAX	-	BCL2-associated X protein
BCL10		B-cell CLL/lymphoma 10
BCL2	TI	B-cell CLL/lymphoma 2
BCL2A1	4	BCL2-related protein A1
BCL2L1	-	BCL2-like 1
BCL2L10	- 11-02 L	BCL2-like 10
BCL2L11	SA B	BCL2-like 11
BCL2L2	÷.	BCL2-like 2
BFAR	-	Bifunctional apoptosis regulator
BID	-	BH3 interacting domain death agonist
BIK		BCL2-interacting killer
BIRC2	-	Baculoviral IAP repeat containing 2
BIRC3	-	Baculoviral IAP repeat containing 3
BIRC5	-	Baculoviral IAP repeat containing 5
BIRC6	-	Baculoviral IAP repeat containing 6
BNIP2	-	BCL2/adenovirus E1B 19kDa interacting protein 2
BNIP3	-	BCL2/adenovirus E1B 19kDa interacting protein 3
<b>BNIP3L</b>		BCL2/adenovirus E1B 19kDa interacting protein 3-like

BRAF	÷	V-raf murine sarcoma viral oncogene homolog B1
CASP1	-	Caspase 1, apoptosis-related cysteine peptidase
CASP10	-	Caspase 10, apoptosis-related cysteine peptidase
CASP14	-	Caspase 14, apoptosis-related cysteine peptidase
CASP2	-	Caspase 2, apoptosis-related cysteine peptidase
CASP3	-	Caspase 3, apoptosis-related cysteine peptidase
CASP4	-	Caspase 4, apoptosis-related cysteine peptidase
CASP5	-	Caspase 5, apoptosis-related cysteine peptidase
CASP6	-	Caspase 6, apoptosis-related cysteine peptidase
CASP7	-	Caspase 7, apoptosis-related cysteine peptidase
CASP8	-	Caspase 8, apoptosis-related cysteine peptidase
CASP9		Caspase 9, apoptosis-related cysteine peptidase
CD27	1	CD27 ligand
CD40		CD40 molecule
CD40LG		CD40 ligand
CD70		CD70 ligand
CFLAR	SA 1	Caspase 8 and FADD-like apoptosis regulator BA
CIDEA	÷.	Cell death-inducing DFFA-like effector a
CIDEB	-	Cell death-inducing DFFA-like effector b
CRADD	2	Caspase 2 and RIPK1 domain containing adaptor with death domain
CYCS	-	Cytochrome C
DAPK1	-	Death-associated protein kinase 1
DFFA	-	DNA fragmentation factor, 45kDa, alpha polypeptide
DIABLO	-	Diablo, IAP-binding mitochondrial protein
FADD	-	Fas (TNFRSF6) associated via death domian
FAS	+	Fas (TNF receptor superfamily, member 6)
FASLG	-	Fas ligand
GADD45A		Growth arrestand DNA damage inducible, alpha

HRK	12	Harakiri, BCL2 interacting protein
IGF1R	ie.	Insulin-like growth factor 1 receptor
IL10	÷	Interleukin 10
LTA	-	Lymphotoxin alpha
LTBR	-	Lymphotoxin beta receptor
MCL1	-	Myeloid cell leukemia sequence 1
NAIP	-	NLR family, apoptosis inhibitory protein
NFKB1	-	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
NOD1	-	Nucleotide-binding oligomerization domain containing 1
NOL3	-	Nucleolar protein 3
PYCARD	-	PYD and CARD domain containing
RIPK2		Receptor-interacting serine-theronine kinase 2
TNF	ST1	Tumor necrosis factor
TNFRSF10A		Tumor necrosis factor receptor superfamily, member 10a
TNFRSF10B	-	Tumor necrosis factor receptor superfamily, member 10b
TNFRSF11B	S A B	Tumor necrosis factor receptor superfamily, member 11b
TNFRSF1A	-	Tumor necrosis factor receptor superfamily, member 1A
TNFRSF1B	- 11	Tumor necrosis factor receptor superfamily, member 1B
TNFRSF21	<b>-</b> C	Tumor necrosis factor receptor superfamily, member 21
TNFRSF25	940) 1940	Tumor necrosis factor receptor superfamily, member 25
TNFRSF9	-	Tumor necrosis factor receptor superfamily, member 9
TNFSF10	-	Tumor necrosis factor (ligand) superfamily, member 10
TNFSF8	-	Tumor necrosis factor (ligand) superfamily, member 8
ТР53	•	Tumor protein p53
TP53BP2	-	Tumor protein p53 binding protein
ТР73	-	Tumor protein p73
TRADD	-	TNFRF1A-associated via death domain
TRAF2		TNF receptor-associated factor 2

TRAF3	-	TNF receptor-associated factor 3
XIAP	-	X-linked inhibitor of apoptosis
АСТВ	-	Actin, beta
B2M	-	Beta-2-microglobulin
GAPDH	-	Glyceraldehyde-3-phosphate dehydrogenase
HPRT1	-	Hypoxanthine phosphoribosyltransferase
RPLPO	-	Ribosomal protein, large, PO
DISC	-	Death inducing signalingcomplex
c-FLIP	-	FLICE-like inhibitory protein
Apaf-1	+	Apoptotic protease activating factor 1
ΑΤΡ	-	Adenosine triphosphate adenosine triphosphate
AIF	-	Apoptosis inducing factor
CAD	-	Caspase activated DNase
CASc	499	Capases catalytic region
PARP	-	Poly(ADP-ribose) polymerase
IAP	$\sim$	Inhibitor of apoptosis proteins
NSCLCs	S-A B A	Non-small cell lung carcinomas AYSIA SABAH
XIAP	-	X-linked inhibitor of apoptosis protein
BRAC1	-	Breast cancer 1, early onset
BRAC2	-	Breast cancer 2
G1	-	Growth phase 1 (Cell cycle)
S	-	Syhthesis phase (Cell cycle)
G2	8	Growth phase 2 (Cell cycle)
м	5	Mitosis
CDK2	-	Cyclin dependent kinase 2
CDK4	-	Cyclin dependent kinase 4
P21	-	Cyclin dependent kinase inhibitor 1
ROS	-	Reactive oxygen species

ER	-	Endoplasmic reticulum
MCF-7	•	Michigan Cancer Foundation-7 (Human breast adenocarcinoma cell line)
MDA-MB231		Estrogen receptor-negative human breast cancer cell line
MCF-10A	-	Normal breast cell line
JC-1	-	Miotchondrial membrane potential assay kit
PI	=	Propidium iodide
АКТ	-	Protein kinase B
Р13К	-	Phosphoinositide 3 kinase
NIX	-	Pro-apoptotic gene that expresses a signaling protein related to BH-3 only family





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- Page Appendix I DNA fragmentation of EA stem, EA leaf and Met stem done 133 by previous study
- Appendix II Effect of MCF-7 cell Proliferation percentage for stem and 134 leaf
- Appendix II quality including 135 The checks which PCR array reproducibility, RT efficiency and genomic DNA contamination for treated and untreated samples.



### **CHAPTER 1**

### INTRODUCTION

#### 1.1 Background of Study

Breast cancer was the top five cancers that lead women and men suffering in worldwide (Tan, Sulaiman, Najimuddin, Samian and Muhammad, 2005: 287). Approximately 14.5% of women and men out of 100,000 cancer cases were reported on April 2016 by Malaysian Deputy Health Minister Datuk Seri Dr Hilmi Yahaya (Arumugam, 2016). Like other cancers, breast cancer is also characterized by the rapid and uncontrolled proliferation of abnormal cells, which form a solid tumor that invade normal cells in the body that able to proliferate throughout the body (Qi, Li, Zhao, Xu, Inagaki, 2013: 654; Wang, Gao, Kokudo, Nakata, and Tang, 2010: 659).

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Variations of this cancer are closely related to gene mutations in oncogenes and tumor suppressor gene, p53, which both of these genes play an important role in cell division and apoptosis. Generally, there are five types of regulatory processes for development of oncogenes in the cell cycle that can lead to cancer cell development. These main five types of regulatory process consist of growth factors, cell surface receptors, intracellular signal transduction components, nuclear DNA binding proteins and components of cyclin (Glinsky, 1998: 72; Rastogi and Mishra, 2012: 1). Other factors such as the abnormal expression level of certain proteins and environmental factors such as pollution also can cause mutation that leading to cancers occurs (Hoffmon, 1999: 239; Mantena, Sharma and Katiyar, 2006: 2018). The present modern technologies such as chemotherapy, radiotherapy and surgical operation in cancer treatment have accompanied with side effect and higher cost. Due to this reason, major trending in research nowadays has taken initiative since the early 1950 in drug discovery from natural product which is rich in secondary metabolites (Merlin, Parthasarathy and Santhoskumar, 2010: 4452). The use of natural product as an anti-cancer drug is also recognized by the US Natural Cancer Institute (Cragg and Newman, 2005: 72; Fouche, Cragg, Pillay, Kolesnikova, Maharaj and Senabe, 2008: 455).

Lack of consuming food based diet and excessive intake of meat may contribute to the high incidence of cancer (Modem, Dicarlo and Reddy, 2013: 177). Several studies have documented that consuming plant products help to reduce the chances to get cancer due to its nature of phytochemical property (Modem *et al.*, 2012: 177; Tasyriq, Najmuldeen, In, Mohamad, Awang and Hasima, 2012: 2). Phytochemicals in natural products are particularly very rich in antioxidants, immune-strengthening and anti-cancerous ingredients could be beneficial to human health (Modem *et al.*, 2013: 177; Dziki, Swiece, Sulkowski, Dziki, Baraniak and Czyz, 2013: 154). Most of the plants which are rich in alkaloids and other phytochemical contents that commonly found in different parts of the plant can be effectively used to cure variety types of disease (Modem *et al.*, 2013: 177).

About 25% to 30% of medicines available are derived from natural products as they provide an important source of bioactive compounds that can be used as an alternative way in treating various types of diseases by affecting different targets of signal transduction pathways that modulate gene expression, cell cycle progression, cell proliferation and cell death (Ramos, 2008: 509). Studies also prove that many biological activities such as antioxidants are due to the presence of high amount of phytochemicals (Ribeiro, Noranha, Ribeiro, Moraes, Santos, Coelho and Chavasco, 2015: 19). Components such as alkaloid, sterol and terpenoid are some examples of phytochemicals that found in most of plants that have been extensively studied for the drug's discovery purpose, especially for anti-

2

cancer (Tasyriq *et al.*, 2012: 1; Kaefer and Milner, 2008: 351; Mohammad and Farimani, 2014: 37).

In addition, cytotoxic activity of chemotherapeutic drugs is also proven to have the ability to induce genotoxic death through apoptosis. Some of the chemotherapy drugs are found to have the ability in inducing apoptosis *in vitro* such as quercetin, polyphenol, epicatechin gallate (ECG), catechin, theaflavin, anthocyanins and curcumin (Ramos, 2008: 514). Besides that, *in vivo* study also provides evidence that chemotherapeutic agents induce apoptotic tumor cell death. Both *in vitro* and *in vivo* studies have brought a great promise in identifying the potential chemotherapeutic agents for cancer treatment through induction of apoptosis (Tan *et al.*, 2005: 287).

Lin, Zhang, Cheng, Tang, Zhang, Zhen, Cheng, Liu, Cao and Dong (2008: 247) have suggested that apoptosis is an ideal mechanism to focus on cancer chemotherapy as it does not lead to cell lysis and inflammatory response which is in contrast to necrosis. Besides that, cancer involves genetic alteration in apoptotic pathway and this provides an insight to a target of treatment to inhibit cancer cells (Wong, 2011: 87).

*Phyla nodiflora* belongs to Verbenaceae family. It has been widely used as medicinal remedies in curing various types of diseases as it contains several of constituents such as triterpenoids, flavonoids, phenols, steroids, halleridone, hallerone and many others (Vanajothi, Sudha, Manikandan, Rameshthangam and Srinivasan, 2012: 287). Due to the presence of varied constituents, this plant is very useful for pharmaceutical purposes (Durairaj, Vaiyapuri, Kanti and Malaya, 2008: 83; Faheem, Wan and Koay, 2011: 102). Previous studies also suggested that this plant has the ability to act as anti-bacterial, parasiticide, anti-inflammation, anti-microbial, cytotoxic and diuretic (Durairaj, Mazumdar, Gupta and Selvan, 2009: 713).

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