STUDY OF CYTOTOXIC MECHANISM AND THE ROLE OF microRNA IN MDA-MB-231 CELL LINES TREATED WITH *Phaleria macrocarpa* (Boerl.) FRUIT ETHYL ACETATE FRACTION (PMEAF)

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UNIVERSITI SAINS MALAYSIA

2017

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by

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Thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

December 2017

ACKNOWLEDGEMENT

First and foremost in the name of God, I would like to express thanks to Almighty for giving me the most gracious, merciful strength and the perseverance to pursue and complete this PhD thesis. It would not have been possible to complete this study without His grace. Secondly I would like to take this opportunity to extend my sincere appreciation to my main supervisor, Associate Professor Dr. Sasidharan Sreenivasan for giving me opportunity to pursue my postgraduate studies. He is a reliable person that I could always count on in times of difficulties and throughout this research and without his knowledge, understanding, patience, guidance and encouragement I would not able to complete my PhD on time. Besides that, his assistance in publications, comments, and suggestions at all times of the research were very helpful to build an excellent research work.

A special note of thanks goes to my Co-supervisor, Dr. Oon Chern Ein from Institute for Research in Molecular Medicine (INFORMM), USM for her guidance and advice. I am thankful to the Electron Microscope Unit (USM), Pn. Jamilah, En. Rizal, En. Johari and Pn. Faizah for helping me on SEM and TEM studies in this research. I am also would like to thank Pn. Norfadhillah Ya'akob from Advanced Medical and Dental Institute (AMDI), USM for her help in flow cytometry analysis and Pn. Uswatun Bahirah from Centre for Chemical Biology (CCB) for her guidance in RNA QC by using Bioanalyzer. Of course not forgetting, Mr. Shie Jie Pang and Mr. Zhi Hui, field experts from Genomax Technologies Pte Ltd, Singapore for providing resourceful information for the analysis of microRNA sequencing data.

I am very grateful to Ministry of Education Malaysia, Government of Malaysia for providing me financial support through the MyPhD fellowship. The library facilities of the USM and computer facilities, as well as the necessary

laboratory equipment‘s offered by Institute for Research in Molecular Medicine (INFORMM) have been indispensable. I also would like to thank all the Staff, postgraduate students and my friends from INFORMM for their friendly supports and assistance since the start of my postgraduate work in 2013, especially the director of INFORMM, Professor Norazmi Mohd Nor. I would like to thank Vijaya, Priya, Priscilla and Cell Culture Team of INFORMM for their support, advice, inspiration and help throughout my project.

Last and not least, my high gratitude goes to my mother as a ‘single-parent’ for her love, care and responsibility in raising me to whom I am today. Special thanks to my husband, Mr.Muhendran for being there as a friend to support and encourage me all the time until I complete my PhD research. My Biozone friends (Darishiani, Kalpanah, Hazeeq, and Mike), thanks for 12 years of friendship bonding and being there at time of difficulty or stressed. Finally my close relatives, Mrs. Gouri family, Ms. Ahci, Mrs. UmaParam, Mrs. Santhy, Mr. Sweneson family, cousin sisters Thatchiayani, Sandtya and my grandmother Madam Papathy for being my backbones.

KAVITHA D/O NOWROJI

Institute for Research in Molecular Medicine

Universiti Sains Malaysia

DECEMBER 2017

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LIST OF ABBREVIATIONS

AIF	Apoptotic inducing factor
ANOVA	Analysis of Variance
AO	Acridine Orange
ATP	Adenosine Triphosphate
BID	BH3 interacting-domain death agonist
BAX	BCL-2-Associated X Protein
BCA	Bicinchoninic acid assay
BCL-2	B-Cell Lymphoma 2
BCL-w	BCL-2-like protein 2
CCCP	Carbonylcyanide-*m*-chlorophenylhydrazone
CV	Cell viability
DAVID	Database for Annotation, Visualization and Integrated Discovery
DCF	2′, 7′-dichlorodihydrofluorescein
DCFH-DA	2′,7′-dichlorodihydrofluorescein diacetate
DE	Differential expression
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco Modified Eagle Medium
DNA	Deoxyribonucleic acid
e.V	Electronvolt
FACS	Fluorescence-Activated Cell Sorting
FDR	False discovery rate
FC	Fold change
FBS	Fetal Bovine Serum
GAE	Gallic Acid Equivalent
GC-MS	Gas Chromatography–Mass Spectrometry
GO	Gene Ontology
GRCh38	Genome Reference Consortium Human Build 38
H_2O_2	Hydrogen peroxide
IC_{50}	Inhibition Concentration by half
JC-1	5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide

KEGG	Kyoto Encyclopedia of Genes and Genomes
MAPK	Mitogen-activated protein kinase
miRNA	microRNA
MTT	3-(4,5-dimethylthiazoylyl)-,5-diphenyltetrazolium bromide
NGS	Next generation sequencing
NIST	National Institute of Standard and Technology
OD	Optical Density
P21	Cyclin-dependent kinase inhibitor
P27	Cyclin-dependent kinase inhibitor 1B
P53	Tumor suppressor protein
PBS	Phosphate Buffer Saline
PMEAF	*Phaleria macrocarpa* ethyl acetate fraction
PCR	Polymerase chain reaction
PI	Propidium Iodide
POS	Positive Control Signal
Rb1	Retinoblastoma1 protein
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SEM	Scanning Electron Microscope
SI	Selective Index
SMAC	Second Mitochondria-derived Activator of Caspases
TEM	Transmission Electron Microscope
TPC	Total Phenolic Content
TPM	Transcripts per million
UTR	Untranslated Region
UV	Ultraviolet
WHO	World Health Organization
XIAP	X chromosome-linked IAP

KAJIAN MEKANISME SITOTOKSIK DAN PERANAN mikroRNA DALAM SEL MDA-MB-231 YANG DIOLAH DENGAN FRAKSI ETIL ASETAT BUAH *Phaleria macrocarpa* (Boerl.) (PMEAF)

ABSTRAK

Phaleria macrocarpa merupakan tumbuhan ubatan yang terkenal yang mempamerkan sitotoksiti terhadap pelbagai jenis sel kanser. Objektif kajian ini adalah untuk mengenalpasti kesan sitotoksik secara *in vitro* dengan mengunakan ujian biokimia melalui pengawalaturan miRNA dalam MDA-MB-231 yang diolah dengan PMEAF. Jumlah fenolik dalam PMEAF adalah sebanyak 14.91 ± 0.97 mg GAE/g. Keputusan ujian sitotoksisiti MTT dan CyQuant menunjukkan bahawa PMEAF adalah agen anti-kanser yang berpotensi dengan nilai purata IC_{50} sebanyak 18.10 µg/mL. Ciri-ciri kematian sel apoptotik termasuk pengecutan sel, blebs membran, kondensasi kromatin dan pembentukan badan apoptotik dapat diperhatikan melalui pelbagai teknik mikroskopi seperti mikroskop cahaya, mikroskop hologram, mikroskop elektron transmisi (TEM) dan imbasan (SEM). Pewarnaan AO/PI dan analisis aliran sitometri dijalankan terhadap sel-sel MDA-MB-231 yang dirawat dengan PMEAF menunjukkan bahawa kematian sel secara perencatan apoptosis. Analisis aliran sitometri tentang kitaran sel menunjukkan bahawa pengumpulan sel-sel MDA-MB-231 yang dirawat dengan PMEAF dalam fasa $G0/G_1$ dan G_2/M. Tambahan pula, aktiviti sitotoksisiti PMEAF juga meningkatkan penghasilan ROS dalam sel MDA-MB-231 dan secara konsisten merangsang kehilangan potensi membran mitokondria ($\Delta\psi_m$). Keputusan kajian tentang profil protein membuktikan bahawa PMEAF meningkatkan penghasilan 9 jenis protein bersifat pro-apoptotik

(Bax, Bid, caspase 3, caspase 8, cytochrome *c*, p21, p27, p53 dan SMAC) dan megurangkan penghasilan 4 jenis protein bersifat anti-apoptotik (Bcl-2, Bcl-w, XIAP dan survivin) dalam sel MDA-MB-231. PMEAF menyebabkan apoptosis dalam sel MDA-MB-231 melalui laluan intrinsik dengan penyertaan oleh caspases, perencatan kitaran sel pada fasa G_0/G_1 dan G_2/M dengan mekanisma diperantara oleh p53.

Kajian mengenai pengawalaturan miRNA dalam sel MDA-MB-231 yang dirawat dengan PMEAF telah mengenalpasti 10 miRNAs dengan peningkatan pengawal aturan dan 10 miRNA dengan penurunan pengawal aturan dalam sel-sel MDA-MB-231 dengan menggunakan perkhidmatan IIlumina, platform Hi-Seq2000 daripada Next Generation Sequencing (NGS). Sebanyak 606 gen sasaran oleh 10 miRNA peningkatan pengawalaturan dan 517 gen sasaran oleh 10 miRNA dengan penurunan pengawalaturan telah diramalkan berdasarkan kaedah analisis berkomputer dan telah disahkan dalam pangkalan data “miRGate”. Sementara itu, keputusan daripada Sumber Bioinformatik v6.8 DAVID telah menentukan fungsi anotasi penglibatan miRNA dengan peningkatan pengawalaturan berperanan untuk mengurangkan experasi onkogen dalam laluan kanser dan miRNA dengan penurunan pengawal aturan berperanan dalam meningkatkan eksperasi gen perencatan tumor dalam laluan apoptotic. Kesimpulannya, kajian ini membuktikan bahawa PMEAF adalah agen anti-kanser yang menunjukkan tahap sitotoksisiti yang tinggi terhadap sel MDA-MB-231 dan merangsang kematian sel bersifat apoptotik melalui pengawalaturan miRNA. PMEAF mungkin menjadi calon yang baik untuk penyediaan drug anti-kanser atau sebagai makanan tambahan bagi pencegahan kesan buruk kemoterapi.

STUDY OF CYTOTOXIC MECHANISM AND THE ROLE OF microRNA IN MDA-MB-231 CELL LINES TREATED WITH *Phaleria macrocarpa* (Boerl.) FRUIT ETHYL ACETATE FRACTION (PMEAF)

ABSTRACT

Phaleria macrocarpa is a well-known medicinal plant which exhibited cytotoxicity against various cancerous cells. The objective of this study was to determine the *in vitro* cytotoxicity effect by biochemical assay through the regulation of miRNAs on MDA-MB-231 treated with *Phaleria macrocarpa* ethyl acetate fraction (PMEAF). Total phenolic content of PMEAF was expressed as 14.91 ± 0.97 mg GAE/g. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and CyQuant Cell Proliferation assay results indicated that PMEAF is a potential anticancer agent with the average IC_{50} values of 18.10 µg/mL. The characteristic of apoptotic cell death includes cell shrinkage, membrane blebs, chromatin condensation as well as the formation of apoptotic bodies and these were observed through light microscopy, holographic microscopy, transmission electron microscopy (TEM) and scanning electron microscope (SEM). The AO/PI staining and flow cytometric analysis of MDA-MB-231 cells treated with PMEAF showed apoptotic cell death. The cell cycle analysis by flow cytometry analysis revealed that the accumulation of PMEAF treated MDA-MB-231 cells in G_0/G_1 and G_2/M-phase of the cell cycle. Moreover, the PMEAF exert cytotoxicity by increasing the ROS production in MDA-MB-231 cells which consistently stimulated the loss of mitochondrial membrane potential ($\Delta\Psi_m$). The PMEAF stimulated the expression of nine pro-apoptotic proteins (Bax, Bid, caspase 3, caspase 8, cytochrome *c*, p21, p27, p53 and SMAC) and suppressed the four anti-apoptotic proteins (Bcl-2, Bcl-w, XIAP

and survivin) in MDA-MB-231 cells. PMEAF induced apoptosis in MDA-MB-231 cells through intrinsic pathway with the participation of caspases, G_0/G_1 and G_2/M-phases cell cycle arrest by p53-mediated mechanism. The PMEAF treatment against MDA-MB-231 cells identified 10 upregulated and 10 downregulated miRNAs by using IIlumina, Hi-Seq2000 platform of Next Generation Sequencing (NGS). A set of 606 target genes of 10 upregulated miRNAs and 517 target genes of 10 downregulated miRNAs were predicted based on computational and validated databases by using miRGate DB Query. Meanwhile, results from DAVID Bioinformatics Resources 6.8 specified the functional annotation of the upregulated miRNAs involvement in cancer pathway by suppressing the oncogenes and down regulating miRNAs by expressing the tumour suppressor genes in regulation of apoptosis pathway. In conclusion, the results of this study proved that PMEAF is a promising anticancer agent with high cytotoxicity against MDA-MB-231 breast cancer cells and it induced apoptotic cell death mechanism through the regulation of miRNAs. PMEAF might be the best candidate for developing more potent anticancer drugs or chemo-preventive supplements.

CHAPTER 1.0: INTRODUCTION

1.1 Overview and rationale of the study

Breast cancer is commonly diagnosed as invasive cancer in women worldwide and the second leading cause of cancer death which affects about 400,000 patients every year (Gluz *et al*., 2009; Siegel *et al*., 2016). Breast cancer occurs due to the abnormal cells growth surrounding the breast tissues and proliferates into neighbouring cell or progression of secondary malignant tumour growths known as 'metastasis". In recent years, breast cancer incidences are increasing vastly due to difficulty in treating cancer patients since different stages of tumour exhibited different treatment responses. Most of the chemotherapeutic drugs, namely Tamoxifen, Doxorubicin, Docetaxel are only effective in one third of breast cancer patients (Fisher *et al*., 2005). Thus, searching for new alternative and complementary medicine which are less toxic as a cure for breast cancer is in great need. In a review focusing on the survival of breast cancer patients and their intake of fruits and vegetables, five of the eight cohort studies showed a direct relationship between vegetable and fruit intake and survival of breast cancer patient, with a 20 - 90% reduction in death risk (Rock and Demark-Wahnefried, 2002). Moreover, phytochemicals from fruits and vegetables are primary contributors in preventing the cancer cells progression and increasing the survival rate of breast cancer patients (Jung *et al.,* 2013). This awakens the interest of researchers in isolating the bioactive compounds from fruits, vegetables and natural products as competent anticancer drugs. Although the outcome of crude medicinal plants is usually slower in onset compared to commercial drugs, patients may benefit through less side effects of herbal medicines which are an alternatives to drug based medicine (Gurib-Fakim, 2006).

In view of that fact, this study was conducted to pave a way in developing new anticancer agents from the traditional medicinal plant, *Phaleria macrocarpa (Scheff.)* Boerl *(Thymelaceae)* for the prevention of breast cancer development. *P. macrocarpa* is a popular medicinal plant rich with various phytochemicals and commonly used in the traditionally medicine to treat various diseases including cancer, impotency, heart disease, kidney disorders, diabetic mellitus and skin diseases (Zhang *et al.,* 2006). Native Indonesians have been practising the consumption of *P. macrocarpa* stem, fruit, seed or leaf in boiled water extract which revealed that the plant has no side effects and showed that it is harmless (Faried *et al.,* 2007). This plant species produces an ample of secondary metabolites such as tannins, terpenoids, alkaloids and flavonoids which are favourable for drugs development or nutritional supplements (Hendra *et al*., 2011). Gallic acid isolated from the fruits of *P. macrocarpa* has proven to inhibit the proliferation of various cancerous cells including human esophageal cancer (TE-2), gastric cancer (MKN-28), colon cancer (HT-29), breast cancer (MCF-7), cervical cancer (CaSki), and malignant brain tumor (CGNH-89 and CGNH-PM) (Faried *et al.,* 2007). To date, there is no detailed study reporting on the mechanism of actions of *P. macrocarpa* fruit extracts particularly through the regulation of miRNAs. Therefore, this study was done to investigate the cytotoxic activities and cell death mechanism regulated by miRNA in relation to the cytotoxicity of *P. macrocarpa* against MDA-MB-231 breast cancer cells as a model.

MicroRNA (miRNA) is a noncoding RNA with 18 to 25 nucleotides, which plays pivotal role as dysregulator of gene expression in biological activities including apoptosis, cellular differentiation and cancer cell proliferation (Garzon *et al*., 2010). The miRNA known as 'oncogene' when overexpressed in tumorigenesis while miRNAs also react as 'tumour suppressor' by downregulating the cancer-related gene

(Iorio and Croce, 2012). In order to identify the roles of miRNAs in tumorigenesis and development, it is worth scrutinizing the related miRNAs and putative target genes in the anticancer mechanism of *P. macrocarpa* ethyl acetate fraction (PMEAF) in MDA-MB-231 breast cancer cell lines. Thus, high-throughput sequencing using next generation sequencing (NGS) technologies were used in this study to analyse data from Illumina (NGS Sequencing Data Library) based on mapping to miRBase v21 for detection of differentially expressed miRNAs in tumorigenesis (Wang *et al*., 2016). Subsequently, the target prediction database of miRGate Database Query was used through free online algorithms to identify putative target genes involved in mediating the cytotoxic effect of *P. macrocarpa* in MDA-MB-231 breast cancer cell lines. Besides, PhenomiR 2.0 database was used to analyse dysregulation of selected miRNA expression in diseases and other biological processes. Lastly, to finalize the selected upregulated and downregulated miRNAs, David Functional Annotation Tools v6.8 was used for gene-annotation enrichment analysis, functional annotation clustering and KEGG pathway analysis.

This study has been conducted by using MTT and CyQuant cytotoxicity assay, various microscopy methods, flow cytometry (Annexin V/PI) analysis, cell cycle analysis, Reactive Oxygen Species (ROS) determination and Mitochondrial Membrane Potential assay. Human Apoptosis Array Kit was used to analyse the expression profiles of apoptosis-related proteins such as Bcl-2, Bax, Bid, Bcl-w, Cytochrome *c*, p21, p27, p53, Caspase 3, Caspase 8, Survivin, XIAP and SMAC. The miRNA sequencing by Illumina Hi-Seq was done to explore the role of the putative miRNAs and their targeted genes as well as the involvement in the pathway of cell death mechanism regulated by *P. macrocarpa* fruit ethyl acetate fraction (PMEAF).

1.2 Research Objectives

Therefore the current study was undertaken with the following objectives:

1. To evaluate the cytotoxicity of *P. macrocarpa* ethyl acetate fraction (PMEAF) against MDA-MB-231 and Vero cells.
2. To investigate the preliminary cytotoxicity mechanism of *P. macrocarpa* ethyl acetate fraction (PMEAF) on morphology and cytology of MDA-MB-231 cells by light, holographic, scanning and transmission electron microscope.
3. To study the detailed cytotoxicity mechanism induced by *P. macrocarpa* ethyl acetate fraction (PMEAF) on MDA-MB-231 cells.
4. To identify the putative miRNAs and target genes regulated by *P. macrocarpa* ethyl acetate fraction (PMEAF) on MDA-MB-231 cells through next generation sequencing (NGS) and bioinformatics tools.

CHAPTER 2.0: LITERATURE REVIEW

2.1 Plants as potential natural product

Natural product to be used in medicine is derived from various sources including native plants, microorganisms, marine organisms, terrestrial vertebrates and invertebrates (Chin *et al*., 2006). Medicinal plants have played an important role throughout the world in treating and preventing diseases since ancient times. The discovery of pure compounds as active principles in plants was first described at the beginning of the 19th century, and the art of exploiting natural products has become part of the molecular sciences. Plants do not only provide food and shelter but also help in curing human diseases and act as a splendid source of bioactive compounds with anticancer, antioxidant, antimicrobial and antiparasitic activity which has been of recent interest among researchers (Khan *et al*., 2010). The developments of natural products as medicine are due to their pharmacological activities and potential therapeutic uses. Indirectly, natural plant medicine represents a way to rescue valuable aspects of traditional culture. Drugs that are derived from natural products are effective in treating various diseases at specific characteristics with less or no side effects (Shah *et al*., 2013). This is due to the bioactive compounds in natural products which give benefits to the body by improving the immune system. Indirectly, naturally-derived drugs enable patients to withstand higher and more effective dosage of treatments such as chemotherapy without additional side effects (Bhadury *et al*., 2006). Besides that, patients from low income developing countries claim that natural products medicine are cheaper, effective and have less side effects if compared to synthetic drugs (Ahsan *et al*., 2009).

Research on medicinal plants has been supported worldwide. The major target of the research is the identification of the values of active compound in medicinal plants and the pharmacological investigation of the extracts which enhance their safety, effectiveness and constant activity. The World Health Organization (WHO) estimates that 80% of people in developing countries are on traditional medicine for their primary health care needs and about 85% of traditional medicine involves the use of plant extracts as sources of drugs.

2.2 *Phaleria macrocarpa*

2.2.1 General Description

Phaleria macrocarpa (Scheff.) Boerl, a plant from Thymelaeceae family was first designated by Scheffer as *Drimyspermum macrocarpum* according to fruiting specimens collected by Teysmann near Dore, in western New Guinea (Angiosperm Phylogeny Group, 2003). *P. macrocarpa,* is commonly known as God's crown or Mahkota Dewa, is one of the Indonesian's native medicinal plants that grow on the island of Papua. It is believed to have the ability to treat various diseases with an abundance of benefits. The plant has also been used traditionally by traditional healers in medical and health treatments (Azmir *et al.,* 2014). The name "God's crown" given to this fruit implies that it descends from heaven, as godsend from divine powers to help mankind.

2.2.2 Botanical Description

It is a tree (Figure 2.1), including stem, leaves, flowers, fruits and thrives in loose, fertile soil at an altitude of 10 to 1200 m above sea level. The tree height ranges from 1 to 18 m with sap exuding 1 m long root and its bark are brownish green and it has

white wood. The leaves are green in colour with the length from 7 to 10 cm and width ranging from 3 to 5 cm. The flowers are typically with 2 to 4 petals of green to maroon color. Seeds exist as 1 to 2 seeds per fruit and are brown, ovoid and anatropous. Although the herb is being used in both un-processed and processed form, however, the former can be poisonous and toxic (Yosie *et al*., 2011). *P. macropcarpa* fruit is of eclipse shape with a diameter of 3 cm. Fruits are green when unripen and become red on ripening (Hendra *et al*., 2011) where the flesh is white, fibrous and watery. It can be propagated by grafting and use of seeds (generative).

Figure 2.1: *Phaleria macrocarpa***.**
(1) A bunch of red ripe fruits, **(2)** *Phaleria macrocarpa* tree, **(3)** Green fruit and leaves. The pictures are taken from ECO HUB, Pusat Repositori Kearifan Tempatan, Universiti Sains Malaysia.

2.2.3 Taxonomical Classification

Table 2.1: Taxonomic classification of *Phaleria macrocarpa.*

CATEGORY	CLASSIFICATION
Kingdom	Plantae
Subkingdom	Tracheobionta
Superdivision	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Rosidae
Order	Myrtales
Family	Thymelaeaceae
Genus	Phaleria
Species	*Phaleria macrocarpa*

Source: Angiosperm Phylogeny Group, 2003.

2.2.3(a) Common Names

The Javanese referred to this tree as Makuto Dewo, Makuto rojo, Makuto queen, Makuta god, and 'Pau' (Susilawati *et al.*, 2011). It is also called 'Simalakama' in Sumatra, Depok (West Java), 'Mahkota Dewa' in Malay, Crown of God or God's Crown in English (Harmanto, 2005).

2.2.3(b) Synonyms

The Latin name of plant is *Phaleria macrocarpa* (Scheff.) Boerl and synonyms are *Phaleria papuana* Warb var. Wichanii (Val) Back (Hou, 1960). *Phaleria calantha* Gilg, *Phaleria papuana* Warb. ex K. Schum. Lauterb., and *Phaleria wichmannii* Valeton.

2.2.4 Distribution

P. macrocarpa is an indigenous plant from Papua Island (Irian Jaya) or Papua New Guinea, more specifically in the area of Maprik about 110 km journey from the town of Wewak. A God's Crown tree was found at about nine meters in height bearing fruit on every branch. Centuries ago samples of the Mahkota Dewa tree were transported from the island of Papua by traditional Javanese medicine men and planted in the palace grounds of Solo and Jogyakarta. Its native habitat is terrestrial primary rainforest; it grows well in tropical areas especially in Malaysia and is known as a popular herbal plantation in the South Asian countries.

2.2.5 Ethnomedicinal Uses

Each part of this plant including fruits, seeds, stems and leaves have their own healing power. The fruits of *P. macrocarpa* have the ability to treat flu, rheumatism, heart diseases and cancer while the leaves are used to treat dysentery, allergy, tumour and impotency. The stems are beneficial in the treatment of bone cancer (Tjandrawinata *et al*., 2010). A decoction of the dried fruit is taken orally to control breast cancer, cervix cancer, lung disease, liver, and heart diseases. Seeds are used as external medicine for the treatment of skin problems and mainly for cultivation as a traditional bio-pesticide (De Padua *et al*., 1999). These proven findings are advancing current scientific

research in developing various herbal formulations to inhibit the growth and spread of breast cancer (Nagaprashanthi *et al*., 2012). Figure 2.2 is a summarized diagram of most bioactive compounds from *P. macrocarpa* with their respective biological activity.

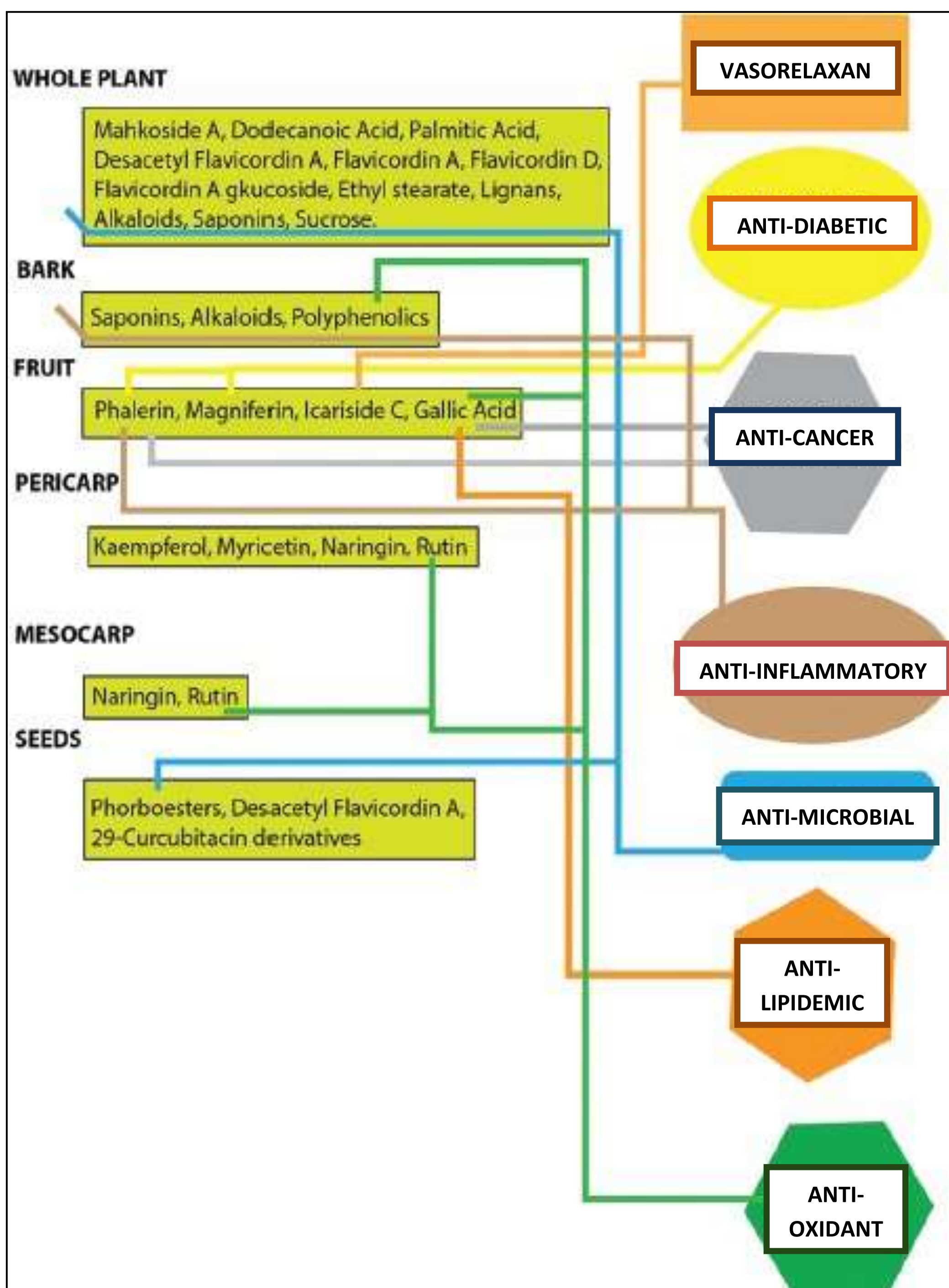

Figure 2.2: Phytochemical constituents isolated from *Phaleria macrocarpa* with their respective biological activity.
Source: Altaf *et al*., 2013.

2.2.6 Phytochemistry

Phytochemical studies of *P. macrocarpa* have proven that various parts of the plant contain diverse chemical constituents. Mahkoside A (4,4′ dihydroxy-2-methoxybenzophenone-6-O-β-D-glucopyranoside), magniferin (xanthonoid), kaempferol-3-o-β-D-glucoside, dodecanoic acid, palmitic acid, ethyl stearate, and sucrose were isolated from the seeds (Zhang *et al*., 2006). The bark is rich in saponins, alkaloids, polyphenolics, phenols, flavonoid and lignans meanwhile the fruit is rich in tannins, cariside C3, magniferin, gallic acid and phalerin (Oshimi *et al.,* 2008). Phalerin, known as benzophenone glycoside (3,4,5, trihydroxy-4-methoxy-benzophenone-3-O-β-D-glucoside) was first isolated from leaves of *P. macrocarpa* (Hartati *et al*., 2005). The pericarp of fruit contains kaempferol, myricetin, naringin and rutin. Naringin and quercitin are found in mesocarp as well as seeds (Hendra *et al*., 2011). Phorboesters, des-acetyl flavicordin-A and 29-norcucurbitacin derivatives have been isolated from seeds (Kurnia *et al*., 2008). Structures of representative secondary metabolites isolated from *P. macrocarpa* are shown in Figure 2.3.

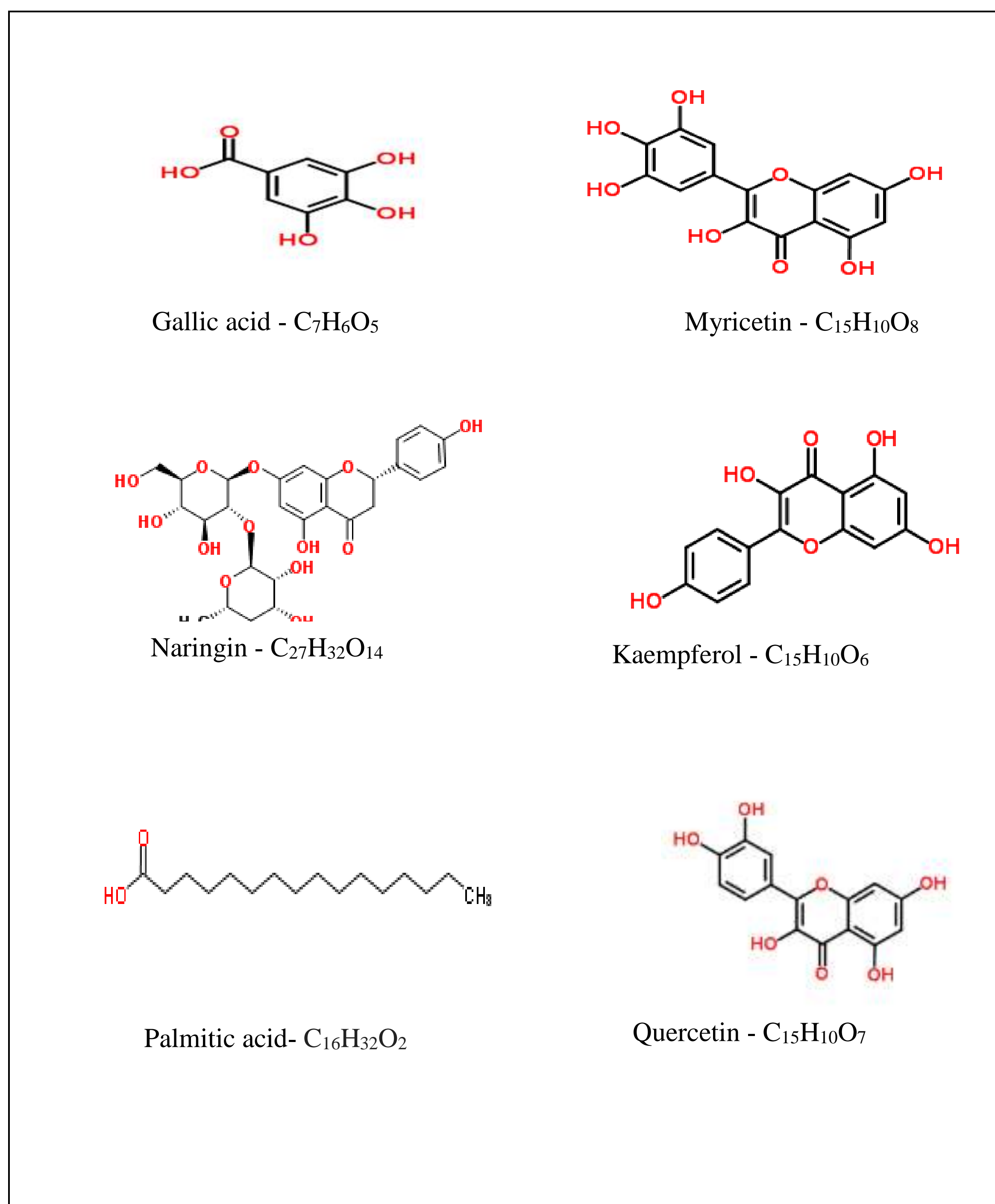

Figure 2.3: Chemical structures of some known compounds found in *Phaleria macrocarpa.*
Source: http://www.chemspider.com/chemical+structure

2.2.7 Pharmacological Activities

P. macrocarpa was commonly used for the treatment of various diseases in folk medicine and various pharmacological activities were reported in literature including anticancer, antidiabetic, anti-inflammation, antibacterial, antioxidant, and antifungal effects (Hending and Ermin, 2009).

2.2.7(a) Anticancer activity

Every part of *P. macrocarpa* including leaves, bark, stem, seed and fruits are widely used as traditional medicine since ancient time in treating different types of cancer especially against breast cancer (Faried *et. al*., 2007; Hendra *et. al*., 2011). Many studies have been proven scientifically that gallic acid showed significant anticancer activity by inhibiting cell proliferation in different cancer cell lines such as human melanoma cell (Lo *et al*., 2010), human hepatocellular carcinoma cell (Sun *et al*., 2016), human small lung cancer cell (Wang *et al*., 2016) and ovarian cancer cell (He *et al.,* 2016). For example, Faried *et al*. (2007) have evaluated the isolated GA from *P. macrocarpa* which inhibited cancer cell proliferation and induced apoptosis in esophageal cancer cell (TE-2). Besides that, ethyl acetate fraction of *P. macrocarpa* (PMEAF) was reported to inhibit cell proliferation by inducing cell death in MDA-MB-231 breast cancer cell (Tjandrawinata *et al*., 2010) and also has proven its capability as an anti-proliferative agent and initiates apoptotic cell death in MCF-7 cell which is an estrogen-dependent and fast-growing cell (Tjandrawinata *et al*., 2010).

2.2.7(b) Antidiabetic activity

P. macrocarpa, traditionally known for its anti-diabetic properties and has been found to decrease the post-prandial hyperglycemia in diabetic patients. The bioassay-guided

fractions of *P. macrocarpa* fruit were examined for α-glucosidase and α-amylase activity to discover the anti-diabetic mechanism and potential attenuation action on post-prandial glucose increase. The study revealed that *P. macrocarpa* can lower hyperglycaemia in both *in vitro* and *in vivo* experiments by effectively inhibiting carbohydrate-hydrolysing enzymes. The natural compounds from the extract have a therapeutic effect on type 2 diabetes mellitus (Ali *et al*., 2013). Moreover, few studies suggested that the natural compounds from *P. macrocarpa* fruit extract work as healing treatment for type 2 diabetes mellitus (Kim *et al*., 2010). Another study has reported to decrease the blood glucose due to the presence of magniferin in the most active n-butanol sub-fraction of methanol extract of *P. macrocarpa* fruit pericarp (Easmin *et al*., 2015).

2.2.7(c) Anti-inflammatory activity

Current researches are more focused on developing drugs or dietary supplements using secondary metabolites of *P. macrocarpa* such as phalerin, saponins, and alkaloids which indicated anti-inflammatory properties. Hendra *et al*. (2011) have done anti-inflammatory *in vitro* assays by using *P. macrocarpa* methanolic fruit extract treated against macrophage RAW 264.7 cell lines induced by LPS/IFN-γ. The results showed inhibition of inducible nitric oxide synthesis in macrophage and indicating their notable anti-inflammatory potential. Meanwhile, *in vivo* anti-inflammatory studies were conducted on animal model; Wistar female rats to determine the effect of dominant compound in *P. macrocarpa*; hydroxyl benzophenon glucoside. The result showed that the inflammation in rat treated with hydroxyl benzophenon glucoside at 22.5 mg/kg per body weight had decreased two-fold compared to normal drug (Mariani *et al*., 2010).

2.2.7(d) Antibacterial activity

Empirically, microorganisms have shown resistance to synthetic antimicrobial agents and this resistance are the current issues in the medical field. Therefore, investigations of alternative medicines from natural products are required to solve those complications. Flavonoids are classified under phenolic groups in plants which have been known to possess antimicrobial activity. The antibacterial assay of *P. macrocarpa* fruit extracts was carried out by the disc diffusion method and tested against Gram-negative bacteria (*Enterobacter aerogenes, Escherichia coli, Klebsiella pneumonie, Pseudomonas aeruginosa)* and Gram-positive bacteria (*Bacillus cereus, Bacillus subtilis, Micrococcus luteus,* and *Staphylococcus aureus*) (Hendra *et al.,* 2011). This study elucidated that the flavonoids compounds of *P. macrocarpa* fruit may possess antimicrobial activities which can be used as an alternative antimicrobial agent in pharmaceutical and cosmetic products (Hendra *et al*., 2011).

2.2.7(e) Antioxidant activity

Various scientific reports have proven that *P. macrocarpa* is a rich source of a polyhydroxyphenolic compound known as GA which is a natural antioxidant. Furthermore, phenolics compounds from the extract of *P. macrocarpa* have also indicated to have biological function as an antioxidant. Lay *et al*. (2014) have examined the antioxidant activity of *P. macrocarpa* fruit extract and fractions by determining the DPPH free radical scavenging property using the UV spectrophotometric method. The results revealed that an ethyl acetate fraction of *P. macrocarpa* exhibited the highest free radical scavenging activity followed by the methanol extract, hexane fraction, chloroform fraction and water fraction. A recent study was undertaken by treating fructose fed male Sprague-Dawley rats with

methanolic extract of *P. macrocarpa* resulted in the prevention of fructose-induced oxidative stress in rats and decreased endogenous antioxidant activity (Yanti *et al.*, 2015).

2.2.8 Toxicological Assessment

Scientific information and evidence on toxicology study is very crucial in terms of safety, quality, associated toxicity and on the side effects of long term use of the products. A toxicity assessment provides an estimate of how much of a chemical substance is needed to cause harm, in addition to the types of harm it causes. The right dosage differentiates a poison from a remedy (Ernest, 2011). There are different procedures to assess carcinogenic or non-carcinogenic effects which can elucidate the consequence and importance of a toxicity assessment. Toxicological studies are conducted by exposing animal (*in vivo*), cells or tissues (*in vitro*) to chemicals. In addition, intake of medicinal plants without assessing its efficacy and safety can cause unpredicted toxic effects that may damage the organs in the human body. Liver and kidney are the main targets in toxicological evaluation due to the metabolic activity and excretion of chemical components.

Even though *P. macrocarpa* has been claimed for its abundance of valuable medicinal properties as therapeutic agents, it may show toxicity effect at high concentrations. Due to the possibility of toxicity effect, supportive toxicity study on *P. macrocarpa* is needed to evaluate the efficacy and safe concentration to produce promising data of *P. macrocarpa* in curing diseases. Chong *et al.* (2011), reported that *P. macrocarpa* exhibited fetotoxicity effect in female mice when fed at dose of 27 mg/kg. Besides that, the fresh fruit of *P. macrocarpa* is taken orally as traditional medicine for treatment of ulcers by the Indonesians (Easmin *et al.*, 2015). Butanol

extracts of ripened fruits of *P. macrocarpa* is reported to cause mild necrosis of proximal convoluted tubules in mice kidney at a dosage higher than 85 mg/kg (Altaf *et al*., 2013). Moreover, toxicological assessment will give a very good indication and the confidence to move the research to clinical trials with suggested bioactive compound of *P. macrocarpa* in future.

2.2.9 Precautions/Safety for Usage

Almost all traditional medicinal plants usage is based on knowledge, skills, practices and beliefs of indigenous people of different culture, and is not scientifically validated for its safety and effectiveness. Each drug derived from plants need appropriate scientific knowledge and information about therapy to prescribe and administer accurately. Fundamentally, some important precaution should be taken to ensure the plant parts are not sprayed with weed killer or pesticides. Then the samples are needed to be washed thoroughly or soaked in water to remove unwanted pollutants before being further processed as dietary or supplements.

The bioactive components extracted from natural plant or herbs with therapeutic activity need to be identified and the preparation should be standardized by quantifying chemical constituent through acceptable analytical methods. In order to analyse the causes of adverse effects of the drugs, it needs a specific technical expertise, facilities and suitable analytical laboratories to investigate the products concerned. For example, the WHO guidelines on safety monitoring of herbal medicines in pharmaceutical industries were used to analyse the herbal products (WHO, 2014). Certain imperative challenges regarding effective observation of natural or herbal derived medicine safety are critically important. Therefore, an adequate protection of public health can be provided by focusing on related regulatory

agencies involved in producing material safety data sheet (MSDS) of the herbal products (Ekor, 2014).

2.3 Cancer

Cancer is currently the second leading cause of death after heart diseases worldwide (WHO, 2014). In Malaysia, cancer is a major cause of morbidity and mortality among Malaysian population. According to National Cancer Registry Data, it was estimated that there are nearly 40,000 new cases per year and a cumulative lifetime risk of about 1:4. In addition, reports from the National Cancer Society of Malaysia (NCSM) projects presented that one in every four Malaysians is likely to develop cancer by the age of 75 years.

There are plenty of known cancer types characterized by its origin such as breast cancer, bladder cancer, lung cancer, brain cancer, melanoma, non-Hodgkin lymphoma, cervical cancer, ovarian cancer, colorectal cancer, pancreatic cancer, oesophageal cancer, prostate cancer, skin cancer etc. Typically, cancer can be initiated through genetic disorders when a defective gene in a particular chromosome is passed to the next generation or when imperfections in DNA replication are found in inherited genes (Schmid, 1963; Van Loo and Voet, 2014). An increase in the ageing population, obesity, physical inactivity, nutrition intake and environmental risks such as the annual haze in Malaysia are some of the additional factors. Several cancers are associated with infectious virus and bacteria such as hepatitis B virus (HBV), human papillomavirus (HPV), human immunodeficiency virus (HIV), and *Helicobacter pylori* (bacteria).

Cancer is the abnormal cell growth that forms tumours and these tumours can be divided into ‘benign’ (non-cancerous cells) and ‘malignant’ (cancerous cells) that

invade and destroy healthy tissue in a process called invasion. In recent years, there is advancement in cancer treatments where more than cures, scientists are enthusiastically finding possibility of early detection and prevention of cancer (Osaki *et al*., 2015). Since, cancer requires a long time to develop, therefore, there is opportunities to prevent cell proliferation, mutation and cancer progression at an early stage. The most common type of cancer treatment is surgery which implies the primary treatment option for most types of cancer is to remove solid tumours. In addition, another two common treatments are radiotherapy that uses high-energy X-rays and chemotherapy that uses powerful cancer-killing medications. Although modern treatments aim to eliminate the cancer cells but side effects still arise with the use of synthetic drugs in cancer treatment (Bertrand *et al*., 2014).

2.3.1 Breast Cancer

Breast cancer affects women worldwide and it is the leading cause of fatality in Asia but it occurs less frequently in men. Originally, breast cancer cells are formed in the tissues of the breast and divide at an abnormally faster rate to form a lump. There are two types of breast cancer namely ductal carcinomas beginning in the tubes (ducts) and lobular carcinoma in the parts of the breast (lobules). Breast cancer rates are increasing with age of most women above 50's and treatments vary depending on stage (Stage I, II, III and IV) of cancer (Mahmood *et al.,* 2013). The stages of breast cancer are crucial factors in determining prognosis before treatment starts. Staging involves clinical examination, mammogram, biopsy, and certain imaging investigation such as chest radiograph (CXR), liver ultrasound (LUS), bone scan (BS), computed tomography scan (CT scan) and magnetic resonance imaging (MRI) (Graham *et al*., 2014).

Surgery to remove cancerous tissue is known as 'lumpectomy' (remove the cancer and leave the healthy tissue behind) and 'mastectomy' (removal of all the breast gland tissue). Subsequently, chemotherapy treatments are given through injection of 'chemo' medicine such as docetaxel, paclitaxel, vinorelbine, capecitabine, liposomal doxorubicin, gemcitabine, and mitoxantrone to kill cancer cells. Radiation therapy, which involves a high-dose of radioactive substance (brachytherapy) that injected into blood to destroy cancerous tissue. Final follow up with hormone therapy medicine for breast cancer such as tamoxifen, toremifene, and fulvestrant as an oral intake for long period of time with a minimum 5 years (Kuźma-Richert *et al*., 2011). It is a preventive measure to prevent gene alteration in cancer cells which blocks specific hormones that stimulate cancer development. Hormone medicine namely Tamoxifen, is specifically endorsed for women of breast cancer with hormone receptor-positive (ER- positive) breast cancers, but there is no effective medicine for women with hormone receptor-negative (both ER- negative). Example of ER positive breast cancer cell lines such as MCF-7, T-47D, BT-474, BT-483, and 600MPE while ER negative breast cancer cell lines are MDA-MB-231, SkBr3, Hs578T, Evsa-T, BT-549, BT-20, and AU565 (Niemeier *et al.,* 2010).

Unfortunately, most of the treatments cause side effect, different treatment dose response and resistance after prolonged exposure to the treatments. The synthetic drugs applied in chemotherapy and radiation treatment not only kill the cancer cells but also affect the healthy cells by causing side effect such as nausea, anaemia, vomiting, weakening of the immune system, diarrhoea and hair loss. Studies have proven that cancer cells have the ability to change and develop resistance towards chemotherapeutic drugs due to the prolonged intake (Kaur *et al.,* 2016). Therefore current studies focus more on finding novel therapeutic agents derived from natural

products/plants as anticancer agents which effectively treat cancer with less/no side effects.

2.3.2 Plant as Anticancer Agents

Since ancient time, plants possess medical history in the treatment of cancer according to Hartwell (1982), where it has been acknowledged that more than 3000 plant species were discovered as a favourable natural medicine in treatment of cancer but some of this plant species were used without proper references.

Currently there are many experiments being carried out to isolate the bioactive components as anti-cancer agents. The potential new anticancer drugs which are undergoing pre-clinical trial are selected based on molecular targets and are comparable or even have better outcomes than synthetic drugs. There are several plant-derived anticancer drugs that are successfully used namely; vincristine, the camptothecin derivatives, topotecan and irinotecan, etoposide, derived from epipodophyllotoxin, and paclitaxel (Sisodiya, 2013).

2.3.3 Evaluation of Cytotoxicity

Most of the active compounds, either in the form of chemicals or drugs from natural products have the potential to be toxic or poisonous at highest concentration and also depends on individual absorption capacity. Therefore, toxicity evaluation of targeted natural compound is an essential criterion to provide safety data which is useful in further studies or during development of new anticancer agent (Gurib-Fakim, 2006). The suggested anticancer properties of crude extract or fractioned compounds need to be tested for cytotoxicity. Cytotoxicity test is a biological assessment and preliminary screening on tissue cells by *in vitro* methods to observe cell death, proliferation and

morphological effects. There are numerous cytotoxicity assays such as MTT assay, MTS assay, CyQuant, XTT, Trypan Blue and etc., which will indicate the cell viability, inhibitory concentration (IC) or effective concentration (EC) value of drug concentration-treated cell lines. The United States National Cancer Institute permits the anticancer cut off point for IC_{50} value to be below than 20 µg/mL (Kuete *et al.*, 2016).

2.4 Cell Death

The balances between cells division and cells death are significantly important for the normal growth and survival of multicellular organisms. Cell death happens when the cells end normal cellular functions, such as respiration, metabolism, growth and proliferation. Cell death can be distinguished by two different mechanisms namely "non-programmed cell death" due to accidental injury or shock (necrosis) and "programmed cell death" in terms of apoptosis.

In addition, cell deaths are differentiated based on morphological changes (apoptotic, necrotic and autophagic) through various microscopic techniques and biochemical assays. Additionally, the caspase activation, collapse of mitochondrial outer membrane permeabilization and exposure of phosphatidylserine to the outer membrane will induce apoptosis in dying cells (Kroemer *et al.*, 2013).

2.4.1 Apoptosis

Apoptosis or programmed cell death is a normal component of the development and health of multicellular organisms. Homeostasis is maintained through a balance between cell proliferation and cell death. It is a process of controlled cellular death whereby the activation of specific death-signaling pathways leads to deletion of cells