MOLECULAR CHARACTERIZATION AND BIOINFORMATICS ANALYSIS OF MICRORNA-221-5P REGULATED BY STANDARDIZED *Polyalthia longifolia* (Sonn.) Thwaites LEAF EXTRACT IN HELA CELL LINES

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by

SHANMUGAPRIYA

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Δψm	Mitochondrial membrane potential
µg/mL	Microgram per milliliter
µM	Micro molar
2D	Two-dimensional
Å	Angstrom
ADCs	Analog-to-digital converters
AIF	Apoptosis inducing factor
ANOVA	Analysis of variance
AO/PI	Acridine orange and propidium iodide
Apaf-1	Apoptotic protease activating factor-1
APCI	Atmospheric-pressure chemical ionization
APL	Acute promyelocytic leukemia
APLP2	Amyloid beta (A4) precursor-likeprotein 2
AP-MS	Affinity purification–mass spectrometry
APS	Ammonium persulfate
AVD	Apoptotic volume decrease
BCA	Bicinchoninate assay
Bcl	B-cell lymphoma
BIND	Biomolecular interaction network database
BMF	B-cell lymphoma 2 modifying factor
bp	Base pair
BSA	Bovine serum albumin
C. elegans	*Caenorhabditis elegans*
CAD	Caspase-Activated dnase
CARD	Caspase-recruitment domain
CBB	Coomassie brilliant blue
CCDB	Cervical cancer gene database
CDH15	Cadherin-15
CDK	Cyclin-dependent kinase
CDKN1B	Cyclin-dependent kinase inhibitor 1B
cDNA	Complementary DNA

CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1
CID	Collision-induced dissociation
cm	Centimeter
CPEB1	Cytoplasmic polyadenylation element binding protein 1
Ct	Cycle threshold
dATP	Deoxyadenosine triphosphate
DAVID	Database for Annotation, Visualization and Integrated Discovery
DGCR8	*Di George* Syndrome critical region gene 8
DISC	Death inducing signalling complex
DMEM	Dubelcco's minimum essential medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
dsDNA	Double-stranded deoxyribonucleic acid
DSG1	Desmoglein 1
DTT	Dithiothreitol
EBV	Epstein-Barr virus
ECD	Electron capture dissociation
ERK	Extracellular signal-regulated kinase
ESI	Electrospray ionization
ETD	Electron-transferdissociation
FAF1	Fas-associated factor 1
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FDR	False discovery rate
FRET	Forster resonance energy transfer
GO	Gene ontology
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HPRD	Human protein reference database
HPV	Human papilloma virus
IAA	Iodoacetamide
IC_{50}	Half maximal inhibitory concentration

IEF	Isoelectric focusing
IGFBP5	Insulin-like growth factor binding proteins 5
IL18	Interleukin 18
ITGAV	Integrin alpha-V
ITGB1	Integrin, beta 1
JNK	Jun N-terminal kinases
K^+	Potassium
kHz	Kilo hertz
KISS	Kinase substrate sensor
LC–ESI–MS/MS	Liquid chromatography-electrospray ionization-mass spectrometry/ mass spectrometry
LDH	Lactate dehydrogenase
m/z	Mass to charge ratio
MAL	Myelin and lymphocyte protein
MALDI	Matrix-assisted laser desorption/ionization
MAPPIT	Mammalian protein–protein interaction trap
mg/g	Milligram per gram
mg/kg	Milligram per kilogram
mg/mL	Milligram per milliliter
MGB	Minor groove binder
min	Minutes
MINT	Molecular interaction database
miRISC	Mirna-Induced Silencing Complex
miRNA	Microrna
MOMP	Mitochondrial outer membrane permeabilization
mRNA	Messenger RNA
MSN	Moesin
MTT	3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCBI	National Center for Biotechnology Information
NCI	National cancer institute
NFQ	Nonfluorescent quencher
nm	Nano meter
nM	Nano molar

ºC	Degree celsius
OD	Optical density
P. longifolia	*Polyalthia longifolia*
PANTHER	Protein Analysis through Evolutionary Relationships
PAZ	Piwi, Arganoate and Zwille
PBS	Phosphate-buffered saline
PCD	Programmed cell death
PIK3R1	Phosphoinositide-3-kinase regulatory subunit 1
PLA	Proximity ligation assay
Plk1	Polo-like kinase 1
*p*NA	*P* nitroanilide
PPIN	Protein-protein interaction networking
pre-miRNA	Precursor mirna
pri-miRNA	Primary mirna
PS	Phospholipid phosphatidylserine
PTEN	Phosphatase and tensin
PTP	Permeability transition pore
PTPC	Permeability transition pore complex
PUMA	P53 upregulated modulator of apoptosis
Q-TOF	Quadrupole Time-of-Flight
RARG	Retinoic acid receptor gamma
RCF	Relative centrifugal force
RIA	Radioimmunoassay
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RP	Ribosomal protein
RPM	Revolutions per minute
rRNA	Ribosomal RNA
RT	Reverse transcription
RT	Room temperature
RT-qPCR	Real time quantitative polymerase chain reaction
RVI	Regulatory volume increase

SD	Standard deviation
SDG	Sustainable development goals
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscopy
SID	Surface-induced dissociation
siRNA	Small interfering RNA
snoRNA	Small nucleolar RNA
TEM	Transmission electron microscopy
THBS2	Thrombospondin-2
TIMP3	Tissue inhibitor of metalloproteinase-3
Tm	Melting temperature
TNF	Tumor necrosis factor
TOF	Time-of-flight
TRAF-6	Tnf receptor-associated factor 6
TRAIL	TNF-related apoptosis-inducing ligand
tRNA	Transfer RNA
UTR	Untranslated region
UV	Ultraviolet
XIAP	X-linked inhibitor of apoptosis
ΔΔCT	Double delta cycle threshold

PENCIRIAN MOLEKUL DAN ANALISIS BIOINFORMATIK MICRORNA-221-5P YANG DIKAWALATUR OLEH EKSTRAK PIAWAI DAUN *Polyalthia longifolia* (Sonn.) Thwaites DI DALAM SEL HELA

ABSTRAK

Polyalthia longifolia (Sonn.) Thwaites adalah sejenis spesies tumbuhan yang kaya dengan nilai-nilai perubatan. Penyelidikan saintifik terkini ekstrak daripada daun *P. longifolia* telah mendedahkan sifat anti kansernya terhadap sel HeLa melalui pengaruhan apoptosis yang bersandarkan kaspase dengan mengawal ekspresi miRNA. Walau bagaimanapun, belum ada kajian dilaksanakan untuk melaporkan analisis fungsi mikroRNA terkawal-atur dan tiada bukti saintifik pengesahan pengawalan ekspresi mikroRNA dalam sel HeLa yang dirawat denagn ekstrak daun methanolic *P. longifolia*. Oleh itu, kajian ini dijalankan untuk mengesahkan ekspresi miRNA dalam sel HeLa yang dirawat dengan ekstrak daun *P. longifolia* berbanding dengan sel-sel HeLa kawalan yang tidak dirawat. Kajian ini telah memberi butiran yang khusus tentang analisis fungsian dan analisis proteomik terhadap ekspresi miRNA. Dalam kajian ini, ekstrak daun *P. longifolia* yang segar telah disediakan dan ujian MTT telah dilakukan untuk mengenal pasti nilai IC_{50} terhadap sel HeLa. Ekspresi miR-221-5p disahkan dengan menjalankan RTqPCR masanyata TaqMan yang mengesahkan regulasi penurunan ekspresi miR-221-5p dalam sel HeLa yang telah dirawat dengan ekstrak daun *P. longifolia* berbanding dengan sel yang tidak dirawat. Analisis fungsi miR-221-5p dilakukan dengan pendekatan mengaktifkan dan menyahaktifkan fungsi miRNA tersebut bersama-sama dengan menggunakan ujian sel pertumbuhan MTT, ujian pewarna Annexin-V FITC dan propidium iodida dan diukur melalui aliran sitometri dan ujian kaspase-3. Selain itu, perubahan

morfologi struktur ultra sel HeLa telah dikaji melalui mikroskop elektron transmisi (TEM), imbasan (SEM) dan mikroskop pendarfluor bagi sel yang diwarana dengan pewarna akridin dan propidium iodida (AO/PI). Analisis proteomik tentang protein yang dikawalatur oleh miR-221-5p juga dilakukan dengan dengan penggunaan LC-ESI-MS/MS. Di samping itu, analisis bioinformatik telah dilakukan untuk mengenal pasti sasaran mRNA untuk miR-221-5p melalui alat bioinformatik miRGate dan gen ontology telah dikaji dengan penggabungan DAVID dan Enrichr serta rangkaian interaksi protein-protein dianalisis dengan menggunakan alat bioinformatik STRING. Penurunan ekspresi miR-221-5p telah disahkan dengan jayanya dalam sel HeLa yang dirawat dengan ekstrak daun *P. longifolia*. Selain itu, mekanisme molekul komprehensif miR-221-5p yang dikaji melalui analisis fungsi dan proteomik jelas menunjukkan peranan miR-221-5p dalam induksi apoptotik intrinsik yang bersandarkan kaspase oleh rawatan ekstrak daun *P. longifolia*. Memandangkan pengekspresan mikroRNA mengambil bahagian dalam pengawalan/ patogenesis kanser dan kecekapannya dalam terapeutik gen semasa yang menasarkan kanser sebagai pendekatan rawatan klinikal yang boleh diterima, maka ekstrak daun *P. longifolia* boleh menjadi calon ubat antikanser baru yang baik.

MOLECULAR CHARACTERIZATION AND BIOINFORMATICS ANALYSIS OF MICRORNA-221-5P REGULATED BY STANDARDIZED *Polyalthia longifolia* (Sonn.) Thwaites LEAF EXTRACT IN HELA CELL LINES

ABSTRACT

Polyalthia longifolia (Sonn.) Thwaites is an exquisite plant species with rich ethnomedicinal values. Recent scientific investigations on *P. longifolia* leaf extract have also revealed its anti-cancer property against HeLa cells through the induction of caspase-dependent apoptosis by regulating microRNA (miRNA) expressions. However, there were no further investigations performed to report the functional analysis of the regulated miRNA, with absolutely no means of scientific evidence of validation of miRNA dysregulation in HeLa cells treated with the methanolic *P. longifolia* leaf extract. Hence, this study was conducted to validate the miRNA expression in methanolic *P. longifolia* leaf extract treated HeLa cells in comparison with untreated HeLa cells with an intricate elucidation of functional and proteomic analysis of miRNA expression. In this study, methanolic *P. longifolia* leaf extract was freshly prepared and MTT assay was performed to identify the IC_{50} value against HeLa cells. The expression of miR-221-5p was validated by performing Taqman real time RTqPCR which confirmed the down-regulation of miR-221-5p in HeLa cells treated with methanolic *P. longifolia* leaf extract compared to the untreated HeLa cells. The functional analysis of miR-221-5p was conducted through gain-of-function and loss-of-function approach by MTT assay, flow cytometric analysis of Annexin V/ Propidium Iodide assay and caspase-3 assay. Besides, the ultra-structural morphological changes in HeLa cells were investigated through

scanning electron microscopy (SEM), transmission electron microscopy (TEM) and fluorescence microscopy for cells stained with acridine orange and propidium iodide (AO/PI). Proteomic analysis of proteins that regulated by miR-221-5p was carried out by LC–ESI–MS/MS. In addition, bioinformatics analysis was performed to identify the mRNA targets of miR-221-5p by miRGate bioinformatics tool and the gene ontology of the predicted genes was determined by DAVID and Enrichr bioinformatics tool while the protein-protein interaction network was analysed using STRING bioinformatics tool. Conclusively, based on the data obtained from this overall study, expression of miR-221-5p has been successfully validated upon *P. longifolia* leaf extract treatment in HeLa cells with its comprehensive molecular mechanism involved through functional and proteomic analysis which clearly indicated the role of miR-221-5p in the induction of caspase-dependent intrinsic apoptotic pathway of cell death by *P. longifolia* leaf extract treatment. Considering the participation of miRNA expression in cancer regulation/ pathogenesis and its efficiency in current gene therapeutic target for cancer as a clinically admissible treatment approach, *P. longifolia* leaf extract could be a promising novel anticancer drug candidate.

CHAPTER 1: INTRODUCTION

1.1 Overview and rationale of study

Cells as the "building blocks of life" are well organized to balance its proliferation and death to ensure the proper development of healthy tissue and to get rid of the damaged cells, respectively (Mason and Rathmell, 2011). The coordination of the cell proliferation as well as the cell death mechanisms is highly important because an unbalanced process may lead to deadly diseases such as cancer. Cancer occurs when cell growth exceeds cell death due to some alteration in the cell causing them to multiply out of control and invade to other parts of the body. Cancer which is also known as malignancy or malignant neoplasm has been accounted to be one of the leading causes of mortality worldwide. Based on the recently published paper on cancer facts and figures 2019, more than 1.7 million new cancer cases are expected to be diagnosed and about 606, 880 deaths is expected to be recorded in United States of America due to cancer in 2019 (American Cancer Society, 2019).

Cancer can be categorized based on the types of cells it occurs such as the carcinomas (epithelial cells), sarcoma (bone and soft tissues), leukemia (blood cells and bone marrow) and lymphoma (lymphocytes). Among the 100 over types of cancer, cervical cancer has attained an alarming health concern among women globally which needs a compelling prevention. Cervical cancer is the third most prevailing gynecologic cancer in USA which peaks in elderly women, usually in their 50s (Dawkins *et al.*, 2018). In fact, 569, 847 incidences of cervical cancer and 311, 365 deaths due to cervical cancer have been reported by world region (Bray *et al.*, 2018). Recent study estimated fourteen million new cancer cases to occur by

2035 which may consequently lead to challenging preventive medicine structure (Pilleron *et al.*, 2019).

Although there are few general treatments available for cervical cancer like radiotherapy, immunotherapy, chemotherapy and surgery; chemoprevention of cancer through the regulation of miRNA with the utilization of medicinal plants is presently being advanced in cancer therapy field. Since dysregulation of genes involved in the biological processes have been convincingly demonstrated to be associated with cancer, miRNA therapeutic approach is highly trustworthy in cancer treatments (Ji *et al.*, 2017). MiRNAs are small, single stranded, non-coding RNA molecules of 20-22 nucleotides that control the expression of target genes by imperfect pairing to multiple mRNA targets. The incomplete base pairing of miRNAs to their mRNA targets at 3' UTRs causes the degradation of mRNA targets and subsequent down-regulation of gene expression and protein inhibition. The participation of miRNAs in the regulation of gene expression at post transcriptional level and subsequent protein translational repression (Bartel, 2004) clearly substantiate the major role of miRNA in diverse biological processes such as cell death (Xu *et al.*, 2004) and cell proliferation (Cheng *et al.*, 2005). Interestingly, various plants rich in anti-cancer bioactive compounds have been shown to regulate the mammalian miRNA levels (Xie *et al.*, 2016; Gezici and Sekeroglu, 2017).

1.2 *Polyalthia longifolia* as an important medicinal plant

Polyalthia longifolia var. Angustifolia Thw. (Annonaceae) is one of the most important medicinal plants which is found throughout Malaysia and widely used in traditional medicine as febrifuge and tonic (The Wealth of India, 1969). The local name of *P. longifolia* is Glodogan tiang. *Polyalthia longifolia* is a small medium-

sized tree with linear-lanceolate leaves, 1 to 1.5 cm broad, occurring in Sri Lanka and now grown in tropical parts of India along road sides and in gardens for their beautiful appearance (Bose *et al.*, 1998). The diterpenes, alkaloids, steroid and miscellaneous lactones were isolated from its bark (The Wealth of India, 1969). The stem bark extracts and isolated compounds were studied for various biological activities like anti-bacterial, cytotoxicity and anti-fungal activity (Goutham *et al.*, 2010). Study conducted by Jothy *et al.* (2012) reported the anti-oxidant activity and hepatoprotective potential of *P. longifolia*. The results of this study revealed that *P. longifolia* leaf extract could protect the liver against paracetamol-induced oxidative damage. They also reported that the observed hepatoprotective activity of *P. longifolia* in their study might be due to its antioxidant activity, resulting from the presence of phenolic compounds in the extracts. Jothy *et al.* (2013) also tested the genotoxic potential of *P. longifolia* leaf against H_2O_2-radical-mediated DNA damage by using plasmid relation, comet, and *Allium cepa* assay. The results of the *in vitro* tests in their study demonstrated that *P. longifolia* leaf was devoid of a significant genotoxic effect under experimental conditions. Their *Allium cepa* assay results showed that, applied in lower concentrations, the methanol extract of *P. longifolia* leaf could be important for maintaining the genetic stability of the organism. An acute oral toxicity study revealed that *P. longifolia* leaf extract was safe after oral administration as a single dose to female albino Wistar rats with up to 5000 mg/kg body weight (Jothy *et al*., 2013).

The induction of apoptosis by *P. longifolia* treatment has been revealed to cause cell cycle arrest at sub G0/G1, G0/G1 and G2/M phases as well as to increase the mitochondria membrane potential depolarization (Vijayarathna *et al.*, 2017a). The proteomic profiling array conducted by Vijayarathna *et al.* (2017a) has also

demonstrated an up-regulation of pro-apoptotic proteins and down-regulation of anti-apoptotic proteins in HeLa cells upon *P. longifolia* treatment. Besides, morphological study through various microscopic approaches also evidently showed the induction of apoptosis in HeLa cells by *P. longifolia* leaf extract treatment based on typical apoptotic cell morphology observed in *P. longifolia* treated cells (Vijayarathna *et al.*, 2017b). The most recent study showed that *P. longifolia* leaf extract induced apoptosis in HeLa cells through the regulation of miRNAs (Vijayarathna *et al.*, 2017c; Vijayarathna, 2017). However, there are no further reports on the relationship between the cytoxicity of *P. longifolia* leaf extract with the detailed mechanism of apoptosis through miRNA deregulation in HeLa cell lines. There is absolutely no further validation and functional analysis of the deregulated miRNAs which has been previously reported. Hence, the current study was undertaken to validate the role of dysregulated miRNA in inducing the apoptotic cell death in HeLa cells and to identify their targets proteins.

The miRNA deregulation, validation and functional analysis in HeLa cell treated with standardized *P. longifolia* leaf extract was conducted by transfection of miRNA mimic and anti-miR into HeLa cells to over-express and silence the miRNA expression, respectively. Validation of miRNA-221-5p in HeLa cells upon *P. longifolia* treatment was carried out through taqman real time RT-qPCR. Functional analysis of miR-221-5p in relation to *P. longifolia* leaf extract treatment in HeLa cells was carried out through MTT, annexin V/PI and caspase 3 assays. Preliminary study of morphology of HeLa cells upon transfection of miRNA mimic and treatment with methanolic *P. longifolia* leaf extract was accomplished using scanning electron microscopy (SEM), transmission electron microscopy (TEM) and dual-fluorescence nuclear staining method using acridine orange (AO) and

propidium iodide (PI) dyes. The detailed proteomics analysis of miR-221-5p in relation to the *P. longifolia* treatment in HeLa cells was implemented through LC-ESI-MS/MS mass spectrometry systems. Further bioinformatics analysis of miR-221-5p was accomplished with the aid of various bioinformatics tools such as migrate (http://mirgate.bioinfo.cnio.es), The Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8 (https://david.ncifcrf.gov/) and a web-based PPI prediction tool called the STRING resource v10.5 (http://string-db.org/). Figure 1.1 shows the workflow of the overall research.

1.3 Objectives

The study was conducted with the objectives:

1. To validate the expression of miR-221-5p in HeLa cells treated with methanolic *P. longifolia* leaf extract treatment in comparison with untreated control HeLa cells.
2. To study the function of miR-221-5p in inducing the apoptotic cell death in HeLa cells treated with standardized *P. longifolia* leaf extract by using miRNA mimic and anti-miR transfection to enhance and knock down the miRNA expression, respectively.
3. To study the morphological changes in miR-221-5p and miR-484 regulated HeLa cells, alongside with *P. longifolia* leaf extract treatment.
4. To investigate the role of miRNAs and their targets in Hela cells to induce apoptotic cell death after treatment with standardized *P. longifolia* leaf extract using bioinformatics approaches.
5. To identify the target apoptotic related proteins of miR-221-5p in HeLa cells treated with standardized *P. longifolia* leaf extract through proteomic approach.

1.4 Workflow

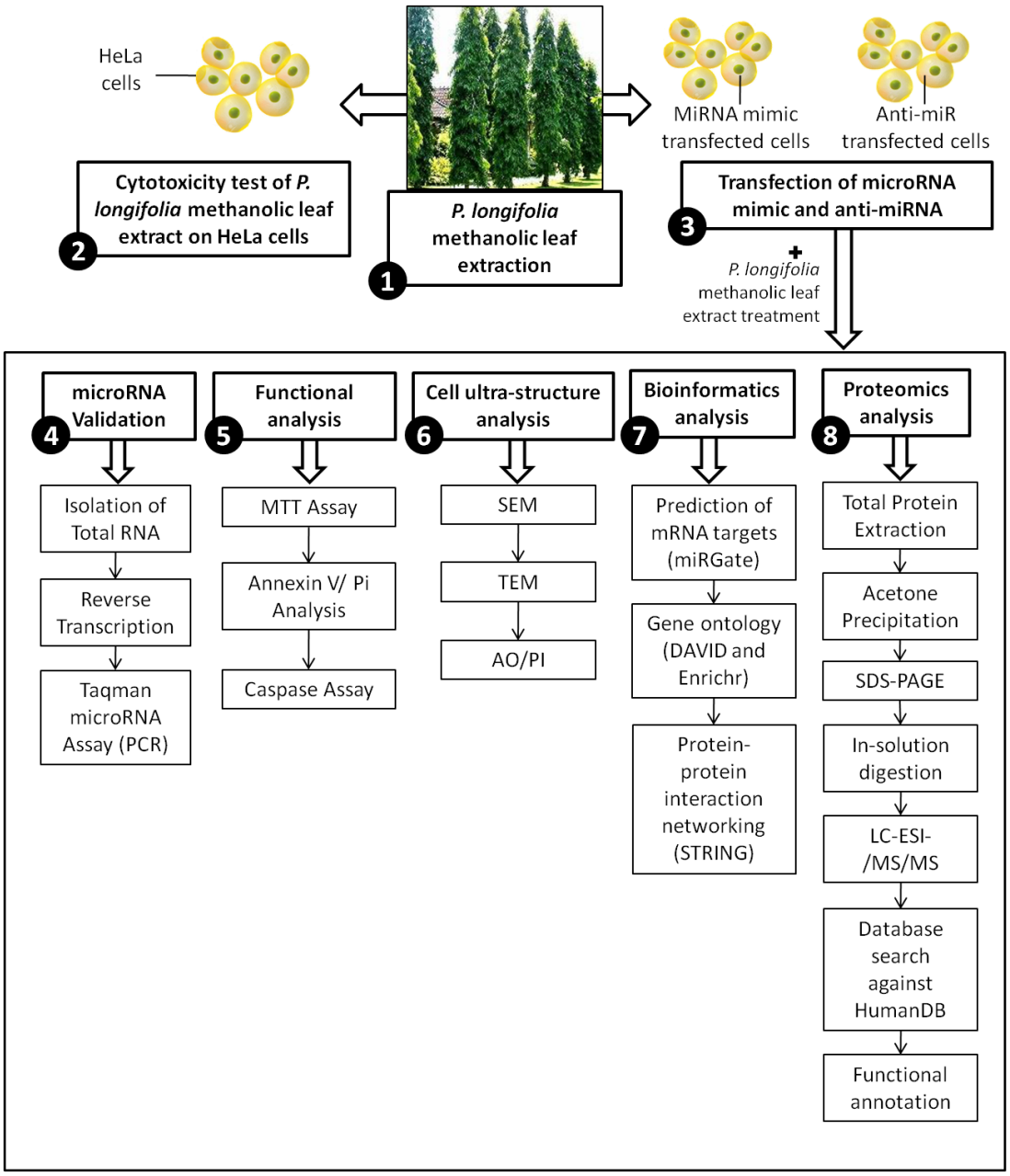

Figure 1.1: Workflow of the overall research

CHAPTER 2: LITERATURE REVIEW

2.1 Cancer

Genetically programmed cell division and differentiation occur in the process of formation of specific tissues and eventually functional organs. However, intermittently the uncontrollable cell division may give rise to tissue masses called tumors, or neoplasms. A single mass of benign tumor is usually not life threatening since it can be cured completely by surgical removal. However, when the cells of a tumor start to invade and interrupt the surrounding tissues, the tumor is said to be malignant and is identified as cancer which can consequently lead to death due to injury to vital organs, secondary infection, metabolic problems, secondary malignancies, or hemorrhage. The place where cancer begins is known as the original or primary site. A malignant tumor can break away from its original location and invade far-away sites through the lymphatic system, forming new tumors. This process is known as metastasis. The uncontrollable growth of cells may occur in any parts of the body leading to more than 100 types of cancer including lung cancer, breast cancer, cervical cancer, stomach cancer, prostate cancer, bowel cancer and ovarian cancer.

According to the American Cancer Society (2019), risk factors for cancer include genetic factors such as inherited genetic mutations and immune conditions as well as the lifestyle of a person such as tobacco use, alcohol use, diet, and physical activity. Other disposing factors to cancer are certain type of infections such as human papilloma virus (HPV), Epstein-Barr virus (EBV), hepatitis B, hepatitis C and *Helicobacter pylori*. Environmental exposures to diverse range of chemicals, radiations and even overexposure to ultraviolet (UV) light from the sun may also

lead to cancer. Smoking and alcohol intake can be associated with several cancers such as the mouth, oral cavity, pharynx, larynx, esophagus, lung, stomach, pancreas and even colon (Schmidt and Popham, 1981). Besides, viral infection can be related to cancer because of their capability to integrate into the DNA of the human stem cell where it mutates and transforms the cell to be the parent of the malignant clone (Doll and Peto, 1981).

2.1.1 Current development in cancer therapy

There are several types of treatments available for cancer including surgery, radiation therapy, chemotherapy, immunotherapy, gene therapy, hyperthermia, and stem cell transplant. However, these treatments have excruciating side effects that vary from person to person depending on the frequency of treatment, the age of the person and other health conditions. Commonly occurring side effects generated by cancer treatments include anemia, alopecia (hair loss), constipation, edema, fatigue, memory problems, peripheral neuropathy, nausea and vomiting (National Cancer Institute, 2018; Wilkes, 2018). Chemotherapy is one of the popular cancer treatments fromthe 1960s as the degree of curing cancer elevated at approximately 33% through radical local treatments. Eventually Cancer Chemotherapy National Service Centre was established in the effort of developing methods to screen chemicals using transplantable tumors in rodents (Devita and Chu, 2008).

The evolving knowledge on cancer mechanisms has expedited the expansion of novel anticancer approaches. One of the most extensive conventions is to slow down or to inhibit the prime characteristic of cancer cells that grow uncontrollably. This can be correlated with the elevation of tendency of the cells to go through the process of cell suicide, or apoptosis. This effective route is eventually achieved

through a mechanistic manner where the cytotoxic drugs are designed so as to impede the DNA replication by damaging the DNA of the cancer cells, subsequently inducing apoptosis.

Crude extracts from plant samples have been established to be selectively toxic to cancer cells after passing through various bioassays including *in vitro* and *in vivo* screenings which led to the plant collection program by the United States National Cancer Institute (NCI), followed by screening of plant species for anticancer activity which resulted in a revelation of enormous number of new anticancer agents such as taxanes and camptothecin (Cassady and Douros, 1980; Shoeb, 2006). Plants rich in pytochemicals such as berberine, curcumin, genistein, daidzein, glyceollin, apigenin, quercetin, baicalein, resveratrol, luteolin, matrine, garcinol, silibinin, mangiferin, doxorubicin, and paclitaxel have been evidently to demonstrated to exhibit anti-cancer property by participating in biological processes including cell differentiation, proliferation and apoptosis (Sala-Cirtog *et al.*, 2015; Biersack, 2016; Devi *et al.*, 2017). The recent evolution of miRNA investigations and its involvement in regulating biological processes such as cell proliferation, and migration, metastasis,apoptosis, and cell differentiation in numerous malignancies by partial complementary binding to mRNA targets (Sethi *et al.*, 2013; Thakur *et al.*, 2014). This has led to a viewpoint concerning to associate phytochemical-rich medicinal plants to regulate the expression of miRNAs involved in tumorogenesis. Regulation of miRNA expression by medicinal plant extracts has become a promising novel strategy for cancer treatment, exclusively through inducing apoptosis and inhibiting cell proliferation in cancer cells (Gezici and Sekeroglu, 2017).

2.2 *Polyalthia longifolia*

Polyalthia longifolia from Annonaceae family is well known for its sophisticated traditional medicinal values. *Polyalthia longifolia* (Sonn.) Thwaites commonly known as False Ashoka, Buddha Tree, Green champa, Indian mast tree, and Indian Fir tree while its synonyms include *Uvaria longifolia* (Sonn.), *Guatteria longifolia* (Sonn.) Wallich, *Unona longifolia* (Sonn.) (Jothy *et al.*, 2013). *Polyalthia longifolia* indegeneously belongs to Sri Lanka and cultivated in Pakistan which is now widely found throughout Malaysia. The classification of *P. longifolia* is as follows:

Kingdom	:	Plantae
Division	:	Magnoliophyta
Class	:	Magnoliopsida
Subclass	:	Magnoliidae
Order	:	Mognoliids
Family	:	Annonaceae
Tribe	:	Annoneae
Genus	:	*Polyalthia*
Species	:	*longifolia*

2.2.1 Botanical description of *P. longifolia*

The evergreen pyramid-like *P. longifolia* trees are known to grow tall upto an altitude of 15 to 20 m. Its dark green glossy leaves are seen to be long and narrow approximately acounting to 7.5-23 by 1.5-3.8 cm (Katkar *et al.*, 2010). The ovate-oblong leaves with wavy margins possess mild fragrance while the mildly pale green flowers with wavy petals are non-fragrant (Lemmens and Bunyapraphatsara, 2003). The oval-shaped ripe fruits are 1.8 to 2 cm long bearing a single smooth and shinny

seed. The *P. longifolia* trees generally bear flowers and fruits during the month of February till June (Wallis, 1985; Yadav and Sardesai, 2000).

2.2.2 Pharmacological activities of *P. longifolia*

The pharmacological properties of *P. longifolia* include anti-microbial, anti-oxidant, anti-cancer, anti-proliferative, radioprotective, anti-inflammatory, and anti-ulcer (Dixit *et al.*, 2014).

2.2.2(a) Anti-microbial activity

Polyalthia longifolia leaves extracted using different solvents have been comparatively tested for anti-microbial activity against microbes such as *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus vulgaris* and *Salmonella typhi* through disc-diffusion method which evidently demonstrated the potential anti-bacterail activity of *P. longifolia* leaf extract (Thenmozhi and Sivaraj, 2010). In another study, methanolic leaf extract of *P. longifolia* was shown to exhibit anti-bacterial activity against *Bacillus* subtilis and *Staphylococcus aureus* reporting diameter of inhibition zones 24 mm and 22.6 mm, respectively (Uzama *et al.,* 2011). In addition, various bioactive components diterpenoids, alkaloids and allantoin isolated from the different parts of *P. longifolia* have also reported to exert significant anti-bacterial and anti-fungal activities against *Aspergillus fumigatus, Saccharomyces caulbequence, Saccharomyces cerevaceae, Candida albicans,* and *Hensila californica* (Rashid *et al.,* 1996; Faizi *et al.,* 2003; Murthy *et al.,* 2005).

2.2.2(b) Anti-oxidant activity

In vitro anti-oxidant activity of ethyl acetate and methanolic *P. longifolia* seed extracts by DPPH, nitric oxide radical, hydroxyl radical, and superoxide radical considering ascorbic acid as standard have been successfully demonstrated by Thonangi and Akula (2018) which was further correlated with total phenolic content assay reporting 114 ± 1.7 and 146.5 ± 2.4 mg/g gallic acid equivalents respectively. Another study also reported the anti-oxidant potential of *P. longifolia* stem bark ethanol extract by analysing the DPPH radicals scavenging effect, ferric ions reduction effect and lipid peroxidation inhibition with IC_{50} values of 18.14, 155.41 and 73.33 µg/mL, respectively (Manjula *et al.*, 2010). *In vitro* antioxidant activity of methanolic *P. longifolia* leaf extract was shown by inhibition of DPPH radical at IC_{50} value of 2.721 ± 0.116 mg/mL (Jothy *et al.*, 2012).

2.2.2(c) Anti-cancer activity

Ethanolic *Polyalthia longifolia* leaf extract was evidently shown to exhibit anti-cancer activity by inhibiting cell viability of several human cancers including colon cancer, liver cancer, prostate cancer, neuroblastoma, with a highest anti-cancer effect on colon cancer cells SW-620 accounting an IC_{50} value 6.1 µg/ml (Verma *et al.*, 2008). Another study reported the anti-cancer activity of *Polyalthia longifolia* stem bark ethanol extract against HeLa and MCF-7 cells with IC_{50} values of 25.24 and 50.49 µg/mL, respectively (Manjula *et al.*, 2010). Recent study conducted by Vijayarathna *et al.* (2017a) revealed the anti-cancer activity of standardized methanolic *Polyalthia longifolia* leaf extract against HeLa cells through MTT assay and CyQuant assay with an average IC_{50} value of 22 µg/mL.

2.3 Plant extraction methods

Plants are highly utilized pharmaceutically due to the rich medicinal values of their phytochemicals such as phenolics and flavonoids. Various plant extraction methods are widely practiced in galenical development such as the maceration method (Figure 2.1 A), soxhlet extraction method (Figure 2.1 B) and ultrasound extraction method (Figure 2.1 C).

2.3.1 Maceration

Maceration is a well-established plant extraction method which involves soaking of the coarse powdered plant material in a closed vessel with an appropriate solvent (Jones and Kinghorn, 2006; Handa *et al.*, 2008). The selection of solvent mainly depends on the bioactive compounds of interest from the plant material and thus the solubility of the compounds in the selected solvent is important to be taken into account while choosing an appropriate solvent. In addition, chemical characterization of the solvent and extraction yield is also equally imperative to consider while choosing a solvent for the plant extraction through maceration method. Commonly used solvents for the maceration process include hexane, chloroform, ethyl acetate, methanol or ethanol (Yan *et al.*, 2008). The maceration process is usually carried out at room temperature for at least three days with occasional agitation to allow the solubilisation of phytochemicals from the plant material. The mixture is then filtered and the final marc is pressed out to completely extract out the dissolved bioactive compounds (Pandey *et al.*, 2014).

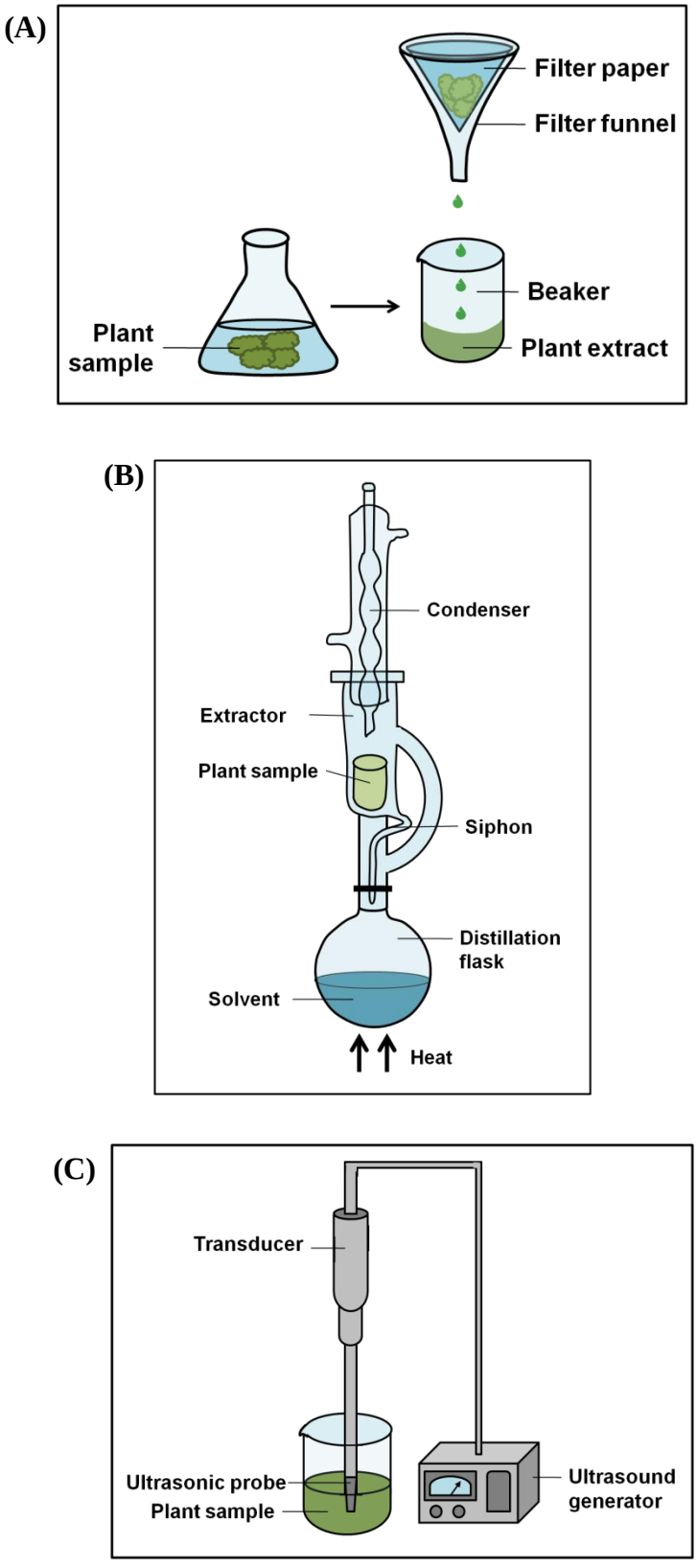

Figure 2.1: Different types of plant extraction methods

(A) Maceration Method**, (B)** Soxhlet Method and **(C)** Ultrasound or Sonication Method

2.3.2 Soxhlet extraction

Soxhlet extraction, also known as the Hot Continuous Extraction was developed by van Soxhlet in 1879 (Soxhlet, 1879). The finely powdered plant material is placed in a "thimble" which is usually attributed from a strong filter paper or cellulose. The sample containing "thimble" is placed in the thimble-holder of the soxhlet extractor while the extraction solvent is filled in the distillation flask at the bottom. When the solvent is heated, the vapourized solvent is condensed and consecutively fills the thimble containing the plant material. When the solvent level rises, a siphon tube aspirates it from the thimble-holder and discharges it back into the distillation flask. This process is continued until the solvent from the siphon tube does not leave residue when evaporated and thus, it is a continuous–discrete plant extraction method (Luque de Castro and Priego-Capote, 2010).

2.3.3 Ultrasound extraction or sonication extraction

This extraction method comprises the utilization of ultrasound with frequencies ranging from 20 kHz to 2000 kHz (Handa *et al.*, 2008). The acoustic effect from the ultrasound causes produces cavitation which in turn increases the permeability of the cell wall promoting the release of phytochemicals from the plant material into the solvent. Although this extraction method is easy and cost-effective, higher ultrasound energy is known to cause undesirable degradation of bioactive compounds.

2.4 MicroRNA

Small endogenous RNA molecules can be classified into several types, including transfer RNA (tRNA), ribosomal RNA (rRNA), small nucleolar RNA (snoRNA), small interfering RNA (siRNA) and micro RNA (miRNA). The endogenous small miRNA molecules which are approximately 20-22 nucleotides long are derived from the double stranded RNA precursor molecules (Ketting, 2010). The breakthrough of miRNA was first discovered in *Caenorhabditis elegans* and the disclosure of small non-coding lin-4 transcript from *C. elegans* which was 22 nucleotides long found to down regulate LIN-14 protein expression via sequence complementary binding to 3' untranslated region (UTR) of lin-14 mRNA (Lee *et al.,* 1993). Since then, miRNA has attained an increasing deliberation and led to detailed investigation of miRNA biogenesis and function in the advancement of molecular biology. Contemporarily, 28645 distinguish hairpin precursor miRNA, expressing 35828 mature miRNA from 223 species have been identified (Griffiths-Jones, 2004; Griffiths-Jones*et al.,* 2006; Griffiths-Jones *et al.,* 2008; Kozomara and Griffiths-Jones, 2011; Kozomara and Griffiths-Jones, 2014) (http://www.mirbase.org/ accessed March 10, 2018). This arising principal class of regulatory genes have been identified by bioinformatics prediction approaches and validated through several experimental methods. The involvement of miRNA in the negative regulation of gene expression at post transcriptional level and subsequent protein translational repression (Bartel, 2004) clearly substantiate the major role of miRNA in diverse biological processes such as cell death (Xu *et al.,* 2004), cell proliferation (Cheng *et al.,* 2005), cell development (Yoo and Greenwald, 2005), cell differentiation (Naguibneva *et al.,* 2006), stress resistance (Dresios *et al.,* 2005), haematopoiesis (Garzon *et al.,* 2006), fat metabolism (Ambros, 2003; Esau *et al.,* 2006) and insulin secretion (Poy *et al.,*

2004). Hence, the evolution of miRNA has exposed a novel and attractive therapeutic target and diagnostic tool for various diseases including cancer.

2.4.1 MicroRNA biogenesis

In like manner of precursor mRNA synthesis, miRNA are also generated by RNA polymerase II by initially producing a lengthy transcript called the primary miRNA (pri-miRNA) (Bartel, 2004). The pri-miRNA transcripts have been evidently validated to possess 5' cap and poly (A) tail at 3' end as any other typical mRNA (Cai *et al.*, 2004; Lee *et al.*, 2004). Previous studies suggest that the length of pri-miRNA transcript can be approximately 1000 nucleotide (Lee *et al.*, 2003; Cai *et al.*, 2004). Considering the length of pri-miRNA is pretty long with complementary bases within the transcript, it is legitimate to form a partially paired stem-loop structure (Treiber *et al.*, 2012). This structure acts as substrate for RNase III class of enzymes, namely DROSHA and *Di George* Syndrome critical region gene 8 (DGCR8) which eventually recognises the hairpin-loop structure of pri-miRNA and catalyzes it into a short precursor miRNA (pre-miRNA) (Denli *et al.*, 2004; Gregory *et al.*, 2004; Landthaler *et al.*, 2004). This first cleavage process is initiated by the binding of the microprocessor complex (complex of DROSHA and DGCR8) to the open-ended part of the stem-looped miRNA and finally the double-stranded cleavage produces a concise hair-pin shaped RNA molecule with a two nucleotide over hang at the 3' end (Han *et al.*, 2004; Han *et al.*, 2006). The double stranded stem-loop structure of pre-miRNA has been identified to be approximately 70-100 bp long (Treiber *et al.*, 2012).

Subsequently, the transportation of pre-miRNA from nucleus to the cytoplasm is mediated by the nuclear export receptor, known as the Exportin 5 (Yi *et*

al., 2003; Lund *et al.*, 2004). Previous studies demonstrated that the Exportin 5 performs its role as nuclear cargo with the aid of RanGTP in which stable complexes of pre-miRNA·Exportin 5·RanGTP are productively exported to cytoplasm down the RanGTP gradient across the nuclear envelope and pre-miRNA and Exportin 5 are dissociated upon the hydrolysis of RanGTP to RanGDP in cytoplasm (Bohnsack *et al.*, 2004). The free Exportin 5 is then returned back to the nucleus to mediate new pre-miRNA exportation.

Instantaneously, the second cleavage in the biogenesis process of miRNA takes place in the cytoplasm by RNase III enzyme called the DICER (Grishok *et al.*, 2001; Ketting *et al.*, 2001). DICER incorporates PAZ (Piwi, Arganoate and Zwille) domain that binds to the two nucleotide 3' overhang and anchors the pre-miRNA in position while placing the stem loop terminal at the positively charged catalytic domain of the DICER (Macrae *et al.*, 2006; MacRae *et al.*, 2007). This arrangement enables the DICER to act as a molecular ruler, thereby assisting the cleavage to occur efficiently at approximately 65 angstrom (Å) from PAZ domain and cleaves off the loop from the pre-miRNA (MacRae *et al.*, 2007; Ketting, 2010; Park *et al.*, 2011). The subsequent shorter double stranded RNA of about 20-25 nucleotides in length, with two nucleotide 3' overhangs at both terminals is known as miRNA duplex or miRNA/miRNA* (MacFarlane and Murphy, 2010).

miRNA duplex is then loaded into the miRNA-Induced Silencing Complex (miRISC) and releases one of the strand while selectively bound to one strand in order to generate an active complex (Bartel, 2004). The strand which is integrated into the miRISC is termed as the guide strand (miRNA) while the strand which is released and degraded is termed as the passenger strand (miRNA*). The Argonaute protein being the major component of RISC acts as the capital for catalytic process.

The Argonaute protein comprises two essential domains, namely PAZ and PIWI. The PAZ domain has been demonstrated to bind to the backbone of the guide strand (Song *et al.*, 2003; Ma *et al.*, 2004) while the PIWI domain acts as the RNase H which breaks down the passenger strand (Martinez and Tuschl, 2004; Song *et al.*, 2004; Ma *et al.*, 2005). Figure 2.2 shows an overview of miRNA biogenesis process.

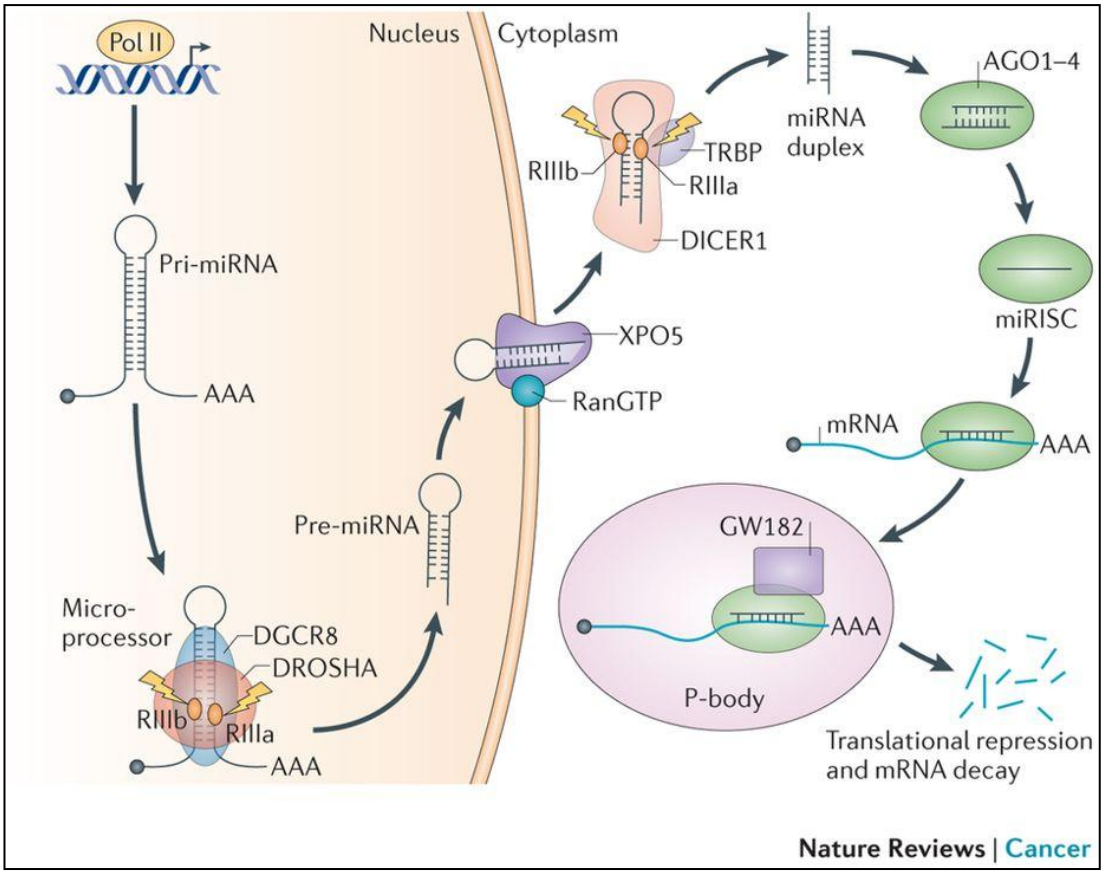

Figure 2.2: Overview of miRNA biogenesis process

(Source: Lin and Gregory, 2015)

Abbreviation: RNA polymerase II (Pol II), Primary microRNA (pri-miRNA), *Di George* Syndrome critical region gene 8 (DGCR8), Precursor microRNA (pre-miRNA), Exportin 5 (XPO5), miRNA-Induced Silencing Complex (miRISC)

2.4.2 MicroRNA and cancer

Ever since the exploration of miRNA and its' correlation with the widespread biological processes mainly including apoptosis and cell proliferation, the fundamental significance of miRNA in tumorigenesis are strongly postulated. Henceforth, the miRNA-mediated molecular mechanism in cancer biology has unfastened a novel dimension for cancer therapeutic targets as well as cancer biomarkers. The miRNA binds to its target mRNA by partial complementary binding, thus silences the gene expression and represses the post translational activity. The means of function of miRNA via alteration of gene expression and consecutive translational expression, pinpoints that miRNAs can act as tumor suppresser genes or oncogenes depending on their target genes (Kent and Mendell, 2006; Zhang *et al.*, 2007).

For instance, up-regulation of specific miRNA targeting the tumor suppressor genes, eventually, promoting cell growth and cancer initiation acts as an oncogene. On the other hand, up-regulation of specific miRNA targeting genes responsible for oncogenic activities which ultimately lead to cancer inhibition or repression acts as tumor suppressor gene (Shenouda and Alahari, 2009). However, the up-growing investigations on miRNA have uncovered the dual role of miRNA in cancer, in which various evidence supports the concept that a same individual miRNA can act as both oncogene and tumor-suppressor gene depending on the cellular environment (Schetter *et al.*, 2012; Sharma *et al.*, 2014; Ding *et al.*, 2018). Based on the literature, extensive studies have reported the correlation between miRNAs and cancer to date (>38, 000 Pubmed hits as of January 2019).

2.4.2(a) Mechanisms involved in microRNA deregulation in cancer

The dysregulation of miRNAs in cancer occur through numerous overlapping mechanisms including chromosomal abnormalities, transcriptional control alterations, epigenetic modulation and disruption in the miRNA processing machinery (Peng and Croce, 2016). For instance, chromosomal alterations may occur due to amplification of a chromosome site harbouring a specific miRNA, leading to an over-expression of the particular miRNA (Hayashita *et al.*, 2005; Tagawa and Seto, 2005) while deletion of the chromosome site may result in down-regulation of the specific miRNA (Calin *et al.*, 2002; Calin and Croce, 2006).

Other than that, various transcriptional factors have been evidently reported to control the expression of miRNAs such as c-Myc (Chang *et al.*, 2008; Han *et al.*, 2013), p53 (He *et al.*, 2007; Hermeking, 2010), myeloid transcription factors PU.1 and C/EBPs (Fukao *et al.*, 2007) and transcription factors NFI-A and C/EBPα (Fazi *et al.*, 2005). Besides, miRNAs have also been reported to undergo epigenetic changes through CpG methylation (Fazi *et al.*, 2007), DNA methylation with histone acetylation inhibitors (Saito *et al.*, 2006), and hypermethylation (Lujambio *et al.*, 2008; Lehmann *et al.*, 2008).

Finally, dysregulation or mutation of any proteins involved in miRNA biogenesis process as described in section 2.1.1 such as DROSHA (Thomson *et al.*, 2006), DGCR8 (Walz *et al.*, 2015), Dicer (Kumar *et al.*, 2009; Iliou *et al.*, 2014), Argonaute proteins (Zhang *et al.*, 2013; Völler *et al.*, 2013), TRBP (Melo *et al.*, 2009) and Exportin 5 (Melo *et al.*, 2010) which leads to miRNA dysregulation.

2.4.2(b) Pathways involved in microRNA regulation in cancer

The current chemotherapy targeting miRNA is hugely attaining interest due to their important participation in cancer pathway. Numerous miRNAs were also evidently shown to regulate tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)-induced apoptosis pathway. Based on the research conducted by Garofalo *et al.* (2008), over-expression of miR-221 and miR-222 was shown to hinder the TRAIL-induced apoptosis in non-small cell lung cancer by suppressing the important apoptotic protein expressions as well as targeting the tumor suppressor $p27^{kip}$mRNAs. The up-regulation of miR-221 and miR-222 was also demonstrated to be over-expressed, leading to the down-regulation of tumor suppressor $p27^{kip}$ in prostate carcinoma (Galardi *et al.*, 2007) and melanoma (Felicetti *et al.*, 2008).

Besides, the involvement of miR-21 regulation in AKT-dependent pathway was also reported to inhibit apoptosis by directly targeting and eventually repressing the expression of FasL (Sayed *et al.*, 2010). Another example of miRNA to be participating in Fas-mediated apoptotic pathway is miR-24 which was shown to target Fas-binding pro-apoptotic protein, namely Fas-associated factor 1 (FAF1), leading to inhibition of apoptosis in different types of cancer (Qin *et al.*, 2010; Schickel *et al.*, 2010). Another cancer pathway, namely the phosphatase and tensin homologue (PTEN) pathway was also shown to be regulated by the expression of miRNAs. For instance, many miRNAs are reported to target and suppress the expression PTEN which is one of the prominent tumor suppressor genes such as miR-17-5p (Xiao *et al.*, 2008), miR-19305p (Xiao *et al.*, 2008), miR-2127 (Sayed *et al.*, 2010) and miR-221 and miR-222 (Chun-Zhi *et al.*, 2010).

There are various microRNAs which have been reported to regulate the cell cycle regulatory pathway, in which oncogenic microRNAs tend to expedite cell cycle progression while the microRNAs with tumor suppressor effect tend to facilitate cell cycle arrest. Exemplary oncogenic microRNA include miR-106b and miR-17-92 families which have been reported to be over-expressed in various cancers are known to target one of the important inducer of G1 arrest, namely p21 from the Cip/Kip family of CDK inhibitors (Ivanovska *et al.*, 2008; Kim *et al.*, 2009). Other studies have also experimentally validated numerous other miRNAs to target other genes involved in cell cycle which eventually regulate the RAS/RAF/MAPK pathway as well as the p53 pathway (Jansson and Lund, 2012). Furthermore, microRNAs are also very well known to target numerous genes involved in DNA damage response in cancer cells. For instance, miR-421 was reported to be highly over-expressed in neuroblastoma and B-cell lymphoma cell lines and was shown to target the apical damage sensor kinase ATM (Hu *et al.*, 2010).

2.4.3 MicroRNA and medicinal plants

Endogenous microRNAs (miRNAs) are short single-stranded RNA molecules that cause mRNA cleavage or translational repression through partial complementary binding to the 3' untranslated region (UTR) of specific protein coding mRNAs (Ling *et al.*, 2013). This plays important role in regulation of various biological activities, thus leading to the break thorough of miRNA-based therapeutic approaches for several diseases. As medicinal plants are highly utilized in the treatment of diseases for centuries due to its rich bioactive phytochemicals, the understanding of its mechanistic actions in regulating the miRNA expressions has recently drawn an