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

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

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SHANMUGAPRIYA
Institute for Research in Molecular Medicine
Universiti Sains Malaysia
May 2019
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<td>Deoxyadenosine triphosphate</td>
<td></td>
</tr>
<tr>
<td>DAVID</td>
<td>Database for Annotation, Visualization and Integrated Discovery</td>
<td></td>
</tr>
<tr>
<td>DGCR8</td>
<td>Di George Syndrome critical region gene 8</td>
<td></td>
</tr>
<tr>
<td>DISC</td>
<td>Death inducing signalling complex</td>
<td></td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s minimum essential medium</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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</tr>
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<td>DPPH</td>
<td>2,2-diphenyl-1-picrylhydrazyl</td>
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</tr>
<tr>
<td>dsDNA</td>
<td>Double-stranded deoxyribonucleic acid</td>
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</tr>
<tr>
<td>DSG1</td>
<td>Desmoglein 1</td>
<td></td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
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</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
<td></td>
</tr>
<tr>
<td>ECD</td>
<td>Electron capture dissociation</td>
<td></td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
<td></td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
<td></td>
</tr>
<tr>
<td>ETD</td>
<td>Electron-transfer dissociation</td>
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</tr>
<tr>
<td>FAF1</td>
<td>Fas-associated factor 1</td>
<td></td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
<td></td>
</tr>
<tr>
<td>FDR</td>
<td>False discovery rate</td>
<td></td>
</tr>
<tr>
<td>FRET</td>
<td>Forster resonance energy transfer</td>
<td></td>
</tr>
<tr>
<td>GO</td>
<td>Gene ontology</td>
<td></td>
</tr>
<tr>
<td>H2O2</td>
<td>Hydrogen peroxide</td>
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</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
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<tr>
<td>HPRD</td>
<td>Human protein reference database</td>
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<tr>
<td>HPV</td>
<td>Human papilloma virus</td>
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<tr>
<td>IAA</td>
<td>Iodoacetamide</td>
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</tr>
<tr>
<td>IC50</td>
<td>Half maximal inhibitory concentration</td>
<td></td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
<td></td>
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<tr>
<td>--------------</td>
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<td></td>
</tr>
<tr>
<td>IEF</td>
<td>Isoelectric focusing</td>
<td></td>
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<tr>
<td>IGFBP5</td>
<td>Insulin-like growth factor binding proteins 5</td>
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<td>IL18</td>
<td>Interleukin 18</td>
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<tr>
<td>ITGAV</td>
<td>Integrin alpha-V</td>
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</tr>
<tr>
<td>ITGB1</td>
<td>Integrin, beta 1</td>
<td></td>
</tr>
<tr>
<td>JNK</td>
<td>Jun N-terminal kinases</td>
<td></td>
</tr>
<tr>
<td>K⁺</td>
<td>Potassium</td>
<td></td>
</tr>
<tr>
<td>kHz</td>
<td>Kilo hertz</td>
<td></td>
</tr>
<tr>
<td>KISS</td>
<td>Kinase substrate sensor</td>
<td></td>
</tr>
<tr>
<td>LC–ESI–MS/MS</td>
<td>Liquid chromatography-electrospray ionization-mass spectrometry/mass spectrometry</td>
<td></td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
<td></td>
</tr>
<tr>
<td>m/z</td>
<td>Mass to charge ratio</td>
<td></td>
</tr>
<tr>
<td>MAL</td>
<td>Myelin and lymphocyte protein</td>
<td></td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix-assisted laser desorption/ionization</td>
<td></td>
</tr>
<tr>
<td>MAPPIT</td>
<td>Mammalian protein–protein interaction trap</td>
<td></td>
</tr>
<tr>
<td>mg/g</td>
<td>Milligram per gram</td>
<td></td>
</tr>
<tr>
<td>mg/kg</td>
<td>Milligram per kilogram</td>
<td></td>
</tr>
<tr>
<td>mg/mL</td>
<td>Milligram per milliliter</td>
<td></td>
</tr>
<tr>
<td>MGB</td>
<td>Minor groove binder</td>
<td></td>
</tr>
<tr>
<td>min</td>
<td>Minutes</td>
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<tr>
<td>MINT</td>
<td>Molecular interaction database</td>
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<tr>
<td>miRISC</td>
<td>Mirna-Induced Silencing Complex</td>
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</tr>
<tr>
<td>miRNA</td>
<td>Microrna</td>
<td></td>
</tr>
<tr>
<td>MOMP</td>
<td>Mitochondrial outer membrane permeabilization</td>
<td></td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
<td></td>
</tr>
<tr>
<td>MSN</td>
<td>Moesin</td>
<td></td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
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</tr>
<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
<td></td>
</tr>
<tr>
<td>NCI</td>
<td>National cancer institute</td>
<td></td>
</tr>
<tr>
<td>NFQ</td>
<td>Nonfluorescent quencher</td>
<td></td>
</tr>
<tr>
<td>nm</td>
<td>Nano meter</td>
<td></td>
</tr>
<tr>
<td>nM</td>
<td>Nano molar</td>
<td></td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
<td></td>
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<tr>
<td>--------------</td>
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<td></td>
</tr>
<tr>
<td>°C</td>
<td>Degree celsius</td>
<td></td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
<td></td>
</tr>
<tr>
<td>P. longifolia</td>
<td><em>Polyalthia longifolia</em></td>
<td></td>
</tr>
<tr>
<td>PANTHER</td>
<td>Protein Analysis through Evolutionary Relationships</td>
<td></td>
</tr>
<tr>
<td>PAZ</td>
<td>Piwi, Arganoate and Zwille</td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
<td></td>
</tr>
<tr>
<td>PCD</td>
<td>Programmed cell death</td>
<td></td>
</tr>
<tr>
<td>PIK3R1</td>
<td>Phosphoinositide-3-kinase regulatory subunit 1</td>
<td></td>
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<tr>
<td>PLA</td>
<td>Proximity ligation assay</td>
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</tr>
<tr>
<td>Plk1</td>
<td>Polo-like kinase 1</td>
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<tr>
<td>pNA</td>
<td>P nitroanilide</td>
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<tr>
<td>PPIN</td>
<td>Protein-protein interaction networking</td>
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<tr>
<td>pre-miRNA</td>
<td>Precursor mirna</td>
<td></td>
</tr>
<tr>
<td>pri-miRNA</td>
<td>Primary mirna</td>
<td></td>
</tr>
<tr>
<td>PS</td>
<td>Phospholipid phosphatidylserine</td>
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</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin</td>
<td></td>
</tr>
<tr>
<td>PTP</td>
<td>Permeability transition pore</td>
<td></td>
</tr>
<tr>
<td>PTPC</td>
<td>Permeability transition pore complex</td>
<td></td>
</tr>
<tr>
<td>PUMA</td>
<td>P53 upregulated modulator of apoptosis</td>
<td></td>
</tr>
<tr>
<td>Q-TOF</td>
<td>Quadrupole Time-of-Flight</td>
<td></td>
</tr>
<tr>
<td>RARG</td>
<td>Retinoic acid receptor gamma</td>
<td></td>
</tr>
<tr>
<td>RCF</td>
<td>Relative centrifugal force</td>
<td></td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
<td></td>
</tr>
<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation assay</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
<td></td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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</tr>
<tr>
<td>RP</td>
<td>Ribosomal protein</td>
<td></td>
</tr>
<tr>
<td>RPM</td>
<td>Revolutions per minute</td>
<td></td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal RNA</td>
<td></td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcription</td>
<td></td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
<td></td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>Real time quantitative polymerase chain reaction</td>
<td></td>
</tr>
<tr>
<td>RVI</td>
<td>Regulatory volume increase</td>
<td></td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
<td></td>
</tr>
<tr>
<td>SDG</td>
<td>Sustainable development goals</td>
<td></td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
<td></td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
<td></td>
</tr>
<tr>
<td>SID</td>
<td>Surface-induced dissociation</td>
<td></td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
<td></td>
</tr>
<tr>
<td>snoRNA</td>
<td>Small nucleolar RNA</td>
<td></td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
<td></td>
</tr>
<tr>
<td>THBS2</td>
<td>Thrombospondin-2</td>
<td></td>
</tr>
<tr>
<td>TIMP3</td>
<td>Tissue inhibitor of metalloproteinase-3</td>
<td></td>
</tr>
<tr>
<td>Tm</td>
<td>Melting temperature</td>
<td></td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
<td></td>
</tr>
<tr>
<td>TOF</td>
<td>Time-of-flight</td>
<td></td>
</tr>
<tr>
<td>TRAF-6</td>
<td>Tnf receptor-associated factor 6</td>
<td></td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
<td></td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer RNA</td>
<td></td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
<td></td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
<td></td>
</tr>
<tr>
<td>XIAP</td>
<td>X-linked inhibitor of apoptosis</td>
<td></td>
</tr>
<tr>
<td>ΔΔCT</td>
<td>Double delta cycle threshold</td>
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PENCIRIAN MOLEKUL DAN ANALISIS BIOINFORMATIK MICRORNA-221-5P YANG DIKAWALATUR OLEHEKSTRAK 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 pengaruh 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 dengan 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\textsubscript{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 menggunakan 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 kecekapanya 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 MICRONNA-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 H₂O₂-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 cytotoxicity 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 from the 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 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 Sekeroğlu, 2017).
2.2 \textit{Polyalthia longifolia}

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

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

2.2.1 \textbf{Botanical description of \textit{P. longifolia}}

The evergreen pyramid-like \textit{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 accounting to 7.5-23 by 1.5-3.8 cm (Katkar \textit{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\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{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-Joneset 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.,
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
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 (Bohsack 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 suppressor 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 p27kip 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 p27kip 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...
enormous interest in the scientific research field. To date, various medicinal plants have been studied and reported to regulate a diversified range of miRNAs.

*Coptidis rhizoma*, a Chinese medicine has been reported to exhibit anti-cancer property by significantly up-regulating miR-21 and miR-23a in human hepatocellular carcinoma MHCC97-L cells through On-chip microarray analysis and Quantitative real-time PCR for miRNA analysis (Zhu et al., 2011). Besides, another traditional Chinese medicinal plant, namely *Icariin* found to induce apoptosis in ovarian cancer A2780 cells via down-regulation of miR-21, which was revealed through qPCR analysis followed by western blot analysis to evaluate the expression of PTEN, RECK and Bcl-2 proteins (Li et al., 2015).

Medicinal plants such as *Arctostaphylos uva-ursi* (L.) Spreng (bearberry), *Vaccinium macrocarpon* Air. (cranberry), *Rhododendron hymenanthes* Makino, *Rosemarinus officinalis*, *Eriobotrya japonica*, *Calluna vulgaris*, *Ocimum sanctum*, and *Eugenia jambolana* are known to be rich in Ursolic acid, 3β-hydroxy-urs-12-en-28-oic-acid (Ngo et al., 2011). Ursolic acid has been extensively and evidently reported to exhibit anti-cancer activity in various cancers like prostate carcinoma (Shanmugam et al., 2011), breast carcinoma (De Angel et al., 2010; Gao et al., 2012), hepatocellular carcinoma (Gayathri et al., 2009; Shao et al., 2011), bladder carcinoma, colorectal carcinoma (Prasad et al., 2012), and leukemia cells (Gao et al., 2012). Urosolic acid treatment has been shown to down-regulate miRNA-21, leading to the induction of apoptosis through activation of caspase-3 in human glioma cell line U251 (Wang et al., 2012a). Another bioactive compound, namely curcumin which is found in many plants was reported to induce apoptosis through the down-regulation of miR-186*, leading to the over-expression of caspase-10 (Zhang et al., 2010).
Medicinal plants rich in folates, retinoids and curcumin have been well explored and revealed to exhibit anti-cancer properties through the regulation of microRNA expression (Davis and Ross., 2008). For instance, folate-deficient human lymphoblastoid cells shown to up-regulate the expression of hsa-miR-222 (Marsit et al., 2006). Retinoids has been reported to down-regulate Ras and Bcl2 through the activation of let-7a and miR-15a/miR-16-1 respectively in acute promyelocytic leukemia (APL) cell lines (Garzon et al., 2007). Besides, curcumin from the rhizome of Curcuma longa was reported to exhibit anti-cancer effect in PxCB-3 pancreatic cancer cell line through the up-regulation of miRNA-22 and down-regulation of miRNA-199a* (Sun et al., 2008).

Viscum album L. is one of the well known medicinal plants for the treatment of various cancers (Tabiasco et al., 2002; Horneber et al., 2008; Khan et al., 2016; Nazaruk et al., 2016). miRNAs involved in the pharmacological activities of Viscum album L. have been identified through high-throughput Illumina sequencing which were subsequently bioinformatically analysed and reported to be interacting with human genes that are highly enriched in KEGG pathways related to various human diseases including cancer (Xie et al., 2017). Medicinal plant Panax ginseng which is rich in various phytochemicals such as triterpene saponins, polysaccharides, phenolics, and flavonoids has been known to exert anti-cancer properties (Gao et al., 1989; Shin et al., 2004; Ali et al., 2005). miRNAs from Panax ginseng have been detected through the next generation high-throughput sequencing technology and subsequent bioinformatics analysis revealed to interact with target genes involved in metabolism, signal transduction and stress responses (Wu et al., 2012).
2.4.4 Validation and Functional Analysis of microRNA

Acknowledging the significant role of miRNA in cancer biology, identification of differentially expressed miRNAs followed by validation and functional analysis through various approaches has become highly intensive in order to further elucidate the molecular mechanisms of miRNAs underlying the cancer biology. Therefore, it can be hypothesized that the evolution of miRNA can be a promising cancer biomarker as well as new drug target and therapeutic means.

2.4.4(a) Validation of miRNA

High throughput technologies to analyse the expression of miRNAs include microarray technology, real time quantitative PCR (RTqPCR) and bead based flow cytometry.

MiRNA expression level can be quantified with the aid of RTqPCR following the development of specific stem-loop primers for the reverse transcription procedure. There are two types of real time quantitative PCR (RTqPCR), namely the SYBR Green and the Taqman. SYBR Green is comparatively cost-effective and easy than taqman because SYBR Green skips the specific probe designing and synthesis (Tajadini et al., 2014). However, absence of specific probe leads to fluorescence emission by SYBR Green dye when it links to any amplification of dsDNA including primer-dimers accounting to false positive results (Orlando et al., 1998; Wong et al., 2005; Wittwer et al., 1997). The utilization of specific probe along with the primers in Taqman real time qPCR overcomes the specificity problem, producing a more accurate quantification of miRNA (Mullis and Faloona, 1987).
The protocol begins with the isolation of total RNA with enrichment of small RNAs using organic extraction method, followed by solid-phase extraction and purification method (Pritchard et al., 2012). It is important to assess the quality and quantity of the isolated RNA to ensure the reliability and accuracy for further functional analysis. Unlike traditional RT PCR, miRNA are not able to anneal to the conventional primers due to short-length and the absence of poly(A) tail in miRNA (Hurley et al., 2012). Hence, the RT primers are designed to comprise a highly stable and complimentary stem-loop structure in order to produce the target cDNA prior to PCR amplification (Kramer, 2011). The specificity of the miRNA is further assured by utilizing specific PCR forward and reverse primers as well as the hydrolysis probe. Relevant melting temperature (Tm) is balanced by including additional 5’ nucleotides to the forward primer. The target miRNA are detected and quantified by the hydrolysis probe which has complimentary sequence between the forward and reverse primer sites. The probe encompass a specific minor groove binder (MGB) linked to a nonfluorescent quencher (NFQ) at its 3’ end and a reporter dye, namely FAM™ dye at its 5’ end. The taqman RTqPCR utilizes the application of Forster resonance energy transfer (FRET), also known as fluorescence resonance energy transfer (Förster, 1946; Förster, 1948) where the excitation of reporter dye is quenched by the quencher dye when the probe is intact. During the polymerization reaction, DNA polymerase tends to cleave the probe and eventually separates the reporter dye from the quencher resulting in fluorescence signal (Didenko, 2001). As the specific probe is only complimentary and hybridized to the target miRNA, nonspecific amplification is not identified nor quantified which ensures the accuracy and reliability of the assay. Figure 2.3 shows the mechanism of Taqman real time qPCR.
Figure 2.3: The mechanism of Taqman real time qPCR
2.4.4(b) Functional analysis of miRNA

Subsequently after identifying and quantifying the miRNA, it is important to further analyse the role of the miRNA and its association to cancer-related biological pathways which can be performed by gain-of-functions and loss-of-functions of miRNA. Advancement in scientific research field has developed wide spread approaches to analyse the effect of over-expression and under-expression of miRNA by artificially synthesising the miRNA mimics and miRNA inhibitors (anti-miR) respectively.

Role of miRNA can be analysed by restoring the expression of miRNA with the aid of double-stranded miRNA mimics. The endogenous-like miRNA are designed and artificially synthesised based on the complementarity of the unique sequences from the 5’ end of the miRNA, popularly known as the “seed site” (Lewis et al., 2003; Doench and Sharp, 2004). This allows the miRNA mimic to silence multiple protein-coding genes and thus repressing the subsequent translational mechanism as effective as the naturally existing endogenous miRNA (Wang, 2010). In order to increase the stability of the non-natural miRNA mimics, chemical modulation like alteration in nucleotides to the passenger strand is practiced (Lim et al., 2005; Garcia et al., 2011).

Knocking down miRNA is one of the well known approaches to study the function of specific miRNA. There are several techniques to silence the specific miRNA. One such way is to introduce the antisense oligonucleotides, commonly known as the anti-miR or miRNA inhibitors.
2.5 Bioinformatics analysis of microRNA

There are several bioinformatics tools to detect miRNA such as the miRBase which compromises the 38589 entries from 271 different species including human, mouse, rat, fly, worm, and Arabidopsis (Griffiths-Jones et al., 2008; Kozomara and Griffiths-Jones, 2011; Kozomara and Griffiths-Jones, 2014; Kozomara et al., 2019). Besides, plant microRNA database (PMRD) software is useful to detect miRNAs particularly from plants (Zhang et al., 2010). Bioinformatics tools such as Rfam (Griffiths-Jones et al., 2003; Gardner et al., 2009), miRDeep2 (Friedländer et al., 2012) and miRcat (Moxon et al., 2008) are designed to discover novel miRNA sequences when a specific miRNA is not found in the available miRNA databases.

Further downstream practical bioinformatics analysis can be performed in order to investigate additional important information such as the mRNA targets, gene expression, pathways involved as well as its downstream proteomics analysis (Brown and Bourke, 2016). There are various computational approaches available to predict the gene target of miRNA based on total number of binding sites of a miRNA at the 3’ UTR, the immediacy to the gene start or the local AU composition. Computational prediction for miRNA-gene interaction analysis can be conducted with the incorporation of freely available bioinformatics tools such as miRonTop (Le Brigand et al., 2010), mirGator (Cho et al., 2013), mirWalk (Dweep et al., 2011), miRanda (Betel et al., 2010), TargetScan (Friedman et al., 2009), Pita (Kertesz et al., 2007), RNAHybrid (Kruger and Rehmsmeier, 2006), microTar (Thadani and Tammi, 2009). Besides, miRGate is web-based software which gives a highly reliable output of miRNA gene target as it compromises 5 different in-house prediction algorithms such as miRanda, Pita, RNAHybrid, microTar and Targetscan as well as four different algorithms to curate experimentally validated miRNA target genes such as
TarBase (Sethupathy et al., 2006), mirTarBase (Chou et al., 2018), miRecords (Xiao et al., 2009) and OncomirDB (Wang et al., 2014).

Following the prediction of gene targets of miRNA, functional annotation, can be analysed with the incorporation of a web-based, high-throughput annotation tools such as the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8 bioinformatics (Huang et al., 2007) or Enrichr bioinformatics (Chen et al., 2013; Kuleshov et al., 2016). These bioinformatics tools aid in the functional annotation of gene targets of miRNA based on Gene Ontology (GO), functional annotation clustering and pathway analysis. The gene targets can also be used to analyse the downstream protein-protein interaction networking (PPIN) with the integration of various web-based bioinformatics tools available such as STRING (Szklarczyk et al., 2015), BioGRID (Stark et al., 2006), Database of Interacting Proteins (DIP™) (Xenarios et al., 2002), The Biomolecular Interaction Network Database (BIND) (Bader and Hogue, 2000), The Molecular Interaction Database (MINT) (Chatr-aryamontri et al., 2007), The Human Protein Reference Database (HPRD) (Peri et al., 2004) and IntAct (Hermjakob et al., 2004).

Bioinformatics analysis provides an insight into the comprehensive fundamental molecular mechanisms, signaling, and pathways that are related to miRNA which is highly beneficial for future investigations (Grün et al., 2005; Maragkakis et al., 2009). Figure 2.4 summarizes the pipeline of the overall bioinformatics analysis.
Figure 2.4: Pipeline of bioinformatics analysis
2.6 Mass spectrometry

One of the greatest advancements in proteomics research is the invention of the powerful technology known as mass spectrometry, which was designed to identify proteins and to investigate their molecular structure based on the mass to charge ratio (m/z) of the molecule. The high-throughput analysis of proteomics by mass spectrometry is hugely utilized in the drug discovery system in order to evaluate the efficiency and safety of the drug by identifying its biomarkers in the biological samples (Glish and Vachet, 2003). Mass spectrometry identifies the proteins based on the motion of a known charged particle, in an electric or magnetic field. This explains the three main constitutional parts of the instrumentation of mass spectrometry, namely the source of ionization, mass analyzer, and the detector which are maintained under high vacuum to allow the movement of ions without any interruption of air molecules, providing an accurate mass spectrum of m/z ratio against ion abundance (Herbert and Johnstone, 2002).

2.6.1 Ionization

The important steps in ionization part are to convert the samples into vaporized form and then to ionize the molecules. Ionization can be carried out by various means such as protonation, cationization, deprotonation, electron ejection or electron capture (Siuzdak et al., 2005). There are two types of ionization sources, namely (1) hard ionization such as electron ionization and (2) soft ionization such as atmospheric-pressure chemical ionization (APCI) (Horning et al., 1973), electrospray ionization (ESI) (Yamashita and Fenn, 1984a; Yamashita and Fenn, 1984b) and matrix-assisted laser desorption/ionization (MALDI) (Karas et al., 1987; Karas and Hillenkamp, 1988; Tanaka et al., 1988). Hard ionization utilizes a highly
energetic source which is considered to be strident, resulting in an extensive fragmentation of the ions and causes destruction of complex biomolecules (El-Aneed et al., 2009). As hard ionization is limited to volatile molecules with low molecular mass, soft ionization is highly preferred over the hard ionization as less fragmentation is produced with the use of less energy. MALDI or ESI are the most commonly employed ionization techniques to date. Briefly, MALDI uses laser beam which is focuses on the mixture dried analyte and matrix, causing the analyte ions to be ejected from the surface as the matrix absorbs the energy from the laser pulse (Awad et al., 2015). On the other hand, ESI uses the nano-spray technology producing vapourized charged droplets from the fine liquid spray (Awad et al., 2015).

The electrospray ionization was first accomplished by Malcolm Dole in 1968 (Dole et al., 1968) which was then improvised by John Fenn to incorporate the idea in investigating the biomolecules such as peptides, proteins, carbohydrates, small oligonucleotides, synthetic polymers, and lipids (Fenn et al., 1989). In ESI technique, the protein samples are solubilized in a liquid polar volatile solvent and sprayed into the mass spectrometry under atmospheric pressure and high voltage. The highly charged droplets are facilitated with coaxially introduced nebulising inert gas like nitrogen gas in order to free the ions from the solvent and vaporize as well as to fragment the analyte ion into smaller size (Iribarne and Thomson, 1976; Kebarle, 2000). Subsequently, the coulombic forces allow these fragmented ions to be directed into the mass analyzer of the mass spectrometry. ESI is considered to be the softest ionization technique, by generating non-covalent complexes in gas phase. In addition, ESI can be easily adapted into liquid chromatography as well as tandem mass analyzers such as quadrupole-time-of-flight.

2.6.2 Mass analyzer
Second part of the mass spectrometry instrumentation is the mass analyser, which is highly important to subsequently separate the ion products from the ionization chamber based on their mass-to-charge (m/z) ratios. There are two types of mass analysers namely (1) beam analysers in which the ions are directed to the detector through the analysing field in a beam and (2) trapping analysers in which ions are trapped in the analysing chamber and are made to be unstable with the use radio frequency before being detected by the detector. The currently employed popular mass analysers include quadrupoles, time-of-flight (TOF) analysers, magnetic sectors, and both Fourier transform and quadrupole ion traps. In addition, there are tandem mass spectrometers which employ more than one analyser known as the hybrid mass spectrometers such as quadrupole-quadrupole, magnetic sector-quadrupole, and more recently, the quadrupole-time-of-flight geometries. The characteristics of these mass analysers vary from one another depending on the range of m/z covered, accuracy, and resolution. Compatibility of the mass analyser to the ionisation source also matters as not all ionization sources are compatible with all mass analysers.

The Quadrupole Time-of-Flight (Q-TOF) which was initially introduced 1996 by combining the concept of quadrupole and ToF analyzer (Morris et al., 1996). Usually, there is an extra quadrupole to focus the ions by providing collision so as to improve the quality of the ion beam (Krutchinsky et al., 1998). Examples of dissociation methods include collision-induced dissociation (CID), surface-induced dissociation (SID), electron capture dissociation (ECD), and electron-transfer dissociation (ETD) and photodissociation (McLuckey, 1992; Wells and McLuckey, 2005; Hart-Smith, 2014). The m/z values of ions are determined by the radio
frequency and direct current voltages applied to the electrodes in the quadrupole which provides an electric field by creating distance variation.

2.6.3 Detection of ions

The final part of the instrumentation is the detector which functions in monitoring the ion current, amplification of the current and transmission of signals to the data system, to plot mass spectra of molecular weight against relative abundance. The high-speed data procurement is supported by an adequate rapid electronics such as the analog-to-digital converters (ADCs) (De Hoffmann and Stroobant, 2007). The popular types of detectors used in mass spectrometry include photomultiplier, the electron multiplier and the micro-channel plate detectors.

The block diagram in Figure 2.5 A summarizes the basic components of a typical mass spectrometry. Figure 2.5 B shows the mechanism of electrospray ionization mechanism while Figure 2.5 C shows the Quadrupole mass analyzer mechanism.
Figure 2.5: Components of mass spectrometry

(A) Block diagram of basic components of a typical mass spectrometry; (B) ESI Ionization mechanism (Konermann et al., 2013); (C) Quadrupole mass analyzer mechanism (Yost and Enke, 1979)
2.7 Mechanisms of cell death

The classification of cell death can be accomplished through the morphological alterations, enzymological benchmarks, utilitarian features or immunological attributes (Melino, 2001; Galluzzi et al., 2007). The two indispensible types of cell death are the apoptosis and necrosis (Buja et al., 1993) which is mainly distinguished by their morphological changes and biochemical pathway.

2.7.1 Apoptosis

Apoptosis is an ancient Greek word which means “falling off”. Apoptosis also known as programmed cell death (PCD) is one of the most sophisticated systems of signalling pathways in cells where the damaged or non-functional cells are destroyed (Campbell et al., 2008). It plays an important role in various biological processes such as embyogenesis, senescence of cells and in developing nervous system as well as in many diseases (Renehan et al., 2001; Alberts et al., 2002). Apoptosis ensue as a defence mechanism, particularly in immune reactions or to destroy and eliminate any damaged cells caused by disease or cytotoxic agents (Norbury and Hickson, 2001).

The morphological characteristics of apoptosis fundamentally exhibit cell shrinkage, denser cytoplasm, chromatin condensation, as well as cell membrane blebbing, and these eventually results in karyorrhexis and fragmentation of cells into apoptotic bodies. The latter process is followed by phagocytosis, whereby macrophages engulf and digest the apoptotic bodies within phagolysosome (Elmore, 2007). However, there are two distinct biochemical pathways of apoptosis, contributing to cell death, namely the intrinsic pathway and the extrinsic pathway (Figure 2.6).
Figure 2.6: The intrinsic and extrinsic pathway of apoptosis
Source: Tan et al., 2014
2.7.1(a) Intrinsic Pathway

Intrinsic pathway of apoptosis is considered to be a receptor-independent pathway which is generally mediated by intracellular signals, particularly the mitochondrial activities to induce apoptosis (Hanahan and Weinberg, 2000). The consummate stimuli for the intrinsic pathway of cell death include radiation, free radicals, viral infection and several cytotoxins which were initially believed to trigger the perturbation of mitochondrial permeability transition pore (PTP) followed by a decline of mitochondrial membrane potential (Δѱm). The opening of mitochondrial PTP is influenced by mitochondrial lipids, proteins of Bcl-2 family, and other proteins that regulate bioenergetic metabolite flux and the conjectural segments found in the permeability transition pore complex (PTPC) (Green and Kroemer, 2004). This in turn results in the leak of plethora of toxin proteins such as cytochrome c, Samc/DIABLO, Omi/HtrA2, apoptotic inducing factor (AIF) and endonuclease G (Saelens et al., 2004).

The release of cytochrome c from the intermembrane space to cytoplasm promptly induces the activation of caspase 3 through the binding of cytochrome c to the C-terminal of apoptotic protease activating factor-1 (Apaf-1) which consequently speed up the linkage of dATP, exposing the N-terminal caspase-recruitment domain (CARD) for oligomerization (Adrian et al., 1999). The CARD-CARD interaction eventually activates the caspase 9 and leads to the formation of cytochrome c/Apaf-1/caspase 9 complexes termed as the apoptosome which activate the executioner caspase 3 resulting in apoptosis (Hengartner, 2000).

Besides cytochrome c, the release of other proteins like Samc/DIABLO and Omi/HtrA2 also involves in the activation of caspase cascade through a different
manner which is by neutralizing the internal inhibitors of caspases known as the inhibitors of apoptosis proteins (IAPs) (Du et al., 2000; Saelens et al., 2004). In addition, Omi/HtrA2 induce apoptosis through a caspase-independent manner, whereby it has been reported to act as protease, facilitating the degradation of X chromosome-linked IAP (XIAP), cellular IAPs (cIAP1, cIAP2) and Apollon (Suzuki et al., 2004) as well as the degradation of antiapoptotic PED/PEA-15 (Trenica et al., 2004). The final set of proteins released from the mitochondria such as the AIF and endonuclease G are translocated into the nucleus which eventually lead to cell death through nuclear chromatin condensation and a massive DNA fragmentation (Cande et al., 2004; Saelens et al., 2004).

2.7.1(b) Extrinsic pathway

The extrinsic pathway of apoptosis exclusively involves the transmembrane receptor protein located on the surface of the plasma membrane. The well-characterized death receptors from the tumor-necrosis factor (TNF) superfamily includes the CD95 (APO-1/Fas), TNF receptor 1 (TNFRI), TNF-related apoptosis-inducing ligand-receptor 1 (TRAIL-R1) and TRAIL-R2 (Scaffidi et al., 1998; Walczak and Krammer, 2000). The activated T cells generate the CD95 receptor protein which allows the aggregation of intracellular death domains which recruit proteins into the death-inducing signalling complex (DISC) (Kischkel et al., 1995). This eventually activates an initiator caspase called caspase 8 followed by the activation of downstream effector caspase called caspase 3 and the activated caspase 3 triggers apoptosis (Hengartner, 2000).
2.7.2 Necrosis

Necrotic cell death is usually stimulated by physio-chemical stress such as hypoxia, ischemia, hypoglycaemia, intense temperature alteration and nutrient constrain. Both morphological changes and the mechanism involved in necrosis are totally different from that of apoptosis. The morphological characteristics reported in the process of necrosis encompass the cell and organelle swelling, reduction in ATP, loss in ion homeostasis, membrane rupture, nuclear lysis, and inhibition of protein synthesis, which also promotes death of neighbouring cells (Mattson and Bazan, 2012). The known necrotic mediators include calcium and reactive oxygen species (ROS) (Vanlangenakker et al., 2008). When oxygen and nutrient supply is inadequate to the cells, the ATP production by the glycolysis is apparently reduced which consequently lowers the pH. In order to counterbalance the drop in pH, the Na\(^+\)/H\(^+\) anti-porter is activated by pumping out the protons and replacing them with Na\(^+\) (Lazdunski et al., 1985). Subsequently, the excessive Na\(^+\) is released via the Na\(^+\)/Ca\(^+\) antiporter as the Na\(^+\)/K\(^+\) ATPase requires energy to pump out the Na\(^+\). The Ca\(^+\) can enter mitochondria through the Ca\(^+\) uniporter and increases the Ca\(^+\) concentration in mitochondrial matrix. The intracellular pH is then restored through an escalation of ROS by the damaged mitochondria and further induces mitochondrial permeability transition pore (PTP) associated with the depolarization of mitochondrial membrane potential and rupture of outer mitochondrial membrane (Szeto et al., 2011) leading to cell death. Figure 2.7 shows the differential in characteristics of apoptosis and necrosis.
Figure 2.7: Types of cell death
Source: Monteiro et al., 2015
3.1 Introduction

Great interest has been drawn in the development of anti-cancer drugs from medicinal plants, ever since the breakthrough of various bioactive compounds present in plant species. Recent studies have evidently proven that these phytochemicals exhibit cancer therapeutic properties by regulating the expression of miRNAs in order to control various cellular pathways involved in cancer pathogenesis such as cell proliferation, cell differentiation, cell invasion, metastasis and apoptosis (Ross and Davis, 2011). *Polyalthia longifolia* species has high sophisticated traditional medicinal values (Gupta et al., 2016) and our lab’s recent study also revealed the anti-cancer property of *P. longifolia* leaf extract by the induction of caspase-dependent apoptosis through dysregulation of miRNAs expression. As current study focuses on the altered expression of miR-221-5p in HeLa cells in association with *P. longifolia* leaf extract treatment in the process of inducing apoptosis, it is necessary to freshly extract the *P. longifolia* leaves in order to be used in our down-stream research involving functional and proteomics analysis of miR-221-5p alongside with the *P. longifolia* treatment.

Hence, this chapter mainly discusses the methanolic extraction method of *P. longifolia* leaves and the *in vitro* cytotoxicity screenings of *P. longifolia* leaf extract in HeLa cells. Maceration method was used to prepare the *P. longifolia* leaf extract while *in vitro* cytotoxicity testing was accomplished using a cell-based assay known as the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. MTT is broadly practiced in cancer research since it is validated to be a feasible,
expeditious and replicable in vitro proliferation assay (Ciapetti et al., 1993). MTT assay was performed to identify the 50% inhibition concentration (IC₅₀) value of the crude extract on HeLa cells which validates the anti-cancer properties of P. longifolia leaf extract. The determination of IC₅₀ value of P. longifolia leaf extract is imperative in this research so as to be used in the down-stream functional analysis and proteomics analysis of miR-221-5p in relation to the P. longifolia leaf extract treatment in HeLa cells.

3.1.1 Objective

Therefore, the main objective of this chapter is as follows:

1) To evaluate the cytotoxicity effect of methanolic P. longifolia leaves extract against HeLa cervical cancer cells.
3.2 Materials and Methods

3.2.1 Sample collection

Polyalthia longifolia leaves were collected from Universiti Sains Malaysia, Penang, Malaysia which has been authenticated at the Herbarium of the School of Biological Sciences, Universiti Sains Malaysia, where a sample was deposited (Voucher specimen: USM/HERBARIUM/11306) (Appendix A). The leaves were washed with tap water and then with distilled water. Washed leaves were dried at room temperature for 7 days. The dried samples were ground into coarse powder using a grinder.

3.2.2 Extraction of P. longifolia leaves

The extraction of P. longifolia leaves was carried out based on Crozier et al. (1997) with some modification. Briefly, air-dried powder of P. longifolia leaves (100 g) was weighed and placed into a conical flask. Five hundred milliliter of 80% of methanol was added and the leaf powder was soaked for 7 days. The macerated extract was filtered using a Whatman No.1 filter paper (Whatman, England) where the filtrate was further concentrated using a vacuumed Rotary Evaporator. The dried extract residue was soaked again in 80% methanol and the filtration process was repeated until the filtrate is seen to be dilute. Any methanol content in the concentrated filtrate was further evaporated in oven to obtain a pure P. longifolia leaf extract.
3.2.3 Cell Lines

The human cervical adenocarcinoma cell line, namely HeLa cell line was obtained from the American Type Culture Collection (ATCC) to be used for this research. The cells were cultured in complete media. This cell line was cultivated as adherent monolayer cultures in cell culture flasks (Corning) in an incubator with humidified atmosphere of 5% CO₂ (Thermo Scientific, Canada) at 37°C. Table 3.1 represents the comprehensive summary of the cell line information.

3.2.4 Revitalisation of frozen cells

General protocol of thawing and propagation of cells were followed, where the vial of frozen cells retrieved from the liquid nitrogen container was thawed by gentle agitation in a 37°C water bath to minimize the toxic effects caused by the cryoprotectant DMSO on thawed cells. The O-ring and the cap of the vial were kept off the water to depreciate contamination. The rapid thawing was done only for approximately 2 to 3 minutes, which was just long enough for most of the ice to melt. One mL of complete medium was added to the 1 mL content of the vial and resuspended delicately which was then totally transferred into a sterile 15 mL centrifuge tube and centrifuged at 200 × g for 5 minutes. The supernatant was discarded and the pellet was resuspended with an appropriate volume of complete media. The resuspended cells were then plated into a culture flask containing pre-warmed complete media. The cells were subsequently incubated in the humidified atmosphere of 5% CO₂ incubator at 37°C overnight allowing the cells to revive.
**Table 3.1:** Origin and the source of the cell line used with its complete growth medium requirements

<table>
<thead>
<tr>
<th>Designated name</th>
<th>Tissue Origin</th>
<th>Source</th>
<th>Complete growth medium requirements</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa</td>
<td>Adenocarcinoma, Cervix, epithelial \textit{Homo sapiens} (Human)</td>
<td>Tissue culture Laboratory, INFORMM</td>
<td>DMEM, 10% FBS, 100 U/mL penicillin and 100 µg/mL Streptomycin (ATCC, 2014)</td>
</tr>
</tbody>
</table>
3.2.5 Subculturing of cells

Subculturing of cells was implemented each time after the cells reached about 70-80% confluence. Firstly, the medium in the flask was discarded and the adherent cells were washed with PBS twice to remove all traces of serum to eliminate the trypsin inhibitors such as calcium and magnesium present in the serum. After removing the wash solution from the culture flask, the cells were treated with Trypsin-EDTA with an amount to just cover the cell layer (relatively 1 mL for 25cm² cell culture flask and 2 mL for 75cm² cell culture flasks) and incubated at 37°C for 5 minutes. The cells were observed under an inverted microscope (Olympus, Japan) to assure the detachment of single cells. The equivalent of twice the volume of pre-warmed complete media was added to the trypsin and the medium was dispersed over the cell layer surface by pipetting several times. The purpose of adding media was to inhibit the trypsin reaction. The cells were then transferred to a sterile 15 mL centrifuge tube and centrifuged at 200 × g for 5 minutes. Upon centrifugation, the supernatant was removed while the pellet was resuspended with 1 mL of complete media. Few drops of resuspended cells were then dispensed into fresh tissue culture flask.

3.2.6 Cell counting

Cell counting was performed using a glass hemocytometer and coverslip. The HeLa human cervical cancer cells were harvested and the pellet was resuspended with 1 mL of complete media. Briefly, 10 µL of cell suspension was transferred into a new eppendorf tube and 10 µL of Trypan Blue was added. The mixture was Pipette up and down several times to ensure a uniform cell suspension using the same pipette tip. After affixing cover slip to the hemocytometer, a small amount of trypan blue-cell suspension is transferred to one of the chambers of the hemocytometer by
carefully touching the cover slip at its edge with the pipette tip and allowing to be filled by capillary action. The chamber was ensured not to be overfilled or underfilled. The cells were viewed under microscope at 100× magnification and viable cells in the four 1 mm corner squares were counted. The number of cells per mL was calculated using the formula below:

$$\text{Number of cells/mL} = \frac{\text{Sum of cell counts in the four corner squares}}{4} \times 10^4 \times 2 \times 1$$

### 3.2.7 In vitro cytotoxicity study of *P. longifolia* leaf extract

Cytotoxicity test of *P. longifolia* leaf extract against HeLa human cervical cancer cells was performed with the employment of MTT assay as reported by Mosmann (1983). Approximately, 3000 to 5000 cells in 100 µL medium were seeded per well in the 96-well plate and allowed to incubate overnight at 37°C with 5% (v/v) CO₂ to achieve attachment of the cells. Subsequently, the cells were treated with different concentrations of *P. longifolia* leaf extract prepared through serial dilution in DMEM ranging from 100 µg/mL to 0.781 µg/mL. The assay for each concentration of the extract was performed in triplicates with additional wells of untreated control cells used to serve as negative control. The plate was incubated at 37°C with 5% (v/v) CO₂ for 24 hours. The following protocol was conducted in dark since MTT is light sensitive. Meticulously, 20 µL of MTT reagent was added to each well followed by further incubation for 4 hours. After 4 hours of incubation period, the media was removed from each well and 100 µL of DMSO was added to each well to dissolve the water-insoluble purple formazon crystals. The plate was kept on a plate stirrer for 30 seconds to completely dissolve the crystals. The absorbance values were measured at a wavelength of 540 nm with the use of a microplate reader (Molecular
Devices Inc., USA). The IC\textsubscript{50} value of \textit{P. longifolia} leaf extract for HeLa human cervical cancer cells was determined and used in the subsequent experiments. The percentage of cell viability was calculated by using the formula below:

\[
Cell \text{ viability}(\%) = \frac{Sample - Blank}{Control - Blank} \times 100\%
\]
3.3 Results

3.3.1 Extraction of *P. longifolia* leaves

The process of the *P. longifolia* methanolic leaf extraction has been demonstrated in Plate 3.1. The dark green filtrate was further concentrated with the incorporation of rotary evaporator which produces a final product of the paste-like dark green leaf extract weighing 7.6 g.
Plate 3.1: *P. longifolia* methanolic leaf extraction process
3.3.2 *In vitro cytotoxicity study of P. longifolia leaf extract*

The cytotoxic effect of *P. longifolia* leaf extract against HeLa human cervical cancer cells was determined with the incorporation of colorimetric MTT assay at a range of 0 to 200 µg/mL after 24 hours of treatment period. The cell viability can be qualitatively assessed by the color intensity of the purple formazan dye. The intensity of the purple formazan dye increases when the concentration of the drug decreases. This indicates that metabolically active cells increases inversely proportional to the leaf extract concentration. The absorbance values obtained from the microplate reader for respective concentrations of the drug were summarized in a graph of cytotoxicity (%) versus concentration of *P. longifolia* leaf extract was plotted (Figure 3.1). The IC$_{50}$ value which is the concentration of the drug resulting in 50% cytotoxicity was determined from the graph. The *P. longifolia* leaf extract showed effective cytotoxic effects with an IC$_{50}$ value of 26.67 µg/mL in HeLa human cervical cancer cells. The percentage of cell viability was calculated by using the formula below:

\[
\text{Cell viability(\%)} = \frac{\text{Sample} - \text{Blank}}{\text{Control} - \text{Blank}} \times 100\%
\]
Figure 3.1: Cell viability of HeLa cells treated with *P. longifolia* leaf extract by using MTT assay

\[
y = -29.559x + 92.144 \\
R^2 = 0.9871
\]

**IC\textsubscript{50} = 26.67 µg/mL**
3.4 Discussion

The fact that over the decades, plants have been the primary source of food and medicines to humans is inevitable. The discoveries of secondary metabolites of plants which are responsible for the health beneficial effects of plants have further increased the attention towards plants (Salim et al., 2008). Ethnomedicinal records of plants as in Ayurveda, “De Materia Medica” and “Pen Ts’ao Chin Classic of Materia Medica” are considered to be the basis of drug discovery from plants (Sneader, 2005).

3.4.1 Extraction of P. longifolia leaves

The preparation of crude extract is an important step as the overall research involving miRNA study is in relation to the P. longifolia treatment. The main steps involved in the extraction process are the drying of leaves, mechanical disruption and the extraction method. The leaves were washed thoroughly under running tap water to clear away soil and other unwanted debris, before placing them separately for drying process. The drying of leaves is an important step which eases the grinding step as well as to minimize the contingency of degradation and alteration of bioactive constituents present in the leaves (Marur and Sodek, 1995). Hindrance of microbial fermentation and enzymatic reactions can be accomplished by maintaining a dry condition (Seidel, 2006). Furthermore, the reason behind maintaining room temperature throughout the drying process was to prevent decomposition of thermoliable metabolites while the purpose of shady environment was to avoid exposure to ultraviolet radiation which may cause chemical changes in the therapeutic components (Jones and Kinghorn, 2006). Mechanical grinding of the
leaves plays an important role to increase the surface area as well as to alleviate the diffusion of solvent during maceration process (Seidel, 2006).

Subsequently, choice of appropriate extraction method is a crucial step to extract the *P. longifolia* leaves in the process of production of galenicals. Plant materials are relatively considered to be impure and hence solvent extraction was used to obtain the therapeutically felicitous part after excluding the inert and impure materials. There are diversified solvent extraction techniques such as maceration, percolation, Soxhlet extraction, pressurized solvent extraction, ultra-sound solvent extraction, extraction under reflux, and steam distillation (Seidel, 2006). The basic principle of this solid-liquid extraction is the diffusion of solvent into the powdered fruit material which then solubilises the medicinal components so that diffuses out from the plant matrix to the solvent under a concentration gradient until equilibrium is reached (Handa, 2008). In order to ensure that therapeutic components are completely extracted out of the leaves, the residue after filtration is endured again to solvent extraction.

The mode of extraction employed in this study is the maceration technique which is widely practiced in galenical development due to its productivity and cost-effectiveness (Jones and Kinghorn, 2006) where the powdered leaves were soaked in the menstrum (methanol) followed by filtration and evaporation. However, it is not prudent to soak the plant extract in the solvent for a long duration for the prevention of artefact formation and decomposition of therapeutic compounds. The final stage of extraction involves the concentration process under reduced pressure using rotary evaporator in which the temperature of water bath was retained below 40°C to preserve the thermoliable components. Besides, the condensed methanol via
condenser of the rotary vapour can also be recycled to macerate the solid residue of the leaf extract, called the marc (Jones and Kinghorn, 2006).

The choice of appropriate solvent for the extraction of plant material is an important criterion to be considered. The most commonly used solvents for the extraction of plant material include methanol, ethanol, acetone, propanol and ethyl acetate. Many investigations have recommended methanol to be used as solvent for the extraction of pharmacologically active components from plants. Protected bioactive components such as those in membrane-bound or compartmentalized in lipophilic structure are extracted with the incorporation of polar organic solvents such as methanol, which is absolutely not possible using aqueous extraction (Shimizu and Li, 2006). Other important advantages of methanol as extraction solvent are that it prevents microbial growth and evaporates faster than water due to its low boiling point. Previous studies have also reported to obtain maximum yield of plant extract in aqueous (80%) methanol (Chatha et al., 2006; Sultana et al., 2009).

3.4.2 In vitro cytotoxicity study of P. longifolia leaf extract

Plants have been the major source of medication since ancient time and nowadays plants serve as the basic foundation in modern medicine. Plants with a long history of traditional medication have drawn increasing attention and are being studied further to investigate their efficiency as chemotherapeutic drugs especially for the treatment of cancer. Although natural products have been utilized traditionally, methodical screening or scrutiny of their cytotoxicity is highly necessary in the drug discovery field particularly for cancer treatment and chemoprevention (Abu-Dahab and Afifi, 2007). Several cytotoxicity tests are available not only for evaluating
hazardous quality of a particular crude extract but also to disclose the molecular mechanisms of action of the extract (Eisenbrand et al., 2002).

Cell lines are extensively used for in vitro cytotoxicity testing rather than the primary cell culture itself due to their robust characterization and accessible culture method. Measurements of cell viability, DNA content, metabolic alterations, synthesis or activity of specific enzymes are the few indices used for the in vitro cytotoxicity testing of crude extracts (Ekwall et al., 1990). The appraisal of these indices is executed by the exposure of cells to different concentrations of the crude extract for a foreordained time period, usually 24 hours (Echobicon, 1997). Besides, the cell doubling time was also demonstrated to be about 24 hours with a plating efficiency of around 60% (Plumb, 2004). The consequence of this experimentation would provide an array of commensurate cytotoxic or inhibitory concentrations.

Several ultra-high throughput cell-based cytotoxicity assays are available and widely being practiced in the drug discovery process such as MTT assay, CyQuant cell proliferation assay, lactate dehydrogenase (LDH) leakage assay, neutral red assay and protein assay which also provide the mechanisms of action against the targeted cell lines (Kroll et al., 2009). In vitro cytotoxicity assays are considered to be uncomplicated, cost-effective, faster and an animal independent assessment with construed toxicity endpoints (Bondesson et al., 1989; Kroll et al., 2009).

In this study, MTT assay has been employed to evaluate the cytotoxicity of the methanolic P. longifolia leaf extract which evaluates through the metabolic activity. According to Fotakis and Timbrell (2006), MTT assay is considered to be the most sensitive cytotoxicity assay because of its sensitivity in encountering early apoptosis. Although there several dyes such as XTT (2,3-bis(2-methoxy- 4-nitro-5-
sulphophenyl)-5-carboxanilide-2H-tetrazolium), MTS (5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazoly)-3-(4-sulfophenyl)tetrazolium, inner salt), and WST-1 ((4-[3-4-iodophenyl]-2-(4-nitrophenyl)-2H-5-tetrazolio)-1,3-benzene disulfonate) which share the same principle of action, MTT is the most commonly used dye to determine cell viability as it is easy to use with a comprehension of a standardized protocol in contrast to other available dyes (Mosmann, 1983; Niles et al., 2008). MTT assay is broadly practiced in cancer research since it is validated to be a feasible, expeditious and replicable in vitro viability assay (Ciapetti et al., 1993).

The basic principle of MTT assay relies on the constant mitochondrial activity in most living cells which eventually relate to the number of viable cells displaying a directly proportional manner with its mitochondrial activity (Meerloo et al., 2011). NAD(P)H-dependent cellular oxidoreductase enzymes may under defined conditions reflect the number of viable cells present. These enzymes are capable of reducing the yellow MTT tetrazolium dye to its insoluble purple formazan. A solubilization chemical is added to dissolve the insoluble purple formazan product into a coloured solution, in which DMSO has been used in this study. Investigation conducted by Wang and his colleagues (2012) on improved solvent for the dissolution of formazan crystals in the MTT assay have revealed DMSO to be one the most efficient solvent for the dissolution of formazan crystals. The optical density (OD) values at 540 nm represent the formazan concentration, detecting the increase or decrease in viable cells. A reference wavelength of 630 nm was also measured in order to nullify the background signals from precipitated proteins, cellular debris, or phenol red which may interfere with the absorbance reading of formazan concentration.
The results obtained from MTT assay double confirmed the cytotoxicity effect of *P. longifolia* leaf extract activity against HeLa human cervical cancer cells at an exposure time of 24 hours as previously reported by Vijayarathna *et al.* (2017a). This was characterized by 50% inhibition (IC$_{50}$) of the HeLa human cervical cancer cell line. The *P. longifolia* leaf extract exhibited cytotoxicity with the IC$_{50}$ values of 26.67 µg/mL. According to the American National Cancer Institute (NCI) plant screening program, the criteria of cytotoxicity activity for the crude extracts is the one with an IC$_{50}$ $<$ 30 µg/mL in the precedent tests (Suffness and Pezzuto, 1990; Gulati *et al.*, 2015).

The result obtained is consistent with previous study conducted by Vijayarathna *et al.* (2017a) who reported an average IC$_{50}$ value of 22 µg/mL for *P. longifolia* leaf extract against HeLa cells from MTT assay and CyQuant assay. Other than this, various studies have reported the anti-cancer property of different parts of *P. longifolia* species by conducting MTT cytotoxicity assay. One such study reported the IC$_{50}$ value of 25.24 µg/mL of ethanolic bark extract from *P. longifolia* against HeLa cells by conducting *in vitro* MTT assay (Manjula *et al.*, 2008). Previous phytochemical analysis of *P. longifolia* has confirmed the presence of bioactive components such as diterpenes and alkaloids which are mainly responsible of its anti-cancer activity (Chen *et al.*, 2000; Lee *et al.*, 2009). The two different clerodane diterpenes isolated from *P. longifolia* leaves, namely viz polyalthialdoic acid and 16 á-hydroxy-cleroda3,13(14)Z-diene-15,16-olide supportively demonstrated its anti-cancer activity on human leukemia HL-60 cells, reporting an IC$_{50}$ value of 21.8 and 13.7 µM respectively (Sari *et al.*, 2013). In addition, another study has also reported the cytotoxic effects of two other bioactive compounds isolated from the ethanolic *P. longifolia* leaves, which are bisclerodane imides Longimide A and Longimide B.
documenting an IC_{50} value of 10.03 and 4.12 lg/mL respectively (Koneni et al., 2010).

Findings obtained from our current study in this chapter revealing an IC_{50} value of methanolic P. longifolia leaf extract which is less than 30 µg/mL against HeLa cells, was not only consistent with the literature background of P. longifolia but also obeys the guidelines set by the American National Cancer Institute (NCI) for crude extract activity. Therefore, the methanolic P. longifolia leaf extract obtained in this study is utilized for the treatment of HeLa cells in our down-stream investigations involving miRNA validation, functional analysis, ultra-structure cell morphological analysis and proteomics analysis which are discussed further in the following chapters.
3.5 Conclusion

In conclusion, the methanolic *P. longifolia* leaf extract exhibited anti-cancer activity against HeLa human cervical cancer cells with an IC\(_{50}\) value of 26.67 µg/mL over a 24 hour treatment. The result is intensely consistent with the IC\(_{50}\) value used in previous study of our lab which reported the induction of apoptosis by *P. longifolia* leaf extract in HeLa cells through the dysregulation of miRNAs expression. Therefore, the crude extract obtained in this study allowed the further investigation of miRNA expression in relation with the *P. longifolia* treatment in the induction of apoptosis in HeLa cells through gain-of-function and loss-of-function analysis. The down-stream analysis can greatly contribute to the finding of an effective anti-cancer agent from a cost-effective and abundantly available natural product with a novel chemotherapeutic approach of miRNA targeting.
CHAPTER 4: VALIDATION AND FUNCTIONAL ANALYSIS OF MIRNA-221-5P EXPRESSION ALONGSIDE WITH P. longifolia LEAF EXTRACT TREATMENT IN HELA CELLS

4.1 Introduction

Recent studies have evidently proven that phytochemicals present in plants exhibit cancer therapeutic properties by regulating the expression of miRNAs in order to control various cellular pathways involved in cancer pathogenesis such as cell proliferation, cell differentiation, cell invasion, metastasis and apoptosis (Bartel, 2009; Friedman et al., 2009; Voorhoeve, 2010; Ross and Davis, 2011). Previous study conducted by Vijayarathna (2017) and Vijayarathna et al. (2017c) demonstrated P. longifolia leaf extract treatment induced apoptosis in HeLa cervical cancer cell through dysregulation of miRNA using mrDeep2 analysis. Based on the statistical significance (p < 0.05) log2 Fold Change and normalized transcripts per million with at least 1000 reads, the dysregulated miRNAs were identified. Among which, miR-221-5p and miR-484 are found to be highly down-regulated in P. longifolia leaf extract treated cells compared to that of untreated cells (Vijayarathna, 2017). Hence, the current study validates and quantifies the expression of miR-221-5p which has been previously reported to be highly down-regulated upon P. longifolia treatment in HeLa cells to induce apoptosis.

Subsequently after validation, it is necessary to investigate the role of miRNAs regulated by P. longifolia leaf extract treatment in the induction of apoptosis in HeLa cervical cancer cells which can be performed through gain-of-function and loss-of-function approach. Gain-of-function can be investigated by enhancing the miR-221-5p expression with the aid miR-221-5p mimic transfection.
while loss-of-function can be done by knocking down the miR-221-5p expression with the aid of miR-221-5p inhibitor tranfection. Hence, the current study was conducted with two important analyses namely quantification of miR-221-5p expression through Taqman RTqPCR analysis and followed by functional analyses of miR-221-5p in association with P. longifolia leaf extract treatment by performing MTT cell viability assay, flow cytometric annexin V/PI analysis and caspase-3 assay.

4.1.1 Objectives

Therefore, the objectives of this chapter are as follows:

1) To validate and quantitate the expression level of miR-221-5p in P. longifolia leaf extract treated HeLa cells in comparison with untreated cells.
2) To study the function of miR-221-5p by enhancement and silencing of miR-221-5p expression in HeLa cells in relation to P. longifolia leaf extract treatment
3) To elucidate the molecular mechanism of miR-221-5p expression in regulating apoptotic cell death in HeLa cells
4.2 Materials and methods

4.2.1 Chemicals and reagents

Accurately 10 mg of methanol extract of *P. longifolia* leaf was resuspended in dimethyl sulfoxide (DMSO) (Sigma-aldrich, USA) to prepare a stock solution of 10 mg/mL to be used in each biological assay. Preparation of the sub-stock solution of the extract was implemented by diluting the stock solution in culture medium, causing the final concentration of DMSO to fall behind 0.5% (v/v) in all the analysis. Dulbelcco’s Minimum Essential Medium (DMEM), fetal bovine serum (FBS), penicillin (100 U/mL)/ streptomycin (100 µg/mL), 0.25% Trypsin-EDTA and phosphate-buffered saline (PBS) tablets were acquired from Gibco (Invitrogen, USA). Taqman MiRNA assay (Applied Biosystems, Foster City, CA, United States) was employed to perform real time RTqPCR. MTT solution was formulated by resuspending the MTT powder (St. Louis, USA) into PBS in the ratio of 5:1. Dimethyl sulfoxide (DMSO) (Sigma-aldrich, USA) was used to solubilise the formazan crystals. FITC Annexin V Apoptosis Detection Kit was purchased from BD Pharmingen™ (BD Biosciences, USA). Caspase-3 Colorimetric Assay Kit was purchased from R & D Systems™, USA. Protein Assay Bicinchoninate Kit was purchased from NacalaiTesque, Inc., Japan.

4.2.2 Transfection of miRNA mimics and anti-miRNA

Mimics of miRNA, namely “Syn-hsa-miR-484 miScript miRNA mimic” and “Syn-hsa-miR-221-5p miScript miRNA mimic” and “miScript miR-221-5p inhibitor” were purchased from Qiagen and resuspended to a final concentration of 20 µM. HeLa cells were seeded in 24-well plate at the density of $1 \times 10^5$ and incubated under
normal growth condition. Meanwhile, 5 nM of each miRNA mimic was prepared by diluting the stock miRNA mimics in 500 µL of culture medium without serum. Three µL of HiPerFect transfection reagent (Qiagen) was then added to the diluted miRNA and mixed by vortexing. The samples were then incubated for 10 minutes at room temperature (25°C) to allow the formation of transfection complexes. The complexes were then added drop-wise on the cells and the plate was gently swirled to ensure uniform distribution. The cells with the transfection complexes were incubated under normal growth condition for 24 hours.

4.2.3 Quantification of miR-221-5p expression

4.2.3(a) Total RNA Isolation

Total RNA was extracted from HeLa cells using the mirVana™ miRNA Isolation Kit (Applied Biosystem) according to manufacturer’s protocol. Briefly, the culture medium was aspirated and discarded, and the cells were rinsed with PBS. The cells were then trypsinized and the cell pellet was washed by gently resuspending in 1 mL PBS and pellet again at low speed. After removing the PBS wash, cells were lysed by adding 300-600 µL Lysis/Binding solution followed by a vigorous vortexing in order to obtain a homogenous lysate. The cell lysate was proceeded to organic extraction step where 1/10 volume of miRNA homogenate Additive was added to and mixed well by vortexing. The mixture was left on ice for 10 min. After that, a volume of Acid-Phenol: Chloroform that is equal to the lysate volume were added and mixed by vortexing for 30-60 sec, followed by centrifugation for 5 min at 10,000 × g at room temperature to separate the aqueous and organic phases. The aqueous phase was carefully removed without disturbing the lower phase, and was transferred into a fresh tube. The sample was proceeded to Total RNA Isolation step
where 1.25 volumes of room temperature 100% ethanol was added to the aqueous phase. A filter cartridge was placed into the collection tubes and the lysate/ethanol mixture was pipetted onto the filter cartridge, followed by centrifugation for ~5 sec at RCF 10,000 × g. The flow-through was discarded and the process was repeated until all of the lysate/ethanol mixture is through the filter. The sample was washed by applying 700 µL of miRNA Wash Solution 1 to Filter Cartridge for 5-10 sec. The flow-through was discarded and washed again with 500 µL Wash Solution 2/3 (working solution mixed with ethanol). The wash step was repeated again. After discarding the flow-through from the last wash, the filter cartridge was replaced again in the collection tube and the assembly was spin for 1 min to remove residual fluid from the filter. The filter cartridge was transferred to a fresh collection tube and 100 µL of pre-heated (95°C) Elution Solution or nuclease free water to the centre of the filter before spinning it for ~20-30 sec at maximum speed to recover the RNA. The elution (which contains the RNA) was collected and stored at -20°C. Quantity and quality assessments of the extracted RNA samples were determined by measuring the absorbance at 260 nm and the ratio of A$_{260}$ to A$_{280}$ nm respectively with the aid of NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, USA). RNA integrity was evaluated from 28S and 18S rRNA bands from 5 µL of total RNA on 1.0% agarose gel electrosis. The gel was stained with ethidium bromide and visualize under UV light. The image was captured using Vilber Lourmet (France).

**4.2.3(b) Reverse Transcription (RT)**

The RT master mix was prepared by scaling the volumes shown in Table 4.1 to the desired number of RT reactions.
Table 4.1: RT Master Mix components

<table>
<thead>
<tr>
<th>Component</th>
<th>Master mix volume/ 15 µL Reaction&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>100mM dNTPs (with dTTP)</td>
<td>0.15</td>
</tr>
<tr>
<td>MultiScribe™ Reverse Transcriptase, 50 U/µL</td>
<td>1.00</td>
</tr>
<tr>
<td>10× Reverse Transcription Buffer</td>
<td>1.50</td>
</tr>
<tr>
<td>RNase Inhibitor, 20U/µL</td>
<td>0.19</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>4.16</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>7.00</strong></td>
</tr>
</tbody>
</table>

<sup>a</sup>Each 15-µL RT reaction consists of 7 µL master mix, 3 µL primer, and 5 µL RNA sample.
The master mix was mixed gently and centrifuged to bring solution to the bottom of the tube which was then placed on ice until the miRNA reactions were prepared. For each 15-µL RT reaction, 7 µL of RT master mix is combined with 5 µL total RNA. The samples are then mixed gently and centrifuged to bring the solutions to the bottom of the tube. Following this, 12 µL of RT master mix containing total RNA was dispensed into a 0.2 mL polypropylene reaction tube and 3 µL of RT primer from each assay set was added into the tubes. The tubes were sealed and mixed gently which were then centrifuged to bring down the solutions. The tubes were incubated on ice for 5 minutes before loading the samples into the thermocycler with the parameter values programmed to the thermal cycler (Agilent Technologies SureCycler 8800, USA) as shown in Table 4.2.

4.2.3(c) Real time Polymerase Chain Reaction (qPCR)

PCR reaction mixture containing the components listed in Table 4.3 was prepared for each sample.

The samples were mixed gently and centrifuged briefly to spin down the contents and to eliminate any air bubbles. The centrifuged strips are then placed in the Applied Biosystems 7900HT Fast Real-Time PCR System and programmed with the parameters shown in Table 4.4.
Table 4.2: Thermocycler set up parameter

<table>
<thead>
<tr>
<th>Step type</th>
<th>Time (min)</th>
<th>Temperature (ºC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HOLD (Ligation)</td>
<td>30</td>
<td>16</td>
</tr>
<tr>
<td>HOLD (Elongation)</td>
<td>30</td>
<td>42</td>
</tr>
<tr>
<td>HOLD (Denaturation)</td>
<td>5</td>
<td>85</td>
</tr>
<tr>
<td>HOLD (Store)</td>
<td>∞</td>
<td>4</td>
</tr>
</tbody>
</table>

The reaction volume was set to 15.0 µL and then the reaction tubes were loaded into the thermal cycler (Agilent Technologies SureCycler 8800, USA) and the reverse transcription run was started.

Table 4.3: PCR master mix components

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µL) / 20-µL Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>TaqMan MiRNA Assay (20×)</td>
<td>1.00</td>
</tr>
<tr>
<td>Product from RT reaction (Minimum 1:15 Dilution)</td>
<td>1.33</td>
</tr>
<tr>
<td>TaqMan 2× Universal PCR Master Mix, NoAmpEraseUNGa</td>
<td>10.00</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>7.67</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td><strong>20</strong></td>
</tr>
</tbody>
</table>

Table 4.4: Applied Biosystems 7900HT Fast Real-Time PCR System set up parameter

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Run Mode</td>
<td>9600 emulation (Default)</td>
</tr>
<tr>
<td>Sample Volume</td>
<td>20 µL</td>
</tr>
<tr>
<td>Thermal Cycling Parameters</td>
<td></td>
</tr>
<tr>
<td>Step</td>
<td>AmplitaqGold® Enzyme Activation</td>
</tr>
<tr>
<td></td>
<td>HOLD</td>
</tr>
<tr>
<td>Time (min)</td>
<td>10 min</td>
</tr>
<tr>
<td>Temp (ºC)</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>60</td>
</tr>
<tr>
<td>Auto Increment Settings</td>
<td>Accept default values. (Default is 0.)</td>
</tr>
<tr>
<td>Ramp RateSettings</td>
<td>Accept default values. (Default is Standard.)</td>
</tr>
<tr>
<td>Data Collection</td>
<td>Accept default values. (Default is 60ºC.)</td>
</tr>
</tbody>
</table>
4.2.4 Functional analysis of miR-221-5p

4.2.4(a) MTT cell viability assay

MiRNA 221-5p and miRNA 484 were functionally analysed by conducting cell viability test. The colorimetric MTT assay as reported by Mosmann (1983) was incorporated to determine the cell viability upon transfection of HeLa cells with miR-221-5p mimic and miR-484 mimic with the presence and absence of methanolic *P. longifolia* leaf extract treatment corresponding to that of untreated HeLa cells.

The miR-221-5p mimics and miR-484 mimics were transfected into HeLa cells following the protocol explained in section 4.2.2. After the transfection period, the cells were treated with IC$_{50}$ value of *P. longifolia* leaf extract (26.67 µg/mL) for 24 hours. After treatment period, cell viability test was conducted using the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) assay reported by Mosmann (1983), with 3 replicates. The following protocol was conducted in dark since MTT is light sensitive. 0.5% w/v MTT (Sigma, USA) was dissolved in phosphate buffered saline and 100 µL of the prepared MTT reagent was added to each well followed by further incubation for 4 hours. After 4 hours of incubation period, the media was removed from each well and 500 µL of DMSO was added to each well to dissolve the formazon crystals. The plate was kept on a plate stirrer to completely dissolve the crystals. The absorbance values were measured at a wavelength of 540 nm with a microplate reader (Molecular Devices Inc., USA).

4.2.4(b) Annexin V/PI assay

The number of cells undergoing apoptosis was quantitatively assessed by flow cytometric Annexin V/propidium iodide (PI) assay for *P. longifolia* treated HeLa
cells, *P. longifolia* treated miR-221-5p mimic transfected HeLa cells as well as the anti-miR-221-5p transfected HeLa cells with the incorporation of FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, USA). Cells were first cultured in T25 flasks, followed by transfection of miRNA mimic and anti-miR independently prior to treatment. After the transfection period, cells were treated with IC$_{50}$ (26.67 µg/mL) value of *P. longifolia* leaf extract for 24 hours. Control HeLa cells and anti-miR transfected HeLa cells were left untreated. After 24 hours, cells were harvested and washed twice with cold PBS by centrifugation at 300 xg for 5 minutes at room temperature (RT). The cell pellet was resuspended with 1x binding buffer at a concentration of 1×10$^6$ cells/mL. Accurately 5 µL of FITC-conjugated Annexin V (Annexin V-FITC) and 5 µL of propidium iodide (PI) were added to 100 µL of cell suspension and vortexed gently. The stained samples were then incubated for 15 minutes at room temperature in the dark. An additional 400 µL of 1x binding buffer was added to each tube before analysing the samples by the flow cytometer (BD FACSCalibur). A total of approximately 10,000 events were acquired for each sample which was distinguished into four different populations accordingly in their quadrants. The sub-populations include the unlabelled cells (viable cells), cells that have been bound to Annexin V-FITC alone (early apoptotic cells), cells that have been stained with PI (necrotic cells) and cells that have been both bound Annexin V-FITC and been labelled with PI (late apoptotic/necrotic cells). The fluorescence distribution was demonstrated as a dot plot analysis and the percentage of fluorescent cells in each quadrant was determined.
4.2.4(c) Caspase-3 assay

The presence of active caspase-3 in synergetic cell sample of miR-221-5p mimic transfectected and *P. longifolia* treated HeLa cells and anti-miR-221-5p transfectected HeLa cells in comparison with untreated and *P. longifolia* treated was validated with the employment of Caspase-3 Colorimetric Assay Kit according to the manufacturer’s protocol (R & D Systems). Briefly, cells were cultured in T75 flasks, followed by transfection of miRNA mimic and anti-miR independently prior to treatment. After the transfection period, cells were treated with IC₅₀ (26.67 µg/mL) value of *P. longifolia* leaf extract for 24 hours. Control HeLa cells and anti-miR transfectected HeLa cells were left untreated. Upon treatment period, the cells were collected by centrifugation at 250 × g for 10 minutes. The supernatant was gently discarded while the cell pellet was lysed by the addition of the 25 µL of cold Lysis Buffer per 1 × 10⁶ cells. The cell lysate was then incubated on ice for 10 minutes and centrifuged at 10, 000 × g for 1 minute. After centrifugation, the supernatant was carefully transferred to a new tube and stored on ice until analysis. Antecedent to caspase assay, the protein content of the cell lysate was estimated by utilizing the Bicinchoninate method (Refer to 4.2.2(d)). Bovine Serum Albumin (BSA) was used as a standard. Once the protein concentrations of the cell lysates were determined, 50 µL of cell lysate (containing 200 µg of total protein) was added in each well of the 96-well flat bottom microplate, followed by the addition of 50 µL of 2× Reaction buffer 3 which was prepared by adding 10 µL of fresh DTT stock per 1 mL of 2× Reaction Buffer 3. Finally, 5 µL of Caspase-3 colorimetric substrate (DEVD-pNA) was added to each reaction well and the plate was incubated at 37°C for 2 hours. After incubation period, absorbance of DEVD-pNA was read at 405 nm in a microplate reader. Absorbance of DEVD-pNA from treated samples was compared
with non-induced cells in order to ascertain the relative caspase-3 activity (expressed as % of control).

4.2.4(d) Bicinchoninate protein assay

The total protein concentration was evaluated by using Protein Assay Bicinchoninate Kit (NacalaiTesque, Inc., Japan) according to the manufacturer’s protocol. Bovine serum albumin (BSA) was used as a standard. Firstly, 2 mg of BSA was measured and diluted in 1 mL of the same Lysis Buffer used in the caspase assay to prepare 2 mg/mL of BSA which was then serially diluted to prepare various concentrations of BSA. Accurately, 25 µL of samples and prepared standards were added into each well with micropipettor. Subsequently, 200 µL of working solution was added into each well which was prepared by mixing Bicinchoninic acid solution to copper sulphate solution in the ratio of 50:1. The reaction samples were mixed with a microplate mixer for 30 seconds, followed by incubation at 37°C for 30 minutes. The absorbance was measured at 562 nm using a microplate reader after restoring the microplate to room temperature.

4.2.5 Statistical analysis

Quantitative data from each assay was expressed as mean ± standard deviation (SD) from the least three independent experiments (n=3 for each experiment). Statistical analysis was performed by analysis of variance (ANOVA) followed by a post-hoc test with Tukey’s test by using SPSS program Version 20. A value of \( p < 0.05 \) was considered to be statistically significant.
4.3 Results

4.3.1 Total RNA Isolation

The absorbance spectrums of RNA were obtained from NanoDrop ND-1000 Spectrophotometer. Spectrums for untreated cells (A), *P. longifolia* leaf extract treated cells (B), miR-221-5p mimic transfected *P. longifolia* leaf extract treated cells (C) and anti-miR-221-5p transfected *P. longifolia* leaf extract treated cells (D) are shown in Plate 4.1 (A), 4.1 (B), 4.1 (C) and 4.1 (D), respectively. The measurements of A260:A280 and A260:A230 ratios for the spectrums are shown in Table 4.5. The absorbance at 260 nm corresponds with the presence of purines and pyrimidines in the RNA while absorbance at 280 nm corresponds with the presence of proteins and phenolic compounds in the RNA.

The absorbance measurement of A_{260}/A_{280} Ratio (µg/µl) indicates the purity of the total RNA extracted from the cell lines, where the value between 1.8 and 2.1 is considered to have a high purity. The results obtained for the RNA isolated from samples A, B, C and D are clearly in agreement with the spectral curve of RNA, which accounts for 2.09, 2.10, 2.09, 2.08, respectively.

In addition, the integrity of the RNA from all four samples was evaluated through 1.0% Agarose gel electrophoresis (AGE). Plate 4.2 shows typical pattern of two specific ribosomal RNA bands which are the 28S and 18S bands for the total RNA extracted from samples A, B, C and D which indicates the successful extraction of intact total RNA from samples.
Plate 4.1: The absorbance spectrums of RNA extracted from untreated cells (A), *P. longifolia* leaf extract treated cells (B), miR-221-5p mimic transfected *P. longifolia* leaf extract treated cells (C) and anti-miR-221-5p transfected *P. longifolia* leaf extract treated cells (D) generated by NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, USA).
Table 4.5: Quantification of total RNA extracted from untreated cells (A), *P. longifolia* leaf extract treated cells (B), miR-221-5p mimic transfected *P. longifolia* leaf extract treated cells (C) and anti-miR-221-5p transfected *P. longifolia* leaf extract treated cells (D) from NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, USA).

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
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<td>5.678</td>
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<tr>
<td>280nm</td>
<td>3.383</td>
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<td>A&lt;sub&gt;260&lt;/sub&gt;/A&lt;sub&gt;280&lt;/sub&gt; Ratio</td>
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<td>Concentration (ng/µl)</td>
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<td>227.1</td>
<td>340.8</td>
<td>279.4</td>
</tr>
</tbody>
</table>
Plate 4.2: Agarose gel electrophoresis of RNA extracted from untreated cells (A), *P. longifolia* leaf extract treated cells (B), miR-221-5p mimic transfected *P. longifolia* leaf extract treated cells (C) and anti-miR-221-5p transfected *P. longifolia* leaf extract treated cells (D). Lane M represents Marker of Promega RNA ladder.
4.3.2 Validation and quantification of miRNA

The miR-221-5p expression levels of the untreated control HeLa cells, *P. longifolia* leaf extract treated HeLa cells, *P. longifolia* leaf extract treated miR-221-5p mimic transfected HeLa cells and *P. longifolia* leaf extract treated anti-miR-221-5p/inhibitor transfected HeLa cells were quantitatively determined by Taqman real time RTqPCR, which is also known as TaqMan MiRNA assay. The amplification plot of the real time RTqPCR is shown in Figure 4.1. The *P. longifolia* leaf extract treatment significantly reduced (P < 0.05) miR-221-5p expression when compared to the untreated HeLa cells (Figure 4.2), with double delta Ct value of 6.32 and an expression fold change reduced up to 0.013. The transfection of miR-221-5p mimics significantly increased the expression of miR-221-5p compared to the treated samples with an expression fold change as high as 0.53 while anti-miR-221-5p transfected HeLa cells shows the most significant decrease in miR-221-5p expression with an expression fold change of 0.011. The expression of housekeeping miRNA, namely RNU6B was shown to be consistent in all the samples except the anti-miR-221-5p transfected HeLa cells, normalizing the data obtained for the miRNA of interest.
Figure 4.1: Amplification plot from real time RTqPCR

(A) and (E) represent the untransfected untreated cells; (B) and (F) represent the untransfected *P. longifolia* leaf extract treated cells; (C) and (G) represent the miR-221-5p mimic transfected *P. longifolia* leaf extract treated cells; (D) and (H) anti-miR-221-5p transfected *P. longifolia* leaf extract treated cells.

A, B, C, D represents the amplification plot for miR-221-5p expression and E, F, G, H represents the amplification plot for RNU6B (housekeeping miRNA) expression.
Figure 4.2: Fold change expression from real time RTqPCR

UT represents untransfected untreated cells; T represents the untransfected *P. longifolia* leaf extract treated cells; mimic+T represents the miR-221-5p mimic transfected *P. longifolia* leaf extract treated cells; anti-miR+T represents anti-miR-221-5p transfected *P. longifolia* leaf extract treated cells.
4.3.3 MTT cell viability assay

To further validate the comprehension of the underlying mechanism of action of miR-221-5p and miR-484 in HeLa cancer cells through *P. longifolia* treatment, MTT assay was performed. MTT assay which is considered as one of the golden standard for cell viability test measures cell viability based on the metabolic activity of viable cells that reduces yellow MTT to purple formazon. Qualitative assessment via the observation of colour intensity of purple formazon dye as shown in Plate 4.3 clearly indicates that more cells are viable after *P. longifolia* treatment when miR-221-5p is over-expressed compared to the miR-221-5p untransfected cells. Although miR-484 over-expressed cells shows slightly more survival rate than the untransfected cells upon *P. longifolia* treatment, the cell viability of miR-221-5p mimic transfected cells is more significant to that of miR-484 mimic transfected cells. This indicates more cells are metabolically active in the presence of specific miRNAs, namely miR-484 and miR-221-5p.

Quantitatively, the absorbance readings of the 24-well microplate was analysed and summarized in Figure 4.3. The cell viability was calculated using the formula below:

\[
\text{Cell viability(\%)} = \frac{\text{Sample} - \text{Blank}}{\text{Control} - \text{Blank}} \times 100\%
\]
Plate 4.3: The 24-well plate used in MTT assay which showed the increasing cell viability resulted in increased purple colouring
Figure 4.3: Cell viability of HeLa cells by using MTT assay

The data are expressed as mean ± SD (n=3). *p < 0.05 is considered to be significantly different
Upon *P. longifolia* treatment, miR-221-5p mimic transfected cells show 12.14% higher cell viability compared to the miR-484 mimic transfected cells. As anticipated, both miRNAs: miR-221-5p and miR-484 over-expressed cells significantly (P<0.05) decreased the apoptotic activity induced by *P. longifolia* treatment in HeLa cells by 29.53% and 17.39% respectively as compared to the treated; miRNA untransfected HeLa cells. The reproducibility between replicates is also good with very low standard errors. In the presence of specific miRNA mimics in methanolic *P. longifolia* leaf extract treated HeLa cells; fewer cells undergo cell death as compared to untransfected methanolic *P. longifolia* leaf extract treated HeLa cells. Among the two specific miRNAs, miR-221-5p is shown to be more effective than miR-484 since higher percentage of cells are viable in miR-221-5p mimic transfected samples compared to the miR-484 mimic transfected samples.
4.3.4 Annexin V/Pi assay

The flow cytometry annexin V/Pi assay is one of the functional analyses which determine viable cells, early apoptotic cells, late apoptotic cells and necrotic cells based on the differences in plasma membrane integrity and permeability was used to study the role miR-221-5p in induction of apoptotic death in HeLa cells treated with *P. longifolia* leaf extract.

Functional analysis of miR-221-5p in relation with the *P. longifolia* leaf extract treatment was performed using gain-of-function and loss-of-function of mir-221-5p by transiently transfecting mature miR-221-5p mimics and anti-miR-221-5p respectively into HeLa cervical cancer cells. Consistent with MTT assay results, the flow cytometry Annexin V/Pi analysis revealed a significant decrease (P<0.05) in early and late apoptosis in miR-221-5p mimic transfected cells compared to that of untransfected and anti-miR-221-5p transfected cells upon *P. longifolia* treatment. The counter plot (Plate 4.4) obtained from the analysis clearly demonstrated a significantly low (P<0.05) viable cells in *P. longifolia* treated cells (78.32%) as compared to the untreated cells (90.44%), concluding that *P. longifolia* treatment induces apoptosis in HeLa cells. However, the apoptotic rate significantly decreased (P<0.05) to 16.76% in miR-221-5p mimic transfected cells and increased to 29.96% in anti-miR-221-5p transfected cells upon treatment as compared to that of untransfected *P. longifolia* treated cells (19.45%). As illustrated in Figure 4.4, the over-expression of miR-221-5p decreases the apoptotic activity induced by *P. longifolia* leaf extract while the knock-down of miR-221-5p further increases the apoptotic activity of *P. longifolia*. 


Plate 4.4: Flow cytometric analysis of HeLa cells by Annexin V/PI double staining.

(A) Represents the untransfected untreated cells, followed by (B) untransfected *P. longifolia* leaf extract treated cells, (C) miR-221-5p mimic transfected *P. longifolia* leaf extract treated cells and (D) anti-miR-221-5p transfected *P. longifolia* leaf extract treated cells.

Quadrant 1 (Q1) shows necrotic cells (An-, PI+), Quadrant 2 (Q2) shows late apoptotic cells (An+, PI+), Quadrant (Q3) shows viable cells (An-, PI-) and quadrant 4 (Q4) shows early apoptotic cells (An+, PI-).
Figure 4.4: Percentage of HeLa cells in viable and apoptosis form based on flow cytometric analysis by Annexin V/PI double staining.

(A) Represents the untransfected untreated cells, followed by (B) untransfected *P. longifolia* leaf extract treated cells, (C) miR-221-5p mimic transfected *P. longifolia* leaf extract treated cells and (D) anti-miR-221-5p transfected *P. longifolia* leaf extract treated cells.

The data are expressed as mean ± SD (n=3). *p < 0.05 is considered to be significantly different.
4.3.5 Caspase 3 assay

The mechanism of miR-221-5p and its role in induction of apoptotic cells death in HeLa cells after treated with *P. longifolia* leaf extract has been further substantiated through caspase-3 assay. The activation of caspase-3 is one of the important hallmarks of apoptosis. The activation of caspase-3 through several other upstream caspase cascade initiates the apoptosis process, also being the coinciding locus for different signalling pathways (Nicholson *et al.*, 1995). Caspase-3 is one of the proteases which comparatively increase in apoptotic cells. Thus, cells were lysed to extract total protein from each sample and protein concentrations were obtained by plotting a typical standard curve from the Bicinchoninate Protein assay (Figure 4.5). Bovine Serum Albumin (BSA) was used as standard. Equal amount of protein from each sample was used to determine the presence of active caspase-3 by using caspase-3 specific substrate, DEVD-pNA.

The results (Figure 4.6) evidently demonstrated a significant increase (P<0.05) of active caspase-3 when the HeLa cells were treated with *P. longifolia* leaf extract as compared to the untreated cells by 11.82%. To uncover the role of miR-221-5p in relation to the *P. longifolia* treatment, miR-221-5p mimic approach has been used to over-express the miR-221-5p level before *P. longifolia* treatment. Interestingly, the level of active caspase-3 was shown to be decreased suggesting a decreased apoptotic effect of *P. longifolia* treatment in miR-221-5p over-expressed HeLa cells. In contrast, level of active caspase-3 in the miR-221-5p knock down samples increased drastically by 30.19% as compared to the control HeLa cells.
Figure 4.5: Typical standard curve from the Bicinchoninate Protein assay for the determination of active caspase-3

\[ y = 1.3774x + 0.5842 \]
\[ R^2 = 0.9956 \]
Figure 4.6: Caspase-3 enzyme activity in HeLa cells treated with *P. longifolia* leaf extract for 24 hours

A represents untransfected untreated cells; B represents the untransfected *P. longifolia* leaf extract treated cells; C represents the miR-221-5p mimic transfected *P. longifolia* leaf extract treated cells; D represents anti-miR-221-5p transfected *P. longifolia* leaf extract treated cells.

The data are expressed as mean ± SD (n=3). *p < 0.05 is considered significant as compared to control.
4.4 Discussion

4.4.1 Transfection of miRNA mimics and anti-miRNA

The role of miRNAs in regulating various cellular processes has attained serious interest in order to utilize their prospective ideals as biomarkers and pharmacotherapy means (Janssen et al. 2013; Caputo et al. 2015; Emanueli et al. 2015). These ~22 base pair (bp) length miRNAs were shown to partially pair with their mRNA targets to effectively regulate the gene expressions and the down-stream protein expressions which subsequently regulate the cellular processes such as cell proliferation, cell invasion and apoptosis (Bartel, 2004; Herranz and Cohen, 2010). Hence, validation and functional analysis of specific miRNA can be performed with the aid of recently developed miRNA transfection techniques which in turn over-express or silence the miRNA by miRNA mimics and miRNA inhibitors or anti-miRNA, respectively.

There are several approaches available to transfect miRNA into cell lines such as the transient transfection, lentiviral infection or plasmid transfection, retroviral infection, transgenic expression and endogenous over-expression of miRNAs. However, transient transfection using miRNA mimics into cell lines was reported to yield an increased molecular weight RNA species and a few hundred fold increase in mature miRNA levels as compared to that of the other approaches where the miRNA expression was only increased by less than 10-fold (Jin et al., 2015). Therefore, transient transfection technique was used in our study with the incorporation of miRNA mimics and inhibitors. miRNA mimics are chemically synthesized double-stranded RNA molecules which imitates naturally occurring endogenous miRNAs after being transfected into cells. On the other hand, miRNA
inhibitors or commonly known as anti-miRNA are single stranded, modified RNA which are designed to specifically pair the miRNA and inhibit its function.

It is necessary to take note that unlike the artificially synthesized miRNA mimics, there are no chemical modifications or nucleotide alterations found in the naturally occurring endogenous miRNAs (Lim et al., 2005; Garcia et al., 2011; Wang, 2011; Thomson et al., 2013). However, these alterations are made only to perk up their stability, to assist guide miRNA loading to RISC, and to selectively exclude the passenger strand as well as to facilitate the delivery of mimics into the cells, circumventing the endogenous miRNA biogenesis pathway but functioning as the natural miRNA (Jin et al., 2015). Transfection of miR-221-5p mimics are meant to specifically over-express the miR-221-5p expression which in turn down-regulates the expression of its mRNA targets and subsequent protein level. Inversely, miR-221-5p inhibitors are transfected into cells to silence the expression of miR-221-5p which will eventually increase the expression of its mRNA targets and proteins.

It is also essential to consider the few draw backs in transient transfection mechanism of these artificial miRNA mimics and inhibitors. Intermittently, miRNA transfection may result in off-target effects, arising from partial pairing of the sense and anti-sense strands to an intended targets. Off-target issue may lead to mRNA degradation, inhibition of translation, or induction of an interferon response and consequently mislead the results, reducing the reliability of the experiments (Jackson et al., 2003; Sledz et al., 2003; Scacheri et al., 2004). However, this limitation was reported to be successfully resolved by minimizing the concentration of miRNA used to transfec into cells (Semizarov et al., 2003; Persengiev et al., 2004). Therefore, our study used a lower concentration of miRNA mimics and anti-miRNA
with the incorporation of HiPerFect transfection reagent, enabling an increased efficiency with as low as 5 nM for miRNA mimic and 50 nM for miRNA inhibitor.

Other than that, important factors such as the incubation time and cell plating density were also highly taken into consideration to allow an optimal transfection of miRNA mimics and miRNA inhibitors. The cell intensity was ensured to be not too confluent and hence, transfection of miRNA mimics and anti-miRNA was performed when the cells are growing in a log-phase proliferative behaviour, allowing cell-to-cell interaction to optimize the transfection efficiency (Radhakrishnan et al., 2010).

Transient transfections of miR-221-5p mimics and anti-miR-221-5p to over-express and knock down the expression of mi-221-5p respectively were validated and quantified by using real time PCR. The similar transfection technique was used for the down-stream analysis including functional analysis and proteomic analysis of miR-221-5p to further investigate the role of miR-221-5p in association with P. longifolia leaf extract treatment in HeLa cervical cancer cells in comparison with miRNA untransfected control cells.

4.4.2 Total RNA isolation

The induction of apoptosis by P longifolia leaf extract treatment in HeLa cervical cancer cells was previously reported to be through the dysregulation of miRNAs, in which miR-221-5p was found to be highly down-regulated and attained inquisition in further investigating its role in cancer cells. In order to validate the involvement of miR-221-5p and further quantify the real time expression level of miR-221-5p in P longifolia treated HeLa cells in comparison with the untreated cells, Taqman MiRNA Assay, putatively known as RTqPCR was incorporated into this study. The
RTqPCR begins with the isolation of RNA of good quality and purity. There are various kinds of naturally occurring RNAs such as ribosomal RNA (rRNA) (80%–90%), messenger RNA (mRNA) (2.5%–5%), transfer RNA (tRNA) and other small length RNA like siRNA and miRNA (Buckingham and Flaws, 2007). Unlike DNA, RNA is an unstable molecule with a very short half-life once extracted (Brooks, 1998). Due to their unstable and heat-stable behaviour, it is highly imperative to maintain a good laboratory technique and RNase-free techniques (Tan and Yiap, 2009). There are different methods to extract RNA from cells depending on purpose of extraction. Few such examples of RNA isolation approaches include the conventional alcohol precipitation or putatively known as Trizol technique, phenol: chloroform followed by solid-phase extraction and solid-phase separation with affinity resin.

Although Trizol reagent is one of the commonly used techniques due to its efficient and reproducibility, incorporation of MirVana RNA isolation kit has been specifically designed to isolate purified miRNA-rich total RNA for studies involving siRNAs and miRNAs (Muljo and Kanellopoulou, 2006). Previous studies evidently reported the a better RNA extraction performance from mirVana isolation kit as compared to Trizol technique in terms of useful reads sequenced, number of miRNA identified, and reproducibility with reduced noise background, thus recommending miRVana over Trizol for miRNA studies (Guo et al., 2014). Other studies also demonstrated the limitations of Trizol technique such as specific loss of miRNA and low GC content in mature miRNA, precursor miRNA, small interfering RNA (siRNA) duplexes, and transfer RNAs (tRNAs) (Kim et al., 2012).

In addition, the important factors to be taken into consideration while extracting RNA include efficient disruption of cells; denaturation of nucleoprotein
complexes; inactivation of nucleases; free from contamination (Doyle, 1996). One of the main challenges in extracting RNA is the damage caused by RNAses present in the cells (Brooks, 1998; Buckingham and Flaws, 2007). This impediment can be resolved by inactivating the RNase in chemical extraction using highly concentrated chaotropic salts in conjunction with acidic phenol or phenol-chloroform solutions. Solid-phase extraction step using spin column plays a role to decrease the affinity of RNA for water and increase its affinity for the solid support used. However, recovering small length RNAs was still found to be challenging.

Therefore, in this study, mirVana isolation kit has been incorporated for the RNA isolation process to be utilized in the RTqPCR, which contemplates all the advantages of conventional methods, at the same time the disadvantage in loss of miRNA is kept at bay. After disrupting the cellular structure and inactivation of RNase steps, the total RNA isolation technique also uses the Acid-Phenol: Chloroform extraction step in order to remove all traces of proteins, lipids, carbohydrates, cell debris and also most of the DNA (Chomczynski, 1987; Sambrook and Russel, 2001; Chomczynski and Sacchi, 2006) which leaves semi-pure RNA sample. This was followed by the final RNA purification by passing the samples through Filter Cartridge containing a glass-fiber filter. This filter functions to immobilize the RNA which was then eluted with a low ionic-strength solution. Total RNA isolation method was chosen over small RNA enrichment method in order to quantitate and evaluate the quality of RNA.

The selection of mirVana isolation kit was supported by recent study conducted by Brown et al. (2018) in which elevated percentage of miRNA yield was shown to be consistently observed in various types of mouse tissues using mirVana as compared with the Trizol method. In the same study, miRNA analysis using
Taqman RTqPCR demonstrated an increased the expression of specific miRNAs from the RNA extracted using mirVana as compared to that of RNA extracted from other methods (Brown et al., 2018). Furthermore, RNA extracted using mirVana was also shown to work efficiently and consistently for the identification and validation of miRNA target genes by revealing highest expression of miRNA gene targets in comparison with other methods (Brown et al., 2018). Overall, total RNA isolation technique using mirVana was shown to be rapid, precise, reproducible and reliable with high yield, purity and minimized contamination.

4.4.3 Taqman RTqPCR

The total RNA extracted from each sample: untreated HeLa cells, *P. longifolia* treated HeLa cells, *P. longifolia* treated miR-221-5p mimic transfected HeLa cells, and *P. longifolia* treated anti-miR-221-5p transfected HeLa cells are utilized to further analyse the miR-221-5p expression. Although there are various methods to analyze RNA, namely Northern blots, RNA dot/slot blots, nuclease protection assays, and in situ hybridization, polymerase chain reaction (PCR) technique still remains the highly used method of choice. RNA can be analysed in terms of semi-quantitative analysis, known as the conventional reverse transcription PCR (RT PCR) or real-time quantitative PCR (qPCR). RT PCR is only used to detect the presence or absence of specific RNA while qPCR technique accurately quantifies the expression of specific RNA in real time (Williams et al., 2006). Real time PCR is considered to provide highly efficient and accurate results as compared to that of the conventional RT PCR (Kubista et al., 2006; Morillo et al., 2003; Novais et al., 2004). There are two popularly used quantitative gene expression techniques (qPCR), namely SYBR Green and Taqman.
Although SYBR Green technique is known for its cost-effectiveness and user-friendly, specificity is the major limitation of this technique because SYBR Green dye binds to any minor groove of double-stranded deoxyribonucleic acid (dsDNA) and emits fluorescence which may lead to false-positive results (Huang et al., 1995; Tajadini et al., 2014). However, Taqman approach employs short sequence-specific hydrolysis probe with dual fluorophore-labelled DNA oligonucleotides, denominated as quencher and reporter at each end (Mullis, 1990; Mullis and Faloona, 1987). The adjacency of the quencher and reporter impede the emission of fluorescence as the quencher absorbs the signal from the reporter based on the fluorescence resonance energy transfer (also called Förster transfer) theory (Valasek and Repa, 2005). When the probe specifically binds to the gene of interest and reporter emits fluorescent signal when it is detached from the quencher by DNA polymerase 5’-nuclease activity, allowing results of quantification data to be more reliable compared to the SYBR Green technique (Tan et al, 1994). Henceforth, Taqman miRNA assay was incorporated in our study to perform the quantification of miR-221-5p expression in HeLa cells. This study can also be used as a validation for the results obtained from DeepSequecing experiment conducted by Vijayarathna (2017) reporting the down-regulation of miR-221-5p upon P. longifolia leaf extract treatment in HeLa cells as normalized with the untreated cells.

Unlike mRNA, miRNAs are found to be challenging to amplify by PCR because of its shorter size and its stable hairpin structure which urged the need to further develop and modify the real time PCR technology (Schmittgen et al., 2008). This led to the discovery of the two-step RT PCR, called the Taqman miRNA Assay. The first step which involves the reverse transcription of total RNA extracted by the mirVana to synthesis cDNA uses a specially designed stem-looped RT primer. This
mature-miRNA specific stem-looped RT primer is designed to encompass the stem extending about 5-8 nucleotides (nt) 3’ overhang sequence complementary to the 3’ end of the mature miRNA, and a loop sequence accommodating a universal 3’ priming site (Zöllner et al., 2013). Contradictory from the standard Taqman qPCR, the advanced stem-looped primer designing enhances the specificity and sensitivity of the miRNA assay.

The subsequent PCR step adopts the conventional Taqman qPCR technique involving the forward and reverse primers along with the miRNA specific Taqman probe. The Taqman probe used in our study consists of FAM reporter dye at 5’ end and a minor groove binder (MGB) linked to a non-fluorescent quencher (NFQ) at the 3’ end of the probe with the PCR target complementary sequence in between which specifically binds to the miRNA of interest. This customized probe elevates the melting temperature (Tm) without extending probe length (Afonina et al., 1997; Costa et al., 2004), granting the construction of shorter probes. As the exonuclease activity of Taq DNA polymerase during amplification cleaves the hydrolysis probe, the reporter molecule emits fluorescence. The emission of fluorescence signal is directly proportional to the PCR products, allowing accurate quantification of miRNA expression in real time. The quantification of miR-221-5p expressions acquired from qPCR analysis was normalized using a standard endogenous reference miRNA, namely RNU6B (U6) (Gee et al., 2011). We used the comparative CT (cycle threshold) method, putatively known as the ΔΔCT approach to analyse the expression of miR-221-5p (Livak and Schmittgen, 2001).

The results obtained from the Taqman qPCR successfully validated the expression of miR-221-5p obtained through miRDeep2 sequencing performed by our previous study. According to our previous study, expression of miR-221-5p reported
to be highly down-regulated upon *P. longifolia* leaf extract treatment in the process of inducing apoptosis in HeLa cervical cancer cells compared to that of untreated cells (Vijayarathna, 2017). Current qPCR analysis using miR-221-5p specific primers and taqman probes confirmed the over-expression of miR-221-5p in untreated HeLa cells in comparison with the *P. longifolia* leaf extract treated HeLa cells. Role of miR-221-5p expression in relation to *P. longifolia* leaf extract treatment was further validated using miR-221-5p mimics and anti-miR-221-5p transfected cells. The data suggests that the down-regulating miR-221-5p expression is a potential therapeutic approach to induce apoptosis in cancer cells, in which *P. longifolia* treatment was shown to be an effective anti-cancer agent to silence the expression of miR-221-5p expression.

According to the literature, the results obtained from the current study are found to be consistent with previous studies on miR-221 analysis. A bunch of studies reported the over-expression of miR-221 in various diseases including cancer. For instance, recent study revealed an over-expression of miR-221 in serum collected from type-2 diabetic patients as compared to the healthy patients through similar RTqPCR approach (Liu *et al.*, 2018a). Another study using SYBR Green real time qPCR also revealed the up-regulation of miR-221 in plasma from Acute Pulmonary Embolism as compared to that of healthy plasma (Liu *et al.*, 2018b). In addition, miR-221 was found to be over-expressed in osteoblasts, leading to increased cell proliferation by regulating ZFPM2 (Zheng *et al.*, 2018). Besides, miR-221 specific TaqMan MiRNA assays performed in tissue samples collected from osteosarcoma patients revealed significantly high expression of miR-221 in comparison with samples from non-cancer tissues (Gong and Gong, 2018).
MiR-221 is reported to play its role as an oncogene in different types of cancer including ovarian cancer (Hong et al., 2013; Li et al., 2017), breast cancer (PLOS ONE Editors, 2017), pancreatic cancer (Xu et al., 2015), prostate cancer (Gordanpour et al., 2011; Yang et al., 2014) and lung cancer (Yamashita et al., 2015). For instance, over-expression of miR-221 was also reported in ovarian cancer tissues and the association of miR-221 with B-cell lymphoma 2 modifying factor (BMF) mRNA was further investigated using miR-221 mimics and anti-miR-221 (Xie et al., 2018). RT-qPCR analysis found that the up-regulation of miR-221 expression decreases the expression of BMF, thus inhibiting the apoptosis process in ovarian cancer cells. Furthermore, similar results were also observed from RTqPCR analysis performed to analyse the miR-221-5p expression in human prostate cancer cells, reporting an increase in cell proliferation by the up-regulation of miR-221-5p expression (Shao et al., 2018). In his study, the molecular mechanism of miR-221-5p was further investigated and successfully validated that miR-221-5p targets one of the tumor suppressor gene, namely the SOCS1, suppressers of cytokine signalling (SOCS) family protein (Shao et al. 2018). These investigations evidently supported our current study to induce apoptosis by P. longifolia leaf extract by targeting miR-221 expression since miR-221 shows tumor-promoting properties.

On the other hand, there are few previous studies which demonstrated various types of cancer cells which over-express miR-221 to show resistance to some of the currently available chemotherapy agents. One such recent study conducted by Du et al. (2017) used similar approach to our current study utilizing the advancement of real time SYBR-Green PCR to study of effect of miR-221 differential expression in regulating the sensitivity of oral squamous carcinoma cells to doxorubicin. In their study, they over-expressed and knock down the expression of miR-221 using miR-
221 mimics and anti-miR-221 respectively in oral squamous carcinoma cells and then treated the samples with doxorubicin in order to study the expression level of miR-221 and further confirmed the participation of miR-221 in chemoresistance of the cells to doxorubicin treatment (Du et al., 2017).

Hence, the literature supports the results obtained in this study indicating the over-expression of miR-221 can be considered to be biomarker for cervical cancer cells. At the same time, targeting or silencing the expression of miR-221-5p can be a promising cancer therapy through miRNA approach. Since P. longifolia leaf extract treatment was successfully validated to down-regulate the expression of miR-221-5p in the process of apoptosis, P. longifolia can be a potential anti-cancer agent for cervical cancer. Functional analysis of miR-221-5p is important to elucidate the mechanism involved in the induction of apoptosis by P. longifolia by targeting miR-221-5p expression.

4.4.4 MTT cell viability assay

Previous findings demonstrated the involvement of miRNAs in the molecular mechanism of apoptosis induced by P. longifolia leaf extract in HeLa cervical cancer cells (Vijayarathna, 2017). Consequently, miR-221-5p and miR-484 gained interest for further studies as they are the most down-regulated miRNA in HeLa cells treated with P. longifolia leaf extract compared to untreated HeLa cells. To attain insight of underlying mechanisms of action of the miR-221-5p and miR-484 in cervical cancer, further in vitro cell viability assay was performed in this study.

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay is one of the most commonly used assay methods in miRNA
functional analysis to evaluate the number of viable cells by using loss-of-function or gain-of-function of specific miRNAs. Cell-based assays are considered to be an established method because receptor-binding and signal transduction activities involving gene and protein expressions in the process of cell proliferation or apoptosis can be studied (Riss et al., 2016). MTT assay evaluates the cell viability based on the mitochondrial enzymatic activity of NAD(P)H-dependent cellular oxidoreductase enzymes present in the viable cells to reduce the yellow tetrazolium compound to purple formazon crystals (Stockert et al., 2012). This concludes that the number of viable cells is directly proportional to the mitochondrial activity of the cells (Meerloo et al., 2011). Mosmann’s development of MTT assay is considered to be the “golden standard” for the determination of cell viability because of its non-radioactive cell counting method with high sensitivity and reproducibility (Mosmann, 1983; van Tonder et al., 2015). Although solubilisation of the water insoluble formazon crystal was found to be a bit challenging, the use of dimethyl sulfoxide (DMSO) resolved the throwback of this assay (Van Rensburg et al., 1997; Wang et al., 2012).

The MTT assay results revealed that the over-expression of miR-484 and miR-221-5p suppressed the apoptotic effect of *P. longifolia* leaf extract treatment, in which miR-221-5p mimic transfected HeLa cells showed highly significant effect as compared to that miR-484 mimic transfected HeLa cells. This has been demonstrated both qualitatively (Plate 4.1) and quantitatively (Figure 4.3) in the result section. *P. longifolia* treatment caused 90.32% cell death as compared with the untreated cells. Interestingly, the over-expression of miR-221-5p and miR-484 through transient transfection of miR-221-5p and miR-484 mimics independently maintained the cell survival rate up to 39.21% and 27.06% respectively even after the *P. longifolia*
treatment. The results from this study suggested the effect of *P. longifolia* leaf extract treatment on HeLa cells to induce apoptosis involves the down-regulation of miR-221-5p and miR-484.

The acquired results supported the previous study conducted by Vijayarathna (2017) indicating the phytochemical-rich *P. longifolia* leaf extract induces apoptosis through miRNA dysregulation in HeLa cervical cancer cells. Through literature investigations, polyphenols from plants have been evidently demonstrated to regulate the miRNA expression in various human cells and play their role in the cell signalling pathways. This supports the results obtained via MTT assay where the involvement of specific miRNAs expression was proven to play its role in HeLa cells treated with *P. longifolia* leaf extract. Furthermore, similar data has been observed while investigating the throwback of currently practiced, U.S. FDA approved breast cancer drug called tamoxifen. Previous study conducted by Miller *et al.* (2008) using similar MTT assay successfully demonstrated an increase in cell survival rate of miR-221/222 over-expressed MCF-7 breast cancer cells as compared to the untransfected cells when treated with 5 µM of tamoxifen. In the same study, it was revealed that over-expression of miR-221/222 inhibits tamoxifen-induced apoptosis by targeting the tumor-suppressor function of p27Kip1 (Miller *et al.*, 2008).

Another study also revealed consistent result indicating the role of over-expressed miR-221/222 in ERα-negative breast cancer cell lines and primary tumors in suppressing the apoptosis effect of tamoxifen by evaluating the cell viability by MTT assay (Zhao *et al.*, 2008). At the same time, Zhao *et al.* (2008) also demonstrated the relationship between miR-221/222 expression and ERα protein expression in ERα-positive breast cancer cell lines and revealed that the knock-down
of miR-221/222 restores the expression ERα protein and thus improves tamoxifen sensitivity to induce apoptosis in breast cancer cells.

Besides tamoxifen, another U.S. FDA approved anti-cancer drug called doxorubicin was found to be less effective in oral squamous carcinoma cells due to the involvement of miR-221 expression. MTT assay was performed and successfully revealed a moderate decrease in cell viability of miR-221 knock down cells and a significant decrease in cell viability of doxorubicin treated miR-221 knock down cells as compared to that of control cells (Du et al., 2017).

4.4.5 Flow cytometric annexin V/PI analysis

Although the role of miR-221-5p over-expression in suppressing the HeLa cell death induced by *P. longifolia* leaf extract treatment was shown by MTT assay, the mode of cell death was further validated through Annexin V/Pi analysis and caspase-3 analysis. The fate of cell death may vary based on the mechanisms undergone to initiate, execute and dispose which can be monitored by morphological and molecular alterations of the cells (Darzynkiewicz et al., 1997; Darzynkiewicz et al., 2001; Darzynkiewicz et al., 2004). These changes can be investigated with the employment of microscopy, flow-cytometry or molecular biology techniques. Flow cytometry is considered to be highly sensitive because various factors can be measured at a time and prompt analysis can be done for thousands of cells per second (Darzynkiewicz et al., 2004). Plasma membrane remodelling is one of the classical apoptosis characteristics which can be studied using flow cytometric Annexin V/Pi analysis. When cells undergo apoptosis, translocation of the phospholipid phosphatidylserine (PS) from inner membrane is exposed to the outer
membrane during early apoptosis stage while permeability of plasma membrane can be observed during late apoptosis stage (Koopman et al., 1994; van Engeland et al., 1998; Hingorani et al., 2011). The translocation of PS to the cell membrane exterior in early apoptotic cells was demonstrated to be detected in flow cytometry by the binding of fluorescent labelled Ca\(^{2+}\) dependent phospholipid-binding protein known as the Annexin V which has high affinity to PS compared to other phospholipid species present in the outer cell membrane such as phosphatidylcholine and sphingomyelin (Vermes et al., 1995). However, PS externalisation coincides with an increased cell membrane permeability in late apoptotic cells which can be quantitatively detected with an incorporation of DNA binding dye, propidium iodide (PI) (Ormerod et al., 1993). Since the cell membrane of early apoptotic cells remain consolidated, PI fails to penetrate the cells. Hence, PI was used in association with Annexin V in order to distinguish late apoptotic cells from early apoptotic cells. However, cell membrane of dead cells are completely destroyed or damaged which eventually allows only the binding of PI. In summary, viable cells show negative results for both Annexin V and PI while cells at early apoptotic phase exhibit Annexin V positive and PI negative, cells at late apoptotic stage show positive results for both Annexin V and PI and finally dead cells show negative result for Annexin V and PI positive (Baskic et al., 2006). Annexin V/Pi double-staining analysis is one of the widely used flow cytometry approaches for the detection of apoptosis in various scientific studies because of its economical, reliability and sensitivity factors (Rieger et al., 2011; Zhang and Liang, 2014).

The results acquired from annexin V assay showed an increase in apoptosis when treated with *P. longifolia* leaf extract as compared to that of untreated cells. However, the functional analysis of miR-221-5p using gain-of-function and loss-of-
function approach by transiently transfecting the miR-221-5p mimics and anti-miR-221-5p respectively prior to treatment, revealed to affect the apoptosis rate. The results revealed that miR-221-5p over-expressed cells decreased the apoptosis cells induced by *P. longifolia* leaf extract treatment in HeLa cells. Contrastingly, cells transfected with anti-miR-221-5p showed an increase in apoptotic cells. This concludes the oncogenic role of miR-221-5p to induce cell proliferation and to inhibit apoptosis.

The acquired results support the microarray analysis conducted by Vijayarathna (2017). Upon revealing the induction of apoptosis in HeLa cells by *P. longifolia* leaf extract treatment, Vijayarathna (2017) further performed miRDeep2 analysis and volcano plot analysis to identify the differentially expressed miRNAs in treated cells as compared to the vehicle control cells. It has been reported that miR-221-5p is one of the highly down-regulated miRNA with log2 Fold Change (FC) of lower than -3 in Hela cells treated with *P. longifolia* leaf extract over vehicle control cells with at least 1000 normalized reads transcripts per million (TPM).

Functional analysis of disparate miRNAs is conducted using annexin V/pi approach to reveal the participation of miRNA in apoptosis. Previous study conducted by Fu *et al.* (2015) demonstrated that knock down of miR-221 expression impedes cell proliferation in a time-dependent manner T24, 5637 and J82 bladder cancer cells. In the same study, flow cytometric annexin V/PI assay was performed to report the induction of apoptosis in miR-221 inhibitor transfected cells as compared to that of control cells (Fu *et al.*, 2015). The over-expression of miR-221 in bladder cancer cells was reported to target the pro-apoptotic gene, namely p53.
upregulated modulator of apoptosis (PUMA) which eventually inhibits the apoptotic signals (Fu et al., 2015).

Furthermore, recent study also demonstrated the role of miR-221 in regulating cell proliferation and apoptosis in ovarian cancer cells by targeting B-cell lymphoma 2 modifying factor (BMF) gene (Xie et al., 2018). Similar to our study, flow-cytometric annexin V/Pi double staining and spectrometric caspase-3 assay were incorporated to detect cell apoptosis in the study of Xie et al. (2018). The results obtained in his study were coherent to our present findings indicating an increased apoptosis rate in anti-miR-221 transfected SKOV3 cells compared to the control cells.

The expression of miR-221 was reported to be inversely proportional to the expression of BMF gene as bioinformatics prediction revealed also revealed that BMF is one of the target genes of miR-221-5p (Hong et al., 2013). Hence, the down-regulation of miR-221-5p expression in HeLa cells by P. longifolia treatment can be deduced to eventually increase the expression of tumor suppressor gene, BMF which sets of the apoptosis process by binding to the anti-apoptosis factors B-cell lymphoma (Bcl)2, Bcl-xL and Bcl-w proteins (Thomadaki et al., 2006) which warrant further detailed studies.

In addition to MTT assay conducted by Du et al. (2017) to reveal the participation of miR-221 in resistance of oral squamous carcinoma cells to doxorubicin drug, annexinV-fluorescein isothiocyanate double staining was also conducted to quantitatively detect cell apoptosis and found similar results. Their study further confirmed that the expression of tissue inhibitor of metalloproteinase-3 (TIMP3) gene and protein level is inversely proportional to the expression of miR-
221 as TIMP3 is one of its mRNA targets. Various studies also reported the contribution of TIMP3 in enhancing the sensitivity of cells to anti-cancer drugs (Garofalo et al., 2009; Gan et al., 2014).

Other than that, ultra-sound mediated down-regulation of miR-221 and miR-21 was found to be a promising gene therapy for liver cancer (Guo et al., 2017). In the study conducted by Guo et al. (2017), decrease in cell viability and increased detection of apoptosis have been successfully demonstrated in anti-miR-21/221 transfected human hepatoma HepG2 cells by MTT and annexin V/PE analysis respectively which is consistent with the results obtained from our present study. Down-regulation of miR-21/221 was found to result in an up-regulation of CDKN1B/p27 and DKN1C/p57 expression while down-regulation of BMF in the process of inducing apoptosis in human hepatocellular carcinoma (Fornari et al., 2008; Gramantieri et al., 2009; Fu et al., 2011; Turato et al., 2014).

Moreover, previous study reported that contribution of miR-221 in cell proliferation of mouse embryonic stem cells by targeting p57 (Li et al., 2014). However, annexin V/pi assay demonstrated that miR-221 inhibitor reduced cell proliferation of embryonic stem cells without inducing apoptosis or necrosis. This suggests that the down-regulation of miR-221 specifically targets cancer cells to induce apoptosis while apoptosis is not observed in miR-221 inhibitor transfected normal embryonic stem cells.

**4.4.6 Caspase-3 analysis**

Activation of caspase cascade is one of the major hallmarks of apoptotic cell death (Porter and Janicke, 1999; Saraste and Pulkki, 2000; Wlodkowic et al., 2009). The
two primitive types of caspases are the initiator caspases (caspase-1, -2, -4, -5, -8, -9, -10, -11, -12) and effector caspases (caspase-3, -6, -7) in which the effector caspases carry out the ensuing apoptosis mechanism via cleavage of cellular substrates and activation of antecedent caspases (Li and Yuan, 2008). Various literatures reported the involvement of miRNAs in the induction of intrinsic apoptotic pathway through the activation of caspase (Pileczki et al., 2016). Unambiguous process of caspase-9 activation comprehend the oligomerization of Apaf-1 through formation of wheel-shaped apoptosome in which seven molecules of Apaf-1 each binding to one molecule of cytochrome c and caspase-9 (Acehan et al., 2002; Yu et al., 2005). Subsequent activation of caspase-9 leads to activation of caspase-7 and caspase-3 that executes apoptosis. The activated caspase-3 catalyses the specific cleavage of several important cellular proteins, thus caspase-3 acts as a death protease (Porter and Janicke, 1999). The activation of caspase-3 is essential for apoptotic chromatin condensation and DNA fragmentation as well as in destroying the cell and involved in the formation of apoptotic bodies in all cell types (Porter and Janicke, 1999). Activation of caspases can be detected by adding their specific substrate thereupon considering the activated caspase to be a biochemical marker for apoptosis (Zhivotovsky, 2003). Hence, investigation of the enzymatic activity of caspase-3 in miR-221-5p mimic and anti-miR-221-5p transfected HeLa cells alongside with P. longifolia leaf extract treatment was performed in this study to further elaborate the functional analysis of miR-221-5p.

The fundamental principle of this assay relies mainly on the cleavage of caspase-3-specific DEVD substrate peptide which is conjugated to the chromophore containing $p$ nitroanilide ($p$NA) by the active caspase-3 present in the cell cytosol (Ibelgaufts, 2012). This action by the active caspase-3 eventually releases the
chromophore pNA which can be quantified spectrophotometrically at a wavelength of 405 nm.

The results obtained from this study manifest an increased level of caspase-3 activation in HeLa cancer cells treated with *P. longifolia* leaf extract in comparison with the untreated cells which is persistent with previous study (Vijayarathna, 2017). The activation of caspase-3 is shown to decrease when miR-221-5p is over-expressed in HeLa cells by transiently tranfecting miR-221-5p mimics before *P. longifolia* treatment. In contrast, miR-221-5p knock down in HeLa cells treated with *P. longifolia* leaf extract shows a drastic increase in activation of caspase-3, suggesting increase in apoptotic rate. This corroborates that function of over-expressed miR-221-5p in cell survival, reducing the effect of *P. longifolia* treatment to induce apoptosis. It also supports the miroRNA analysis conducted by Vijayarathna (2017) in which miR-221-5p was found to be highly down-regulated upon *P. longifolia* treatment.

Furthermore, various studies have successfully reported the anti-cancer properties of medicinal plant extract by investigating the activation of caspase-3. Phytochemicals present in medicinal plants have been demonstrated to regulate the miRNA expression, thus affecting various cellular processes including apoptosis, cell cycle, cell differentiation and cell proliferation (Ross and Davis, 2011). Hence, targeting miRNA using phytochemical-rich medicinal plant is a promising candidate for cancer treatment.

Online prediction revealed that caspase-3 is one of the targets of miR-221 which was then validated by western blot analysis, demonstrating a significant decrease of caspase-3 protein by miR-221 over-expression in HEK293T cells (Jin *et al.* 2019).
al., 2018). In the same study, miR-221 regulated caspase-3 expression was found to affect the TNF Related Apoptosis Inducing Ligand (TRAIL)-induced cell apoptosis in hepatocellular carcinoma. Therefore, a tumor suppressive long non-coding RNA, namely CASC2 was found to contribute in reducing the TRAIL-induced apoptosis resistance by serving as “sponge” of miR-221, which eventually down-regulates miR-221 causing an increase in expression of Casapase-3 to induce apoptosis in hepatocellular carcinoma (Jin et al., 2018).

Study conducted by Gramantieri et al. (2009) revealed similar findings in which anti-miR-221 transfected hepatocellular carcinoma cells were demonstrated to highly increase the expression of BMF, a proapoptotic BH3-only protein and caspase-3 protein expression as compared to that in control cells. Thus, down-regulation of miR-221 is a potential cancer therapy approach to induce apoptosis.

Another study also demonstrated the role of miR-221 expression in regulating caspase-3 expression in osteosarcoma. The study conducted by Hu et al. (2018) revealed that the over-expression of miR-221 expression in osteosarcoma cell lines eventually knocked down the expression of cyclin-dependent kinase inhibitor 1B (CDKN1B). Down-regulation of CDKN1B expression was found to reduce the expression of Bax and caspase-3 and increase the cyclin E, cyclin D1, Bcl-2, Snail, and Twist1, thus decreasing cellular apoptosis in MG63 and U-2OS cell lines (Hu et al., 2018). This finding absolutely supports the results of present study revealing that the down-regulation of miR-221 in HeLa cells through P. longifolia leaf extract treatment induces apoptosis by increasing caspase-3 expression.

Similar results were also found in another study where the down-regulation of miR-221 expression was shown to increase the expression of proteins involved in
Apaf-1 apoptotic pathways including caspase-3, -8 and -9 in laryngeal squamous cell carcinoma cell line, Hep-2 through western blot (Sun et al., 2015). Caspase-3 activity was demonstrated to drastically decrease in miR-221/222 mimic transfected human prostate cancer cells, thus repressing apoptosis and contrastingly miR-221/222 knock down cells were shown to sensitize increased TNF-α/CHX-induced apoptosis (Wang et al., 2015).

Recent study also showed similar results in which over-expression of miR-221 was revealed to induce PC-9 lung cancer cells resistance to gefitinib by down-regulating APAF-1 (Gao et al., 2018). According to that study, the expression of APAF-1 and caspase-3 proteins were found to be significantly down-regulated in gefitinib treated miR-221 over-expressed PC-9 cells as compared to that of treated miRNA untransfected cells (Gao et al., 2018). In another study, knock down of dicer expression was demonstrated to increase chemosensitivity of PC-9 cells to gefitinib through the down-regulation of miR-30b/c and miR-221/222 which subsequently increases the caspase-3 protein level, inducing gefitinib-induced apoptosis.

In addition, elevated miR-221 expression was shown to decrease caspase-3 expression, thus inhibiting apoptosis process in various cancer cell lines, namely SKBR3 (breast cancer), HCC1500 (breast cancer), MDA-MB-231 (breast cancer), A549 (lung cancer), DU145 (prostate cancer), HepG2 (hepatocellular carcinoma), HeLa (cervix cancer) and HGC-27 (gastric cancer) by performing real time PCR (Ergun and Oztuzcu, 2014). Hence, an up-regulation of miR-221-5p expression affects the induction of apoptosis by P. longifolia leaf extract treatment in HeLa cells by reducing the caspase-3 activation can be supported by similar results obtained by these previous studies, explaining the oncogenic role of miR-221 to inhibit apoptosis by blocking caspase-3.
4.5 Conclusion

In conclusion, this chapter successfully validated the down-regulation of miR-221-5p expression by *P. longifolia* leaf extract treatment which was recently reported from the study conducted by Vijayarathna (2016). The current study also confirms that the expression of miR-221-5p plays an important role in regulating apoptosis process induced by *P. longifolia* leaf extract treatment in HeLa cervical cancer cells. Over-expression of miR-221-5p was shown to increase cell viability, decrease apoptosis, and decrease caspase-3 activity; thus, suppressing the apoptotic effect of *P. longifolia* leaf extract treatment in HeLa cells. It can be proposed that the polyphenol-rich *P. longifolia* leaf extract is a potential anticancer drug which is shown to target miR-221-5p and eventually induces caspase-dependent apoptosis in HeLa cells.

Although elucidation of molecular mechanism of apoptosis involving miR-221-5p expression in association with *P. longifolia* treatment using molecular analysis such as Taqman qPCR, MTT assay, Annexin V/Pi assay and caspase 3 assay is highly recommended for the better insight of miR-221-5p role, it is also important to further investigate the ultra-structure morphological alterations that occur in miR-221-5p mimic and inhibitor transfected HeLa cells. In addition, downstream bioinformatics and proteomics study of miR-221-5p is equally necessary to further uphold its oncogenic role in regulating apoptosis.
CHAPTER 5: *IN VITRO ULTRA-MORPHOLOGICAL ASSESSMENT OF MICRORNA OVER-EXPRESSED HELa CELLS IN RELATION WITH* *P.* *longifolia* *LEAF EXTRACT TREATMENT

5.1 Introduction

*Polyalthia longifolia* leaf is reported to be a promising anti-cancer agent which has been evidently demonstrated to induce apoptosis in HeLa cells through down-regulation of miR-221-5p and miR-484 (Vijayarathna, 2017d). This has been successfully further validated in Chapter 4 through various dimensions of functional analyses. Although functional analyses of miR-221-5p has been performed to further investigate the molecular mechanism involved in the induction of apoptosis by dysregulation of miR-221-5p expression, morphological characterization in HeLa cells is also highly essential to be justified as morphological observation is the gold standard for anticancer study. Effects of miR-221-5p and miR-484 expressions in *P. longifolia* leaf extract treated HeLa cells was investigated using scanning electron microscopy (SEM), transmission electron microscopy (TEM) and fluorescence microscopy for acridine orange/ propidium iodide (AO/PI) double staining. Each microscopic analysis differs fundamentally in their way.

Significant morphological changes such as cell shrinkage, cell blebbing, formation of apoptotic bodies, chromatin condensation, nuclear fragmentation (karyorrhexis) in cells undergoing apoptosis have been well-elucidated in previous studies (Kerr *et al.*, 1972; Häcker, 2000; Saraste and Pulkki, 2000; Ziegler and Groscurth, 2004; Amini-Sarteshnizi *et al.*, 2014). As advancement in microscopy techniques allowed us to have a better picture and understanding about the precise and detailed ultra-structural changes that take place in *P. longifolia* leaf extract
treated HeLa cells when the expression of miR-221-5p and miR-484 are elevated by transiently transfecting miR-221-5p mimic and miR-484 mimics respectively. Scanning electron microscopy assessed the cell surface morphology while transmission electron microscopy apparently allowed internal ultra-structural morphological changes in HeLa cells. In addition, acridine orange / propidium iodide double staining supported further cell viability analysis together with morphological changes in a mass number of cells together. These morphological changes further justify the biochemical changes revealed by the functional analysis from Chapter 4.

5.1.1 Objective

The objective of this study is as follows:

1) To observe and analyze the morphological changes of over-expressed miR-221-5p and miR-484 HeLa cells independently in relation to *P. longifolia* leaf extract treatment through different microscopic approaches.
5.2 Materials and methods

5.2.1 Cell culture and miRNA transfection

The HeLa cell culture and transient transfection of miRNA mimics into cells are performed following the protocol explained in the previous chapter: Chapter 4, Section 4.2.2, and 4.2.3.

5.2.2 Scanning Electron Microscopy (SEM)

Preliminary study of surface morphology of HeLa cells upon transfection of miRNA mimic and treatment with PLME was accomplished using scanning electron microscopy (SEM). Briefly, HeLa cells were seeded in culture disks followed by the transfection of miR-221-5p mimic and miR-484 mimic independently. After the transfection period, the cells were treated with IC\textsubscript{50} value of methanolic \textit{P. longifolia} leaf extract (26.67 µg/mL). Additional samples of untreated HeLa cells and methanolic \textit{P. longifolia} leaf extract treated HeLa cells were also prepared. The samples were then fixed in McDowell-Trump fixative at 4°C for 24 h. Then, the samples were washed in buffer for three times before postfixing in 1% Osmium tetraoxide for 2 h. The SEM sample preparation was carried out according to the HMDS technique shown in Appendix B. The samples were then viewed under the scanning electron microscopy (Leo Supra 50 VP Field Emission SEM, Germany).

5.2.3 Transmission Electron Microscopy (TEM)

The ultrastructure as well as the internal composition of HeLa cells was also analysed by transmission electron microscopy (TEM). HeLa cells were treated with IC\textsubscript{50} value of methanolic \textit{P. longifolia} leaf extract (26.67 µg/mL) after being
transfected with miR-221-5p mimics and miR-484 mimics independently. Additional untreated and treated samples were also prepared. After 24 hours of treatment period, the cells were harvested and centrifuged into pellet. The cell pellet was resuspended and fixed with McDowell-Trump fixative. The samples were then centrifuged and washed twice before post-fixing in 1% Osmium tetraoxide for 2 hours. After post-fixation, the samples were washed and incubated in a water bath at 45°C for 30 minutes. The samples were then mixed with 2% agar. The solidified agar containing cells were cut into small cubes and processed through dehydration in ethanol and finally embedded in Spurr’s resin mix and later cut into ultra-thin sections (<90 nm) with the aid of PT-PC PowerTome Ultramicrotomes. The sample sections were adsorbed onto carbon coated copper mesh grids and stained with 2% uranyl acetate and lead citrate before viewing under the electron microscope (Philip CM12, Netherlands). For detailed TEM sample preparation, refer to Appendix C.

5.2.4 Acridine Orange / Propidium Iodide (AO/PI) Staining

Cell viability analysis was carried out through dual-fluorescence nuclear staining method using acridine orange (AO) and propidium iodide (PI) dyes. Basically, HeLa cells were cultured and transfected with miR-484 mimics and miR-221-5p mimics independently. The cells were treated with IC₅₀ value of methanolic P. longifolia leaf extract (26.67 µg/mL) after the transfection period. Additional samples of untreated cells and treated cells were also prepared. Each sample was harvested and centrifuged into cell pellet. The cell pellet was washed and resuspended in PBS. The sample was mixed with 1:1 of AO: PI. The slides were then viewed under BX53 fluorescent microscope (Olympus, Tokyo, Japan) using a FITC filter.
5.3 Results

5.3.1 Scanning Electron Microscopy (SEM)

Surface alteration and general morphological changes of *P. longifolia* leaf extract treated HeLa cells and miRNA mimic transfected HeLa cells compared to the control untreated HeLa cells were observed using scanning electron microscopy (SEM). Investigation by SEM showed that the untreated HeLa cells have the typical morphological characteristics of cervical cancer cell (Plate 5.1). The untreated cells were seen to be intact with slightly flattened epithelioid structures as well as the typical morphological characteristics of lamellipodia and filopodia were also shown under the 500 ×magnification (Plate 5.1C). The *P. longifolia* leaf extract treated HeLa cells produced significant modifications of the cell surface compared to that of untreated HeLa cells, in which the cells were observed to appear in circular shape, partly detached and the surface protrusions and blebbing generating apoptotic bodies can be seen clearly (Plate 5.2). The shortening and disappearance of lamellipodia and filopodia as well as the cell shrinkage can be observed in *P. longifolia* leaf extract treated cells. Remarkably, in the presence of elevated expressions of specific miRNAs, namely, miR-221-5p (Plate 5.3) and miR-484 (Plate 5.4), the effect of methanolic *P. longifolia* leaf extract treatment towards the morphological changes of HeLa cells was reduced. Although the cells have been treated with the same IC50 concentration (26.67 µg/mL), the miRNA over-expressed cells tend to retain the typical morphological attribute of untreated HeLa cells as the cell blebbing and detachment of cells are not severe as compared to that of *P. longifolia* leaf extract treated HeLa cells.
Plate 5.1: Scanning Electron Microscopy micrographs of untreated HeLa cells. (A) 100X magnification; (B) 200X magnification; (C) 500X magnification.
Abbreviations: lamellipodia (la), filopodia (f)
Plate 5.2: Scanning Electron Microscopy micrographs of *P. longifolia* leaf extract treated HeLa cells. (A) 100X magnification; (B) 200X magnification; (C) 500X magnification.

Abbreviations: lamellipodia (la), filopodia (f), cell blebbing (cb)
Plate 5.3: Scanning Electron Microscopy micrographs of *P. longifolia* leaf extract treated miR-221-5p mimic transfected HeLa cells. (A) 100X magnification; (B) 200X magnification; (C) 500X magnification.

Abbreviations: lamellipodia (la), filopodia (f)
Plate 5.4: Scanning Electron Microscopy micrographs of *P. longifolia* leaf extract treated miR-484 mimic transfected HeLa cells. (A) 100X magnification; (B) 200X magnification; (C) 500X magnification.

Abbreviations: lamellipodia (la), filopodia (f)
5.3.2 Transmission Electron Microscopy (TEM)

The ultrastructure studies of miRNA mimic transfected HeLa cells and *P. longifolia* leaf extract treated HeLa cells compared to the control untreated HeLa cells at nano level were demonstrated using transmission electron microscopic characterization. TEM analysis of untreated HeLa cells revealed normal voluminous nucleus and cell morphology with all the general organelles such as nucleolus, nucleus, nuclear membrane (Plate 5.5). The presence of cytoplasmic organelles such as mitochondria, endoplasmic reticulum and ribosomes can also be seen in the untreated HeLa cells (Plate 5.5). On the other hand, *P. longifolia* leaf extract treated HeLa cells contrastingly discloses major vacuolization as well as distinct cell blebbing which protrudes to form apoptotic bodies (Plate 5.6). The micrograph also discloses the condensation and fragmentation of nucleus. Simultaneously, nucleus has also broken down. However, miRNA transfected HeLa cells were shown to have reduced apoptotic morphological alteration although they have been treated with the constant IC$_{50}$ concentration (26.67 µg/mL) of *P. longifolia* leaf extract, with only mild apoptotic morphologies such as formation of very few cell blebbing as compared to the miRNA untransfected *P. longifolia* leaf extract treated cells (Plates 5.7 and 5.8).
Figure 5.5: Transmission Electron Microscopy micrograph of untreated HeLa cell

Figure 5.6: Transmission Electron Microscopy micrograph of *P. longifolia* leaf extract treated HeLa cell

Abbreviations: plasma membrane (pm), microvilli (mv), nucleus (n), nucleolus (nl), nuclear membrane (nm), mitochondria (mt), vacuole (v)

Morphological changes induced by *P. longifolia* leaf extract in Figure 6 include formation of cell blebbing (Red arrow), formation of apoptotic body (black arrow), and nuclear fragmentation (asterisk)
Abbreviations: plasma membrane (pm), microvilli (mv), nucleus (n), nucleolus (nl), nuclear membrane (nm), mitochondria (mt)

Morphological changes include formation of cell blebbing (Red arrow), formation of apoptotic body (black arrow)
5.3.3 Acridine Orange / Propidium Iodide (AO/PI) Staining

Acridine orange (AO) and propidium iodide (PI) fluorescent dyes were used to analyse the cell viability which shows specificity for living, apoptotic and late apoptotic/ necrotic states. Plate 5.9 demonstrates the morphologies seen with AO/PI staining in untreated HeLa cells (Plate 5.9 A); *P. longifolia* leaf extract treated HeLa cells (Plate 5.9 B) and *P. longifolia* leaf extract treated miR-221-5p mimic transfected HeLa cells (Plate 5.9 C) and *P. longifolia* leaf extract treated miR-484 mimic transfected HeLa cells (Plate 5.9 D). Untreated cells show a diffuse green fluorescence while, in *P. longifolia* leaf extract -treated cells confirms the occurrence of apoptosis since clumps of intense green and yellow fluorescent spots can be observed within the cell due to condensed chromatin material (Plate 5.9 B2). The characteristic condensation patterns observed were the crescent shape at the nuclear periphery and the more numerous round clumps. Few late apoptotic cells can also be observed which have been stained red by PI with no evident green fluorescence. However, specific miRNA mimic transfected HeLa cells show lower percentage of apoptotic cells as compared to the untransfected *P. longifolia* leaf extract treated cells (Plate 5.9 C and Plate 5.9 D).
Plate 5.9: Photomicrograph of AO and PI stained HeLa cells. (A1) untreated at 20X magnification; (A2) untreated at 40X magnification; (B1) PLME treated at 20X magnification; (B2) PLME treated at 40X magnification; (C1) PLME treated miR-221-5p mimic transfected at 20X magnification; (C2) PLME treated miR-221-5p mimic transfected at 40X magnification; (D1) PLME treated miR-484 mimic transfected at 20X magnification; (D2) PLME treated miR-484 mimic transfected at 20X magnification. Prominent dead cells (red arrows), apoptotic cells (yellow arrows) and early stage of apoptosis, cell blebbing (white arrows) are pointed out.
5.4 Discussion

5.4.1 Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) is one of the golden standards to capture the detailed ultra-structural changes observed from the cell surface of HeLa cells in three-dimensional (3D) and topographical illustration (Starborg and Kadler, 2015). Utilization of this microscopic technique contributed to investigate the role of miR-221-5p over-expression on the apoptotic effect of P. longifolia leaf extract treatment in terms of cell surface morphological alterations in HeLa cells. SEM generates image by focusing beam of electrons with atoms at various depths in reach the samples, producing signals called secondary electrons (Bozzola and Russell, 1992; Heath, 2005; Stadtländer, 2007). SEM allows us to magnify samples from 10 times to up to 100 000 times, yielding high resolution micrographs with detailed surface structure of the sample (Flegler et al., 1993). Although SEM is considered to be costly and comes along with a small risk of radiation exposure, the results obtained are highly valuable to meticulously demonstrate the morphological changes of samples in the research field including life science, biology, gemology, medical and forensic science, metallurgy (Haguenau et al., 2003).

However, sample preparation for SEM viewing is a bit laborious and requires special attention. The fundamental processes implicated in SEM sample preparation include surface cleaning, preserving the sample with a suitable fixative, rinsing, dehydrating, and drying. The sample is mounted on a metal holder, and coated with an ultra-thin electrically conductive material such as gold, gold/palladium alloy, platinum, iridium, tungsten, chromium, osmium and graphite (Bozzola and Russell, 1992; Suzuki, 2002). In our present study, McDowell-Trump fixative was
specifically used to stabilize the samples as it is highly recommended for the SEM (McDowell and Trump, 1976; Dykstra and Reuss, 2003). Rinsing step after fixing step is highly necessary to wash away the excess fixative followed by dehydration step using graded series of ethanol to gradually remove water by exchanging through liquids with lower surface tensions (Robinson et al., 1985). Besides, gold coating was employed as emphasised to provide optimum resolution for surface imaging (Fourie, 1982).

Micrographs obtained through this SEM analysis extensively elucidated the morphological surface details of HeLa cells that correspond to the hallmarks of apoptotic characterization. *P. longifolia* treated cells demonstrated apoptotic morphological details such as cell shrinkage, membrane blebbing, narrowing of lamellipodia, filopodia disappearance, microvilli disappearance, cytoplasmic extrusions, and separated apoptotic bodies, which are clearly not observed in the untreated HeLa cells. This result is consistent with our previous study conducted by Vijayarathna et al. (2017b), further substantiating the anti-cancer effect of *P. longifolia* to induce apoptosis in HeLa cells. However, our current study revealed the role of miR-221-5p over-expression to suppress the apoptotic effect induced by *P. longifolia* leaf extract treatment in HeLa cells, illustrating almost similar morphology observed in untreated HeLa cells. Although the concentration of *P. longifolia* leaf extract (26.67 µg/mL) used to treat miRNA untransfected and miRNA mimics transfected HeLa cells was kept constant, severe apoptotic morphological changes was only observed in the treated, miRNA untransfected cells.

Numerous studies reported similar morphological changes corresponding to the biochemical and cytological changes in cells (Bonanno et al., 2000, 2002) to support the occurrence of apoptosis as observed in the *P. longifolia* treated cells. For
instance, cell shrinkage which is known as the apoptotic volume decrease (AVD) has been well explained to be due the failure of regulatory volume increase response (RVI) to maintain the ionic balance required for life through activity of the Na+/K+-ATPase (Bortner and Cidlowski, 1998; Lang et al., 1998; Bortner and Cidlowski, 2002). This leads to hyperosmotic condition of cells causing cells to shrink and eventually activates the caspase cascade through externalization of phosphatidylserine, mitochondrial depolarization, and release of cytochrome c (Bortner et al., 1997; Hughes et al., 1997; Dallaporta et al. 1998; Thompson et al., 2001).

Other than that, another signature morphological change is the cell blebbings. These cell blebbings are found to be the result of caspase-3 activation as well as the asymmetry flipping of phosphotidylserines (Janicke et al., 1998; Zheng et al., 1998; Coleman et al., 2001; Sebbagh et al., 2001). The observation of cell blebbing in apoptotic cells is indeed the formation of actin-dependent cytoplasmatic protrusions which later disassemble from the cell to form apoptotic bodies (Mills et al., 1998; van Engeland et al., 1998; Robertson et al., 2000; Lane et al., 2005; Wickman et al., 2013). Furthermore, the breakdown of mirovilli, lamellipodia and filopodia structures in apoptotic cells are reported to be associated with the actin machinery involving the actin bundling proteins (Cohan et al., 2001; Khurana and George, 2011). The filopodia structure is reported to be abundant in metastatic cancer cells and plays important role for its invasiveness by contributing in cancer cell migratory mechanisms (Friedl and Wolf, 2003; Machesky, 2008; Dasgupta et al., 2011). This supports our current results observed in SEM analysis showing the cease of these structures in cells undergoing apoptosis induced by *P. longifolia* treatment.
These apoptotic attributes are minimized in miR-221-5p and miR-484 mimics transfected HeLa cells even after the *P. longifolia* treatment. Consistent function of miR-221 has been observed in previous study in which transfection of miR-221 inhibitor was shown to inhibit the cell migration and invasion in Extra-Hepatic Cholangiocarcinoma through morphological changes (Li *et al.*, 2015). Besides, recent study on anti-miR-221 transfected MDA-MB-231 cells also evidently illustrated fibroblast-like morphology alteration, indicating significant decrease in cell migration and invasiveness compared to control cells which maintained a cobble stone-like structure (Liang *et al.*, 2018). These previous morphological study relating miR-221 expression supports our current findings that apoptosis is induced by *P. longifolia* treatment through down-regulation of miR-221. In addition, biochemical changes involving over-expression and silencing of miR-221 which has been demonstrated in Chapter 4 through MTT, Annexin V/ pi and caspase-3 analyses further evidently explains the morphological changes observed in our current SEM analysis.

### 5.4.2 Transmission Electron Microscopy (TEM)

Transmission electron microscopy (TEM) was incorporated to further analyse the intracellular morphological changes in HeLa cells caused by the up-regulation of miR-221-5p expression in relation to *P. longifolia* leaf extract treatment. Unlike surface analysis through SEM, TEM has advantage over SEM as TEM allows us to investigate the detailed morphological, compositional and crystallographic about internal structure of the cells, with apparently higher magnification and resolution (Pretorius *et al.*, 2006; Stadtländer, 2007). However, sample preparation for TEM is highly tedious, laborious, and time-consuming as compared to that of SEM as TEM
has few additional steps such as postfixation, the embedding of cell samples in resin, the sectioning of samples, and the staining of semithin and ultrathin sections (Stadtländer, 2007). TEM uses high voltage electron beam which are emitted by an electron gun and focused by electrostatic and electromagnetic lenses to transmit through the samples. The electrons exiting from the sample creates high resolution two-dimensional (2D) micrographic image with magnification ranging between 500 to 500,000 times (Flegler et al., 1993).

Apoptotic morphological alterations inside the cell structure can be demonstrated as it allows the observation of sectioned specimens, providing a highly qualitative intracellular ultrastructure image of HeLa cells (Unwin and Zampfgi, 1980; Unwin and Ennis, 1984; van Tendeloo et al., 2012). This eventually permits us to investigate the effect of miR-221-5p over-expression in P. longifolia treated cells on internal cell structure in comparison with untransfected P. longifolia treated cells and untreated control HeLa cells. The results obtained by TEM analysis revealed consistent findings from SEM analysis with further detailed internal cell structure, demonstrating the apoptotic effect of P. longifolia treatment in HeLa cells while the miR-221-5p over-expressed cells tend to have decreased apoptotic effect induced by P. longifolia treatment.

Execution of apoptosis was detected in P. longifolia leaf extract treated HeLa cells by observing ultra-morphological alterations via TEM, where quintessential apoptosis morphology such as cell shrinkage, cell blebbing, formation of apoptotic bodies, condensed mitochondrial cristae, impaired nuclear envelope, nuclear condensation and fragmentation. These apoptotic characteristics are not found in untreated cells. On the other hand, over-expression of miR-221-5p with the transfection of miR-221-5p mimics remarkably inhibited the apoptotic effect of P.
*longifolia* treatment in HeLa cells, promoting resistance to the chemotherapy. Similar morphological aspects have been reported earlier to study the effects of apoptosis through TEM (Kerr *et al.*, 1972; Ziegler and Groscurth, 2004; Burattini and Falcieri, 2013). The apoptotic effects of *P. longifolia* treatment and the anti-apoptotic effects of miR-221-5p over-expression through morphological changes further validates the results obtained from functional analysis, explained in previous chapter. This is because the mechanism and biochemical changes involved in the execution of apoptosis are highly responsible for the morphological changes observed in the cells (Allen *et al.*, 1997; Aschoff and Jirikowski, 1997; Chang and Wong, 1997; Cruchten and Broeck, 2002).

The basic apoptotic morphological aspect observed via both SEM and TEM is the cell blebbing, cell shrinkage observed in the *P. longifolia* treated cells. However, TEM image of treated HeLa cells showed that chunks of nuclei fragments to be found within the cell blebs, generating an apoptotic body to be cleaved from the cell. This particular morphology was well explained in previous studies reporting the contribution of actomysin contractility for the displacement of histones from nuclei into blebs prior to be discharged into the extracellular environment as packaged in apoptotic bodies (Coleman and Olson, 2002; Croft *et al.*, 2005; Wickman *et al.*, 2013). Another study also demonstrated the involvement of Rho effector ROCK I, a serine/threonine kinase for the shortening of actin cytoskeleton structure in the caspase-regulated cleavage of apoptotic body through membrane blebbing (Coleman *et al.*, 2001; Sebbagh *et al.*, 2001). These prominent morphological traits are not observed in untreated HeLa cells. However, miR-221-5p and miR-484 over-expressed HeLa cells showed decreased cell blebbing, indicating the effect of miR-221-5p in inhibiting apoptosis induced by *P. longifolia* treatment.
Besides, nuclear condensation and fragmentation are prominently observed in the *P. longifolia* treated HeLa cells undergoing apoptosis which is hardly observed in miR-221-5p and miR-484 over-expressed treated cells. These morphological changes in apoptotic cells are previously studied and reported to be due the impairment in mitochondrial membrane potential, resulting in the release of a pro-apoptotic proteins called apoptosis inducing factor (AIF) and Caspase-Activated DNAse (CAD) which are responsible for the nuclease activity of DNA (Susin et al., 2000; Joza et al., 2001; Modjtahedi et al., 2006). In addition, another study shows the participation of caspase-3 in activating CAD during apoptosis which subsequently contributes in the degradation of chromosomal DNA leading to chromatin condensation and fragmentation (Sakahira et al., 1998; Enari et al., 1998). Over-expression of miR-221-5p and miR-484 in treated HeLa cells did not show nuclear condensation or fragmentation, suggesting its resistance activity against *P. longifolia* treatment in inducing apoptosis. Therefore, the morphological study via TEM further corresponds to our functional analysis elucidated in Chapter 4, supporting that the down-regulation of miR-221-5p is a potential approach for anti-cancer activity to induce apoptosis through *P. longifolia* treatment.

### 5.4.3 Acridine Orange / Propidium Iodide (AO/PI) Staining

Acridine orange and propidium iodide (AO/PI) is one of the commonly used methods to assess the cell viability based on the cell morphology and fluorescence. Unlike other quantitative cell viability assessments which are time-consuming, laborious and expensive high operator-dependent, AO/PI is considered to be simple, rapid, and cost-effective analysis (Bank et al., 1988). Acridine orange and propidium iodide aids as fluorescent marker to visualize and differentiate both viable and dead
cells within the entire sample, without passing through the histologic or radioimmunoassay (RIA) technique. Acridine orange and propidium iodide analysis is considered to produce more reliable results as compared to that of the traditional trypan blue assay in analysing cell viability (Jones and Senft, 1985; Mascotti et al., 2002). The AO/PI analysis is a commonly used cell viability analysis (Wallen et al. 1980; Darzynkiewicz et al. 1992; Mascotti et al. 2000; Foglieni et al. 2001; Chan et al. 2012, 2013).

Acridine orange is a membrane-permeable intercalating dye that can enter and stain both living and dead cells. Acridine orange is usually used to stain DNA and RNA in live cells by intercalation or electrostatic attraction while PI is impermeable to cell membrane fluorescent dye which only stains the dead cells as it can only enter the membrane disrupted cells (Chan et al., 2015). The AO dye, functioning as an inclusion dye readily enters cell membrane and fluoresce green while the PI dye functions as an exclusion dye that only penetrates to damaged cell membrane and binds to nucleic acid and fluoresce red (Krebs and Gierlach, 1951; West, 1969; Krishan, 1975). Apoptotic cells fluoresce orange due to their condensed chromatin in the nucleus that distinguishes them from necrotic dead cells. These stained cells can be easily differentiated with the incorporation of dark field fluorescence microscopy. Although both AO and PI dyes are capable to enter dead cells, PI is more competent in binding to nucleic acid due to its higher molar concentration with better specificity and affinity than that of AO (Bank, 1988). Furthermore, it can also be explained in terms of fluorescence resonance energy transfer (FRET) phenomenon, where the fluorescence emission of AO is reported to be absorbed by PI molecules when both are present together.
The results obtained in the current study revealed that the HeLa cells of untreated sample mostly fluoresce green, indicating that almost all cells are viable with nucleus inside an intact cell membrane. However, *P. longifolia* treated HeLa cells were shown to exert apoptotic traits as yellowish-orange fluorescence started to show up. These treated cells were also shown to exhibit similar traits like cell blebbing and formation of apoptotic bodies as demonstrated via SEM and TEM which further confirms the induction of apoptosis by *P. longifolia* in HeLa cells. Contrastingly, over-expression of the miR-221-5p and miR-484 in treated HeLa cells independently through miR-221-5p mimics and miR-484 mimics respectively displayed lesser number of apoptotic cells, based on the AO/PI fluorescence emission. Although miRNA over-expressed cells were treated with the same concentration of *P. longifolia* leaf extract, the intensity of cell blebbing and apoptotic bodies were shown to be significantly low as compared to that of in the miRNA untransfected, *P. longifolia* treated cells. The results clearly validates that the expression of miRNAs do play an important role in regulating cellular process such as cell proliferation and apoptosis. In this case, up-regulation of miR-221-5p and miR-484 were shown to inhibit the apoptotic effect of *P. longifolia*. The current findings support the previous miRNA deep sequencing results obtained by Vijayarathna reporting the induction of apoptosis in HeLa cells by *P. longifolia* through down-regulation of miR-221-5p and miR-484. Therefore, targeting these miRNAs may be a promising anti-cancer approach to induce apoptosis in HeLa cells.
5.5 Conclusion

In conclusion, it can be deduced that *P. longifolia* leaf extract treatment exhibit anti-cancer property in HeLa cells by inducing apoptosis through the down-regulation of miR-221-5p and miR-484. All three different types of microscopic observations, namely SEM, TEM and AO/PI analysis collectively demonstrated the typical apoptotic morphological changes such as cell shrinkage, cell blebbing, chromatin condensation and fragmentation in *P. longifolia* treated cells, while these morphological alterations were not significant in the miR-221-5p and miR-484 over-expressed treated cells as compared to the untreated cells. Further validating the functional analysis elucidated in Chapter 4, this chapter also suggests the *P. longifolia* leaf extract can be a promising miRNA based anti-cancer chemotherapy for cervical cancer by inhibiting oncogenic miRNAs such as the miR-221-5p and miR-484.
CHAPTER 6: BIOINFORMATICS ANALYSIS OF MIR-221-5P BY PREDICTION OF GENES AND PROTEIN-PROTEIN INTERACTION NETWORKING FOR MIR-221-5P

6.1 Introduction

Hsa-miR-221 (Accession number: MI0000298) is a subset of non-coding RNA molecules and specifically belongs to the family of miRNAs. miR-221 coexist with miR-222 and are located in a cluster on chromosome Xp11.3 (Zhang et al., 2010). Recently, depth studies conducted by Vijayaratna et al. (2016) has evidently demonstrated the induction of apoptosis in HeLa cells treated with Standardized Polyalthia longifolia methanolic leaf extracts via miRNA regulation in which miR-221-5p expression was found to be highly down-regulated upon P. longifolia leaf extract treatment, leading to apoptosis. Although the expression of hsa-miR-221-5p has been validated and functionally analysed through various experiments, explained in Chapter 4, followed by ultra-morphological study of cells over-expressing miR-221-5p in relation to P. longifolia treatment, explained in Chapter 5, bioinformatics analysis of miR-221-5p is highly necessary to further elucidate the gene targets of miR-221-5p and its subsequent proteins involved in the mechanism behind the induction of apoptosis via regulation of miR-221-5p expression.

Advancement in computational analysis with the application of information accrued in public databases including National Center for Biotechnology Information (NCBI), PubMed and so forth as well as various software developments allowed us to systematically predict and meta-analyse the target mRNAs and proteins of the miR-221-5p based on their sequences and structure (Morya, 2012, Sheehan, 2012; Passetti et al., 2014; Rasheed, 2017). In oncology and
pharmaceutical field, bioinformatics analysis has emerged as a potential method to interpret and better understand the biomedical information by utilizing the \textit{in silico} approach such as mathematical and statistical reasoning (Akhtar \textit{et al.}, 2016; Diniz and Canduri, 2017). In the recent decade, miRNAs are evidently documented to participate in various cellular and pathological mechanisms by partially pairing to mRNA targets which subsequently leads to post-translational, protein expression alterations (Bartel, 2004; MacFarlane and Murphy, 2010). Hence, the role of miR-221-5p in various cellular processes, especially in cancer cells has drawn vast attention to identify the target genes for miR-221-5p which may lead to the exposure of cellular mechanisms and therapeutic approaches regulated by miR-221-5p. This research is aimed to analyze the predicted target genes and biological pathways for miR-221-5p as well as to investigate the protein-protein interaction networking using authoritative bioinformatics tools.

6.1.1 Objectives

The specific objectives of this study are as follows:

1) To identify the mRNA targets of miR-221-5p based on various computational algorithm approaches and experimental validated database searches.

2) To analyse role of miR-221-5p in biological pathways through meta-analysis.

3) To investigate the miR-221-5p regulated protein-protein interaction networking (PPIN)
6.2 Materials and methods

6.2.1 Identification of mature sequences of miR-221-5p

Mature sequences of miR-221-5p in different species were analyzed from miRBase Database (http://mirbase.org/index.shtml). The location and the conserved structure of hsa-miR-221-5p were retrieved from gene database (Gene ID: 407006) of NCBI and Rfam 13.0 database (http://rfam.xfam.org/) respectively.

6.2.2 miRNA-gene interaction analysis of miR-221-5p

In order to identify the target genes of miR-221-5p, miRGate (http://mirgate.bioinfo.cnio.es) database accommodating contemporary computational predicted miRNA–mRNA pairs with the utilization of highly integrated algorithms was used. On the miRGate open resource homepage, query parameters has been set accordingly by choosing the organism as *Homo sapiens*, followed by the type of list and type of ID as only miRNAs and MirBase respectively and finally entered the miRNA ID as miR-221-5p. Thenceforth in the advanced options, ENCODE principle isoforms was checked, with the grouping threshold of 2. As for the gene biotypes, protein coding, processed transcript and processed pseudogene were checked. Finally, all the computational prediction methods, namely Rnahybrid, Miranda, Pita, Targetscan, and Microtar as well as all the validated prediction methods, namely Mirtarbase, Mirecords, Tarbase and OncomiRDB were checked before proceeding to the query run. Co-predicted target genes by at least two of the 5 different algorithms combined with the experimentally validated genes were selected for further target analysis.
6.2.3 Gene enrichment analysis

The Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8 (https://david.ncifcrf.gov/) was incorporated in order to further understand the biological significance of the large list of genes analyzed by the MiRGate database (Huang et al., 2009). Under the start analysis panel, the list of co-predicted target genes by at least two of the 5 different algorithms combined with the experimentally validated genes as well as predicted apoptotic genes were pasted followed by selecting the identifier as “OFFICIAL_GENE_SYMBOL” and finally list type was selected as gene list before submitting the list. Under the gene list manager, species was selected as *Homo sapiens*. Once the DAVID IDs were detected, default selections were checked for the annotation categories in Disease (OMIM), Gene ontology (Biological Process, Molecular function, cellular component), Pathways (BBID, BIOCARTA, KEGG) and Protein domains (INTERPRO, PIR_Superfamily, SMART) databases before analysing the functional annotation clustering of the gene targets of hsa-miR-221-5p.

As for the functional annotation clustering, the classification stringency was set high with kappa similarity and multiple linkage thresholds were 0.5 respectively while the enrichment threshold was 1.0. EASE scores from each term member contribute to the overall enrichment score in which the enrichment scores more than 0.5 was believed to play a more enriched role.

6.2.4 Protein-Protein Interaction Network (PPIN) Analysis

The same set of predicted gene targets of hsa-miR-221-5p used in up-stream analysis was entered protein analysis, where the copredicted protein coding genes by at least
two computational methods combined with all the experimentally validated protein coding genes plus the apoptotic genes predicted by at least one computational method, accounting to 849 unique protein coding genes were utilized. The Investigation of protein-protein interaction network (PPIN) was accomplished with the incorporation of web-based PPI prediction tool called the STRING resource (Version: 11) which is available online, at http://string-db.org/. Firstly, the option “multiple proteins” was selected from the side option bar and subsequently the list of official gene symbols were pasted in the “list of names” box. After that, organism was selected as Homo sapiens before submitting query. As a result, STRING database showed all the associated proteins corresponding to the interaction sources. PPIN can be further accustomed by setting options. Basic setting was fixed so as to illustrate the type of interaction by the line colour of the network edges, while the all the interaction sources were checked namely text mining, experiments, databases, co-expression, neighbourhood, gene fusion and co-occurrences. Furthermore, interaction score was set to be in high confidence (0.90).
6.3 Results

6.3.1 Identification of mature sequences of miR-221-5p

Mature sequences of miR-221-5p in various species have been retrieved from miRBase Database (Table 6.1). The location of miR-221-5p in *Homo sapiens* was found to be located at chromosome X (NC_000023.11); positioning from 45,746,157 - 45,746,266 (Figure 6.1). The conserved secondary structure of miR-221 (RF00651) is shown in Figure 6.2 which was extracted from Rfam 13.0 database available online, at http://rfam.xfam.org/ (Kalvari *et al.*, 2017).
<table>
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<th>Description</th>
<th>MiRNA ID</th>
<th>Sequence</th>
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**Figure 6.1**: Location of miR-221 in Chromosome X
Figure 6.2: Conserved secondary structure of miR-221

(Source: Kalvari et al., 2017)
6.3.2 miRNA-gene interaction analysis of miR-221-5p

The miRGate tool predicted a total of 4910 protein coding gene targets for miR-221-5p (Supplementary 1). Among the total predicted genes, only three genes were co-predicted by 4 different computational algorithms while 51 genes and 625 genes were supported by at least 3 and 2 computational methods respectively. There were 5 genes which have been experimentally validated among the total predicted protein coding genes, namely GATAD2B, BMF, ICAM1, CDKN1C and PIK3R1, among which BMF has been computationally predicted to have two different target sites. Besides, 326 apoptotic genes were predicted by at least one computational method. The Venn diagram (Figure 6.3) summarizes the number of gene targets predicted by several computational algorithms attained through miRGate bioinformatics tool. The Venn diagram (Figure 6.4) summarizes the predicted genes which were selected for further analysis. Among the 577 genes predicted by atleast 2 computational algorithms, 55 genes are apoptotic genes, while among the 326 apoptotic genes predicted by at least 1 computational method, 4 genes were found to be experimentally validated genes. The empty overlapping area between the set of genes predicted by atleast 2 computational algorithms and the set of experimentally validated genes, suggests that although the target genes of miR-221-5p are predicted by only a single computational method, they were found to experimentally validated and interesting 4 of them are found to be in the set of apoptotic genes. Table 6.2 shows the representative target genes predicted by miRGate bioinformatics tool.
Figure 6.3: Protein coding genes predicted by different computational algorithms
Figure 6.4: Summary of target genes for further analysis
Table 6.2: Representative target genes predicted by miRGate bioinformatics tool

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<th>Computational Predictions</th>
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6.3.3 Gene enrichment analysis

Gene enrichment analysis was performed for the co-predicted target genes for miR-221-5p by at least two databases, combined with the experimentally validated gene targets. Additionally, apoptotic genes predicted by at least one database were included for the gene ontology analysis. Resultantly, a total number of 849 predicted target genes of miR-221-5p were meta-analysed in DAVID. According to the annotation analysis carried out with the adoption of DAVID (on 5th August 2018), 800 unique DAVID IDs has been detected. Among which, 90.6% (725 genes) are involved in biological process, 94.8% (758 genes) are involved in cellular component and 91.1% (729 genes) are involved in molecular function. Gene ontology terms with FDR threshold less than 0.05 for biological process, molecular function and cellular components are represented in Tables 6.3, 6.4 and 6.5 respectively. Gene ontology was further confirmed by analysing the list of genes in Enrichr (Figure 6.5).
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### Table 6.4: GO terms for molecular function adapted from DAVID bioinformatics

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Table 6.5: GO terms for cellular component adapted from DAVID bioinformatics

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Figure 6.5: Gene ontology bar chart adapted from Enrichr bioinformatics represented based on combinatory scores. (A) Gene ontology for biological process, (B) Gene ontology for molecular function and (C) Gene ontology for cellular component
6.3.4 Protein-Protein Interaction Network (PPIN) analysis

Protein-protein interaction network statistical analysis by STRING bioinformatics tool revealed an overall 822 nodes with 1276 edges as shown in Figure 6.6. The line colour of the network edges represents the type of interaction evidence. Hence, more lines for a specific network shows higher confidence indicating a strong data support from the active interaction sources. The PPI enrichment p-value accounts 1.11e-16.
**Figure 6.6:** Protein-protein interaction networking generated by STRING bioinformatics tool
6.4 Discussion

6.4.1 miRNA-gene interaction analysis

The discovery of miRNAs and their important roles in the expression of target genes by partial complementary binding (Bartel, 2004; Filipowicz et al., 2005, Sontheimer and Carthew, 2005) has led to an increasing focus on predicting and validating miRNA gene targets. MiRNA being an imperative regulator of the gene expression, it inevitably impacts almost all the fundamental cellular processes including cell proliferation, cell differentiation, and apoptosis (Ameres and Zamore, 2013). According to our previous investigations of the oncogenic role of over-expressed miR-221-5p and its involvement in cellular processes including apoptosis, bioinformatics analysis is believed to provide insight of proteomics and PPIN involving miR-221-5p in greater extent. Briefly, P. longifolia induced apoptosis by the down-regulation of miR-221-5p was reported Therefore, it is highly necessary to accurately analyze and validate the target genes of miR-221-5p which consequently led to the utilization of several experimental as well as computational approaches based on the basic principles and assumptions.

However, miRNA target prediction remains challenging as the size of the miRNA is very short to accommodate adequate information for specificity which obscure them to identify their complementary sequences (Ekimler and Sahin, 2014). Furthermore, another major challenge in recognising the target genes of specific miRNA is the characteristic of the miRNA to regulate gene expression even through partial complementary pairing which may accommodate expansive range of genes by a single miRNA (Bartel, 2009). This characteristic makes the target gene predicting process more complicating because prediction may also include false targets of the
miRNA (Pasquinelli, 2012). Identifying gene targets of miRNA through experimental analysis may be less reliable as some miRNA targets that are predominantly repressed at the protein level is probably to be omitted out (Barbato et al., 2009).

In accordance to these obstacles, numerous computational approaches have been developed over the years with the incorporation of distinctive algorithms to predict the miRNA target sites in silico (Thomson et al., 2011). The divergent algorithms are often developed based on empirical evidences with statistical scores, p-values and energy or conservation values for reliability check. There are several bioinformatics tools that predict the miRNA target genes which includes miRonTop (Le Brigand et al., 2010), mirGator (Cho et al., 2013), mirWalk (Dweep et al., 2011), miRanda (Betel et al., 2010), TargetScan (Friedman et al., 2009), Pita (Kertesz et al., 2007), RNAHybrid (Kruger and Rehmsmeier, 2006), microTar (Thadani and Tammi, 2009) and so forth each consolidating distinctive benchmarks. As the ability to predict miRNA target genes with high confidence level remains challenging due to paucity of overlap between the different predictions algorithm methods and weak reliability outcome through experimental validation tests.

Therefore, a web-based miRNA-mRNA prediction database enclosing both computational and experimental validated miRNA-mRNA pairs from different sources with various well established algorithms was used in this study. The in-house prediction algorithms include miRanda, Pita, RNAHybrid, microTar and Targetscan. MiRanda incorporates an effective computational algorithm which score alignments based on the complementarities of nucleotides, allowing G-U wobble pairs by using a threshold of thermodynamic stability energy more than 120 kcal (Betel et al., 2010). Pita which stands for probability of interaction by target
accessibility computes the free energy of the unbound and bound double strand in primary complete reciprocal seeds for each miRNA in the mRNA (Kertesz et al., 2007). In order to provide more room for certainty in prediction accuracy, Pita also filters out less conserved predicted target sites with the application of phylogenetic hidden Markov mode known as Phastcons (Siepel et al., 2005). Actively most applicable hybridization sites are predicted by the RNAHybrid prediction analysis which ignores the intramolecular hybridization as well as evaluating the Poisson approximation of multiple binding sites and calculation of effective numbers of orthologous targets in comparative studies of multiple organisms (Kruger and Rehmsmeier, 2006). Besides, microTar prediction method employs the Vienna package to predict the target genes through mRNA sequence complementarities and RNA duplex energy as well as analyzing the significance of miRNA binding on complete mRNA molecules (Thadani and Tammi, 2006). Finally, TargetScan computation predication tool analyses intact seed pairing utilising the mammals EnsEMBL alignments and scores the prediction based on the seed match, local AU contribution and mRNA binding site localization (Friedman et al., 2009).

MiRGate database also accommodates the experimental validated target genes via external data description compiling four disparate public databases including TarBase (Sethupathy et al., 2006), mirTarBase (Chou et al., 2018), miRecords (Xiao et al., 2009) and OncomirDB (Wang et al., 2014). TarBase anchors manually curated experimentally validated miRNA target genes supplemented with function-related data along with general information extracted from external databases such as UniProt, Ensembl and RefSeq (Vergoulis et al., 2012). Research articles associated to functional analysis of miRNA were refined by data mining process of the text methodically in order to manually review the relevant literature so
as to compile the miRNA-mRNA pairing in MirTarbase (Hsu et al., 2014). Likely, miRecords also curates the target genes of miRNA from selected publications via a systematic documentation strategy (Xiao et al., 2009) while the OncomirDB mainly annotates the experimentally validated oncogenic and tumor-suppressivemiRNA from published abstracts with direct evidences (Wang et al., 2014).

Contemporarily, miRGate is the only feasible tool that integrates five well-established computational predictions as well as four experimentally validated databases which consequently reduces the false positive results (Andres-Leon et al., 2015). Since the downstream analysis is exclusively on proteomics related cancer, the gene biotypes included protein coding and processed transcripts as well as processed pseudogene considering the essential function of pseudogenes in cancer biology (Xiao-Jie et al., 2015). Although the Homo sapiens species specific curated database analysed 4919 gene targets altogether for miR-221-5p, gene targets predicted by at least two computational prediction methods combined with the entire experimentally validated targets as well as the predicted apoptotic genes were picked for the downstream analysis which accounts 849 gene targets. Genes predicted by more than one algorithm is believed to increase the reliability of the target genes while reducing the false positive results. The finalized number of genes predicted by at least two computational methods combined with the entire number of experimentally validated genes accounts accurately 631 genes and 582 genes, before and after the elimination of duplicates from the list respectively. Experimentally validated genes are considered to be more reliable compared to that of bioinformatically analysed because they are experimentally proven with sufficient physical evidences. Hence, it is important to take note that all the experimentally validated genes are all predicted by only one computational approach and among the
5 validated genes, 4 genes were known to be apoptotic genes. Since our major concern in this study is to investigate the role of miR-221-5p in apoptosis, apoptotic genes predicted by at least one computational method is also included in our downstream analysis, which accounts to 267 genes. Therefore, total of 849 genes was used to study the downstream analysis.

Among the 849 miRGate predicted genes, 37 genes were found to be in the cervical cancer gene database (CCDB) (Agarwal et al., 2011). CCDB is an integrated database to document the genes that participate in cervical carcinogenesis found with biomedical evidences based on the molecular and genetic effects such as methylation, gene amplification, mutation, altered expression and polymorphism. Some gene targets of miR-221-5p predicted by miRGate were previously reported to be down-regulated in cervical cancer in comparison with normal cells (Agarwal et al., 2011). These genes includes amyloid beta (A4) precursor-likeprotein 2 (APLP2) (Ahn et al., 2004), Cadherin-15 (CDH15) (Vazquez-Ortiz et al., 2005), Carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) (Albarran-Somoza et al., 2006; Shadeo et al., 2007), Cytoplasmic Polyadenylation Element Binding Protein 1 (CPEB1) (Hansen et al., 2009), Desmoglein 1 (DSG1) (Wong et al., 2006; Manavi et al., 2007), E3 ubiquitin-protein ligase (HUWE1) (Ahn et al., 2004), insulin-like growth factor binding proteins 5 (IGFBP5) (Hudelist et al., 2005; Manavi et al., 2007), Interleukin 18 (IL18) (Cho et al., 2001; Wong et al., 2006), Integrin alpha-V (ITGAV) (Wilting et al., 2008), Integrin, Beta 1 (ITGB1) (Manavi et al., 2007; Hudelist et al., 2005; Ahn et al., 2004); Myelin and lymphocyte protein (MAL) (Hatta et al., 2004; Wong et al., 2006; Wilting et al., 2008), Moesin (MSN) (Morozova et al., 2006), Retinoic Acid Receptor Gamma (RARG) (Xu et al., 1999), and Thrombospondin-2 (THBS2) (Kodama et al., 2001). Thus, results obtained
through miRGate bioinformatics analysis evidently support our findings that targeting miR-221-5p by *P. longifolia* treatment is a promising approach to inhibit cervical cancer. This is because knock down of miR-221-5p eventually over-expresses the expression of the genes predicted by miRGate.

Furthermore, one of the miRGate predicted, experimentally validated mRNA targets of miR-221-5p are Bcl-2-modifying factor (BMF) which is a pro-apoptotic gene (Giam *et al.*, 2008). Very recent study conducted by Xie *et al.* (2018) exceptionally revealed the induction of apoptosis by over-expressing BMF through miR-221 knock-down. Other than that, abundant other studies have reported the apoptotic effect of BMF in various cancer cells through different mechanism. One such recent study demonstrated the BMF induced apoptosis in breast cancer cells in miRNA-dependent manner by mediating STARD13 3’UTR (Guo *et al.*, 2018). Another cytotoxicity study for a platinum blue complex revealed its anti-cancer property through induction of apoptosis in cancer cells by increasing pro-apoptotic gene including BMF (Adiguzel *et al.*, 2017). These experimentally validated genes coincide with the previous study reporting the induction of apoptosis by *P. longifolia* leaf extract treatment through the up-regulation of pro-apoptotic proteins such as BAX, BAD, cytochrome c, caspase-3, p21, p27 and p53 in HeLa cells (Vijayarathna *et al.*, 2017).

Another experimentally validated mRNA target of miR-221-5p is CDKN1C which is also known as p57KIP2. CDKN1C is one of the cyclin-dependent kinase (CDK) inhibitors of the Cip/Kip family which is known to exhibit as a tumor-suppressor gene by negatively regulating cell proliferation (Lee *et al.*, 1995; Yan *et al.*, 1997). Previous study revealed its function to promote drug-induced apoptosis in HeLa cells through mitochondria-mediated and subsequent caspase-dependent
manner (Samuelsson et al., 2002; Vlachos et al., 2007). Previous study conducted by Vijayarathna et al. (2017) evidently demonstrated the molecular mechanism involved in the induction of mitochondria-mediated caspase dependent intrinsic apoptosis in HeLa cells by *P. longifolia* leaf extract treatment. *P. longifolia* leaf extract treatment was also shown to trigger cell cycle arrest at G1 phase of cell cycle in HeLa cells leading to the induction of apoptosis when the DNA damage beyond repair (Vijayarathna et al., 2017a).

In addition, phosphoinositide-3-kinase regulatory subunit 1 (PIK3R1) was also predicted by miRGate bioinformatics analysis with experimental validation evidence. Significant down-regulation of PIK3R1 expression has been evidently reported in various cancer types as compared to that of normal samples. For example, PIK3R1 was reported to be under-expressed in prostate cancer (Tomlins et al. 2007; Grasso et al., 2012; Munkley et al., 2015), breast cancer (Varambally et al., 2005; Jiao et al., 2007), lung cancer (Gao et al., 2014), hepatocellular liver cancer (Massie et al., 2011), and glioblastoma tumor cells (Rajan et al., 2014). Therefore, over-expressing PIK3R gene by targeting miR-221-5p through *P. longifolia* treatment is a potential approach to inhibit cancer.

The data obtained from the miRGate collectively supports the previous findings reported by Vijayarathna et al. (2017) demonstrating the induction of apoptosis by *P. longifolia* leaf extract treatment through the regulation of miRNA expressions. As the predicted gene targets of miR-221-5p are known to be similar from the previous microarray findings, further gene enrichment analysis may provide a better insight of the participation of these genes in the biological processes.
6.4.2 Gene enrichment analysis

In order to associate the gene targets of miR-221-5p with their pertinent functional annotation, gene-annotation enrichment has been analysed with the incorporation of a web-based, high-throughput annotation tool known as the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8 bioinformatics. DAVID comprises fourteen annotation categories including the three main gene ontology (Biological process, Molecular function and Cellular component), KEGG Pathways, BioCarta Pathways, Swiss-Prot Keywords, BBID Pathways, SMART Domains, NIH Genetic Association DB, UniProt Sequence Features, COG/KOG Ontology, NCBI OMIM, InterPro Domains, and PIR SuperFamily Names to measure global annotation profiles of the genes with Cohen’s Kappa statistical analysis which represents the degree of annotation co-occurrence (Huang et al., 2007).

Gene ontology has been accepted as a universal language with potent and reserved terminology to annotate the functional role of genes (Ashburner et al., 2000). The application of the functional analysis of genes may be ambiguous since genes perform integral part in biological process, molecular function and cellular component. As a result, gene ontology has been independently categorised into these three classifications. The specific characteristics of the genes based on the biological knowledge can be assigned through these ontologies independently. Biological process indicates the contribution of the genes to chemical or physical conversion in order to accomplish the biological process such as ‘cell growth’, ‘apoptosis’, ‘cell signalling’ and so forth while molecular function of the genes refers to biochemical activities of the genes stating its potential functional terms such as ‘enzyme’, ‘transporter’ or ‘ligand’ (Ashburner et al., 2000). The third category of Gene Ontology namely cellular component refers to location of the active gene product in
the cell structure which includes terms such as ‘ribosome’, ‘nuclear membrane’ or ‘Golgi apparatus’ (Ashburner et al., 2000).

Based on gene ontology analysis obtained through both DAVID and Enrichr, biological process involving positive regulation apoptosis process and negative regulation of cell proliferation is evident. Other than this, gene ontology analysis for biological process by DAVID also revealed that the involvement of target genes in lipopolysaccharide-mediated signaling pathway (GO: 0031663) with a high enrichment score (6.51) which is also reported to be involved in apoptosis. Previous studies have reported the Lipopolysaccharide Signal transduction can induce endothelial apoptosis TNF Receptor-Associated Factor 6 (TRAF-6)-Mediated Activation of c-Jun NH2-Terminal Kinase (Hull et al., 2002). Lipopolysaccharide Signal are also reported to depolarise mitochondria membrane potential, leading to release of cytochrome c and the subsequent caspase activation to execute apoptosis (Tucsek et al., 2011; Smith et al., 2015; Sumegi et al., 2017). This eventually supports and relates our previous study conducted by Vijayarathna (2017) and our current study on *P. longifolia* inducing apoptosis via miR-221-5p down-regulation in HeLa cells.

Another biological process predicted by DAVID which has to be taken into consideration is the negative regulation of cysteine-type endopeptidase activity involved in apoptotic process (GO: 0043154). Cysteine is a type of protease among the five classes of endopeptidases which have been evidently proven to participate in cancer progression, metastasis and invasion by inducing the expression of the endopeptidase in the neighbouring normal cells to result tumour expansion (Zucker et al., 2000; Yang et al., 2009). As the functional annotation clustering of the gene targets of hsa-miR-221-5p suggests that the down regulation of hsa-miR-221-5p
elevates the expression of Proteinase inhibitor I25, cystatin as well as inversely regulates the cysteine-type endopeptidase activity. Hence, down regulation of hsa-miR-221-5p can eventually inhibit cancer invasion or metastasis through the contradictory activity of cysteine protease. Cathepsins are cysteine proteases which have been indicated to be over-expressed in breast cancers (Kandalaft et al., 1993), cololectral cancers (Hirai et al., 1999), tongue cancers (Saleh et al., 2006), pancreatic cancer (Michl, 2011) and laryngeal carcinoma (Macabeo-Ong et al., 2003). Corresponding to the functional annotation clustering by DAVID, these cathepsin lysosomal proteases can be inhibited by cysteine protease inhibitor which has been predicted by the INTERPRO database. Several studies indicated that cysteine protease inhibitor called the cystatin is not only responsible for the inhibition of cathepsins (Henskens et al., 1996) but has also been experimentally proven to prevent in vitro invasiveness of cancer cells (Troy et al., 2004). Reviews have suggested that specific protease inhibitors may contribute cancer treatment in combinations with the anti cancer drugs (Rakashanda et al., 2012).

Besides, another biological process based gene ontology classification, namely positive regulation of tumor necrosis factor production (GO: 0032760) was predicted by DAVID which has high enrichment score (5.39) with a FDR as low as 0.001. The family of tumor necrosis factor (TNF) such as TNF-receptor 1 (TNF-R1, p55, CD120a), Fas (CD95, APO-1) and TNF-related apoptosis-inducing ligand receptors (TRAIL-R1, DR4; TRAIL-R2, DR5, APO-2) are also involved in apoptosis process (Guicciardi and Gores, 2009). For instance, several evidence points have demonstrated that initiation of apoptosis specifically in cancer cells can occur through recombinant human Apo2L/TRAIL (Ashkenazi et al., 1999; Kelley et al., 2004). Ion channels are highly involved in the regulation of cell proliferation and
apoptosis. The formation of the death inducing signalling complex (DISC) is initiated by the binding of ligands to the death receptors which consequently activates caspase 8 (Walczak and Sprick, 2001). As activation of caspase 8 leads to the activation of other components involved in intrinsic apoptotic pathway, both extrinsic and intrinsic pathways are interrelated (Li et al., 1998; Yin et al., 1999). Overwhelming evidences show the implication of various ion channels in the regulation of apoptosis such as the calcium permeable channels, potassium channels, sodium channels and chloride channels (Kondratskyi et al., 2014). For instance, an elevation of cytoplasmic calcium ions may induce apoptosis through alteration in mitochondrial outer membrane permeabilization (MOMP) leading to depolarization of mitochondrial membrane potential which induces the release of cytochrome c, thereby activating the caspase-dependent apoptosis (Hajnoczky et al., 2006; Kroemer et al., 2007; Giorgi et al., 2012). Convincing studies suggest that ion channel targeting can be a promising anticipation in cancer therapy.

This can also be associated with another notable biological process annotated by DAVID bioinformatics, particularly negative regulation of NF-kappaB transcription factor activity (GO: 0032088) because NF-kappaB transcription activity is known to antagonise the previously discussed TNF-induced programmed cell death and render chemoresistance in cancer (Wajant et al., 2003; Papa et al., 2006). Various other studies have also demonstrated its participation in cell proliferation and carcinogenesis and mainly suppressive action against cancer treatment including chemotherapy and radiation (Karin et al., 2005; Basseres and Baldwin, 2006; Karin, 2006). Hence, negative regulation of NF-kappaB transcription factor activity is a promising approach for cancer treatment (Kim et al., 2006; Mauro et al., 2009).
Another noteworthy gene ontology analysed by DAVID bioinformatics is the positive regulation of JNK cascade (GO: 0046330). Activation of Jun N-terminal kinases (JNK) through phosphorylation of Thr- and Tyr-residues eventually initiates apoptosis by over-expressing the pro-apoptotic genes (Dhanasekaran and Reddy, 1998). JNK executes apoptosis through transactivation of specific transcription factors including c-Jun or by directly regulating the activities of mitochondrial pro- and anti-apoptotic proteins (Dhanasekaran and Reddy, 1998). Other studies have also reported the contribution of JNK in the phosphorylation of p53 protein-induced apoptosis (Fuchs et al., 1998; Oleinik et al., 2007) as well as in the induction of p53-independent apoptosis (Davis, 2000). Besides, JNK is reported to be easily translocated to mitochondria, so as to initiate mitochondria-mediated apoptosis (Kharbanda et al., 2000; Chauhan et al., 2003). This subsequently leads to the release of cytochrome c and caspase activation (Chinnaiyan, 1999; Hill et al., 2004).

In addition, one more biological process categorized gene ontology includes the positive regulation of ERK1 and ERK2 cascade (GO: 0070374). Ras/Raf/extracellular signal-regulated kinase (ERK) isoforms namely, ERK1 and ERK2 have been successfully shown to contribute in the induction of apoptosis by withdrawal of potassium (K⁺) (Subramaniam et al., 2004). ERK also promotes neuronal apoptosis by causing potassium deprivation, leading to plasma membrane and DNA damage (Cheung and Slack, 2004). Mechanisms involved ERK-mediated apoptosis include mitochondrial depolarization (Kim et al., 2003; Nowak et al., 2006; Zhuang et al., 2007) and up-regulation of tumor suppressor gene p53 (DeHaan et al., 2001; Woessmann et al., 2002; Yeh et al., 2004; Liu et al., 2008).

There are numerous evidences to demonstrate ERK-mediated apoptosis in various cells, including cancer cells. For instance, various studies demonstrated the
contribution of ERK in induction of apoptosis in Human cervix adenocarcinoma HeLa by Cisplatin (Wang et al., 2000; Kim et al., 2005), Apigenin (Llorens et al., 2004), and Shikonin (Wu et al., 2005). Pro-apoptotic function of ERK activity is well-documented in cell death induced by several currently available chemotherapy compounds such as etoposide (Stefanelli et al., 2002; Tang et al., 2002; Lee et al., 2005) and doxorubicin (Tang et al., 2002; Fernandez et al., 2004; Yeh et al., 2004; Alexia et al., 2006; Lee et al., 2006).

The biological process obtained from DAVID bioinformatics are apparently inter-related to each other and the interpretation of the biological process evidently elucidates the direct or indirect participation of gene ontology results obtained for molecular function and cellular components. For instance, molecular function obtained from DAVID bioinformatics include protein binding (GO: 0005515), transcription factor binding (GO: 0008134), protein kinase binding (GO: 0019901), protein kinase activity (GO: 0004672), NF-kappaB binding (GO: 0051059), protein serine/threonine kinase activity (GO: 0004674), cysteine-type endopeptidase inhibitor activity involved in apoptotic process (GO: 0043027), death receptor binding (GO: 0005123) to name a few. On the other hand, cellular components obtained from gene ontology results of DAVID bioinformatics are undoubtedly involved in all the biological process to take place such as external side of plasma membrane (GO: 0009897), cell surface (GO: 0009986), membrane (GO: 0016020), cytoplasm (GO: 0005737), nucleus (GO: 0005634), plasma membrane (GO: 0005886), nucleoplasm (GO: 0005654), protein complex (GO: 0043234), mitochondrion (GO: 0005739), and mitochondrial outer membrane (GO: 0005741).

Therefore, the gene ontology analysis evidently holds up our findings explained in previous chapters, indicating the successful of induction of apoptosis by
*P. longifolia* leaf extract treatment in HeLa cervical cells through dysregulation of miRNAs. This is because the gene ontology analysis via bioinformatics for the cluster of gene targets of miR-221-5p are successfully documented in this chapter to contribute in the apoptotic process as the genes are over-expressed by the knock down of miR-221-5p by *P. longifolia* treatment.

### 6.4.3 Protein-Protein Interaction Network (PPIN) analysis

MiRNA regulates gene expression that directly or indirectly tunes the synthesis of proteins. Protein-protein interaction is constitutionally essential to implement a specific function in biological processes (Snider *et al.*, 2015). A comprehensive mapping of PPIN is highly neccessary to understand the complex process as well as to discover novel proteins to aid therapeutic inventions.

Heretofore, there has been a drastic development of intriguing high-throughput experimental techniques for the identification and characterization of protein-protein interaction networks such as the yeast two hybrid method (Hamdi and Colas, 2012; Ferro and Trabalzini, 2013; Stasi *et al.*, 2015), Luminescence-based mammalian interactome mapping (Blasche and Koegl, 2013), Affinity purification–mass spectrometry (AP-MS) (Dunham *et al.*, 2012), membrane yeast two-hybrid (MYTH) assay (Snider *et al.*, 2010), Mammalian protein–protein interaction trap (MAPPIT) (Sahni *et al.*, 2015), Kinase substrate sensor (KISS) (Lievens *et al.*, 2014), Proximity ligation assay (PLA) (Koos *et al.*, 2014) and so forth, each accommodating disparate approaches and principles. In order to catch up the rapidly increasing genomic informations, several computational approaches have been developed to predict the interactions between proteins with the aid of the high-
throughput technologies. Well-known PPI repository include STRING (Szklarczyk et al., 2015), BioGRID (Stark et al., 2006), Database of Interacting Proteins (DIPTM) (Xenarios et al., 2002), The Biomolecular Interaction Network Database (BIND) (Bader and Hogue, 2000), The Molecular Interaction Database (MINT) (Chatr-aryamontri et al., 2007), The Human Protein Reference Database (HPRD) (Peri et al., 2004) and IntAct (Hermjakob et al., 2004). The STRING database (http://string-db.org) was used to analyse the PPIN of the 849 gene targets of the hsa-miR-221-5p in this study.

The PPIN analysis conducted with the aid of the web-based STRING bioinformatics tool revealed consistent revelation obtained by DAVID bioinformatics tool on biological process. Notewrothyly, the top five functional enrichment of the protein network are regulation of apoptotic process (GO: 0042981), regulation of cell death (GO: 0010941), regulation of programmed cell death (GO: 0043067), negative regulation of biological process (GO: 0048519), and negative regulation of cellular process (GO: 0048523). One of the binary interaction with high interaction confidence (0.99) is the interaction between CASP3 and APAF1. The interaction clearly elucidates the mitochondria-dependent apoptotic pathway where the cytochrome c released from mitochondria to cytoplasm, initiates the oligomerization of APAF-1 which subsequently activates the caspase cascade to promote apoptosis as discovered in mammals, C. Elegans and D. Melanogaster (Li et al., 1997; Qin et al., 1999; Qi et al., 2010; Favaloro et al., 2012; Cheng et al., 2016). In another recent study, the interaction between APAF1 and CASP3/9 through caspase recruitment domain (CARD) independent to cytochrome c release by mitochondria has been reported during apoptosis in starfish (Tamura et al., 2018).
According to the STRING PPIN analysis, the highest combined score (0.99) was recorded by the interactions between various ribosomal proteins (Supplementary 2). Interestingly, overwhelming number of studies have reported the important role of ribosomal proteins in various cellular process. Numerous independent ribosomal proteins have been experimentally proven to be stimulating the tumor suppressor p53 pathway, thus attenuating tumor cell growth and cell proliferation (Zhou et al., 2015). For instance, one of the binary interaction with highest interaction confidence score (0.99) shown by STRING is the interaction between RP11 and RP23. Remarkably, a very recent study conducted by Hu et al. (2017) experimentally proved the association of robosomal protein L11 (RPL11) and ribosomal protein S23 (RPS23) to be involved in the molecular mechanism of cytokine-induced killer (CIK) therapy in breast cancer patients. RPS23 was reported to increase the activity of RPL11 by promoting ribosomal pressure which in turn upregulates the expression of p53 tumour suppressor gene (Vijayakumaran et al., 2015). Besides, RPS3 was experimentally proven to apoptosis via caspase 8/ caspase 3 activation (Jang et al., 2004). Several other ribosomal proteins have been previously reported to be inhibit tumor cell proliferation by either stimulating tumor suppressors or by suspending the oncoproteins (de Las Heras-Rubio et al., 2014). Furthermore, neighbouring ribosomal proteins predicted by STRING bioinformatics tool have also been reported to induce apoptosis via cell cycle arrest. For instance, RPL34 was identified to interfere cell cycle by restraining cyclin/cyclin-dependent kinases (Cdk), namely Cdk4 and Cdk5 as well as shown to inhibit the apoptosis suppressor gene p35-activated kinase (Moorthamer and Chaudhuri, 1999).

However, there are protein-protein interactions which demonstrated a contradictory inference from our findings revealing negative regulation of apoptosis
and positive regulation of cell proliferation. Hence, it is necessary to understand that one miRNA can target more than 1000 genes (Enright et al. 2003; Stark et al. 2003; John et al. 2004; Rajewsky and Socci 2004; Brennecke et al. 2005; Grun et al. 2005; Lewis et al. 2005) and one gene can be regulated by multiple miRNA (Enright et al. 2003; Lewis et al. 2003, 2005; John et al. 2004; Rajewsky 2006). *P. longifolia* treatment dysregulates a cluster of miRNAs. A cluster of miRNAs are reported to be up-regulated, causing their mRNA targets to be under-expressed while a set of miRNAs are found to be down-regulated, causing over-expression of their mRNA targets. Hence, this explains the contravert results which may be due to the impact of miRNA–target gene networks. Further investigations and validation is highly required to analyse the oncogeneic role of miR-221-5p in cancer cells.
6.5 Conclusion

The bioinformatics results summarizes the comprehensive flow of miRNA and mRNA targets as well as its subsequent protein-protein interaction networking involved in the apoptosis mechanism. Although the dual role of miRNA was shown through the mRNA target prediction and its subsequent PPIN analysis based on computational approaches, this chapter has also successfully revealed an abundant number of apoptotic genes and proteins which may be responsible for the induction of apoptosis by *P. longifolia* through the down-regulation of miR-221-5p, supporting our hypothesis. The comprehensive analysis of interactions between miRNA and mRNA targets and subsequent protein expression using various bioinformatics tools incorporated with different computational algorithms uses quantitative prediction scores to overcome the challenge with uncertainty in their accuracy. Further experimental proteomic analysis using mass spectrometry can be conducted in order to identify and characterize the proteins involved in relation to over-expression and knock down of miR-221-5p alongside with *P. longifolia* treatment.
CHAPTER 7: PROTEOMIC ANALYSIS OF MIR-221-5P TARGET PROTEINS ALONGSIDE WITH P. longifolia leaf EXTRACT TREATMENT IN HELA CELLS BY MASS SPECTROMETRY

7.1 Introduction

The major function of miRNAs in regulating various cellular processes including cell proliferation, cell differentiation and apoptosis is mainly through partial complementary binding to their mRNA targets. The partial pairing allows a single miRNA to target to multiple genes, which leads to silencing of the gene expressions or degradation of the mRNA targets at translation level, eventually suppressing the target proteins (Friedman et al., 2009). Although bioinformatics study has been conducted for miR-221-5p to predict the mRNA targets and its subsequent protein-protein interaction networking (PPIN) involved using computational approaches based on various algorithms of prediction, it is also highly necessary to conduct a further proteomic analysis for miR-221-5p alongside with the methanolic P. longifolia leaf extract treatment. The technical advances in proteomics mass spectrometry have beneficially allowed the researchers to identify the multiprotein systems in human in order to understand the distinct proteins and its participation in the overall networked system. Mass spectrometry is one of the most widely utilized high-throughput proteomics analytic tools. Mass spectrometry based proteomics analysis uses the “bottom-up” strategy in which protein complexes from the total protein sample are digested into peptides through enzymatic cleavage which are then separated based on chemical and physical properties followed by analysis using mass spectrometer.
Hence, this chapter focuses on the extraction of total proteins from the untreated HeLa cells, methanolic *P. longifolia* leaf extract treated HeLa cells as well as the miR-221-5p mimics and anti-miR-221-5p transfected HeLa cells, alongside with the *P. longifolia* treatment. The proteins present in each sample are identified with the incorporation of LC-ESI-MS/MS and are functionally analysed using web-based bioinformatics tool, namely PANTHER to understand the participation of differentially identified proteins in various biological processes which in turn elucidates the effect of *P. longifolia* treatment in the induction of apoptosis as well as to understand the role of miR-221-5p expression in the protein expression.

### 7.1.1 Objectives

Therefore, the main objectives of this chapter are as follows:

1) To identify the proteins in HeLa cells regulated by methanolic *P. longifolia* leaf extract treatment and the miR-221-5p target proteins alongside with *P. longifolia* leaf extract treatment, using LC-ESI-MS/MS.

2) To perform functional classification for the proteins identified from untreated HeLa cells, methanolic *P. longifolia* leaf extract treated HeLa cells as well as the miR-221-5p mimics and anti-miR-221-5p transfected HeLa cells, alongside with the *P. longifolia* treatment, using PANTHER Bioinformatics tool.
7.2 Materials and methods

7.2.1 Sample preparation

HeLa cell culture was performed following the methods explained in Chapter 3, under the section 3.2.5 Subculturing of cells. This was followed by the transfection of miR-221-5p mimics and anti-miR-221-5p into independent HeLa cell samples, which has been explained earlier in Chapter 4, under the section 4.2.2 Transfection of miRNA mimics and anti-miRNA.

7.2.2 Extraction of total protein using RIPA lysis buffer

Radioimmunoprecipitation assay (RIPA) buffer (Nacalai tesque, Inc., Japan) was used to lyse the cultured HeLa cells, following the manufacturer’s protocol. Briefly, the medium was discarded and the cells were washed twice with cold PBS. After removing the PBS, the cells were trypsinized and scraped completely with a cell scraper which was then pelleted down. The cell pellet is then washed with PBS and 200 µL of RIPA lysis buffer was used to resuspend the pellet obtained from the final wash. The samples were then incubated on ice for 15 minutes. After 15 minutes, the cell lysate was centrifuged at 10,000 × g for 10 minutes at 4°C. Finally, the supernatant containing the total protein extracts was transferred to a fresh tube for further analysis.

7.2.3 Acetone precipitation and buffer exchange

Ice cold 100% acetone which was pre-chilled at –20°C for at least 2 hours was added to the sample at a volume ratio of 5:1. The mixture was then incubated at –20°C for 6 hours, followed by centrifugation at 15,000 × g for 10 minutes. Then, the
supernatant was carefully discarded without disturbing the pellet. The precipitated protein pellet was then resuspended in 50 mM ammonium bicarbonate with half the volume of the original sample volume.

7.2.4 Determination of protein concentration

The total protein concentrations of the samples were evaluated by using Protein Assay Bicinchoninate Kit (Nacalai Tesque, Inc., Japan) according to the manufacturer’s protocol. The protocol has been well documented earlier in chapter 4, under the section 4.2.4(d) Bicinchoninate Protein Assay.

7.2.5 Buffer preparation and SDS-PAGE

7.2.5(a) 10% SDS

Five grams of sodium dodecyl sulfate (SDS) was dissolved in 25 mL of ddH₂O was added to make a final volume of 50 mL.

7.2.5(b) Resolving buffer (1.5 M Tris-Cl, pH 8.8)

An amount of 18.17 g tris base was dissolved in 80 mL of ddH₂O. The solution was then adjusted to pH 8.8 by adding 2 M hydrochloric acid (HCl). Finally, ddH₂O was added to make a final volume of 100 mL.
7.2.5(c) Stacking buffer (0.5 M Tris-Cl, pH 6.8)

An amount of 7.3 g tris base was dissolved in 50 mL of ddH₂O. The solution was then adjusted to pH 6.8 by adding 2 M HCl. Finally, ddH₂O was added to make a final volume of 100 mL.

7.2.5(d) Ammonium persulfate (APS), 10% (w/v)

Ten milligrams of ammonium persulfate was dissolved in 100 μL of ddH₂O. The solution was freshly prepared prior to use.

7.2.5(e) Loading buffer, 5×

Accurately 0.76 g of tris base and 1 g SDS was dissolved in 25 mL of ddH₂O. The pH of the solution was then adjusted to 6.8 with 2 M HCl. After that, 10 mL of glycerol and 0.025 g of bromophenol blue were added into the solution. Subsequently, ddH₂O was added to reach a final volume of 50 mL. The loading buffer without β-mercaptoethanol was aliquoted in 900 μL and stored at -20°C for later use. The 5 × loading buffer was freshly prepared by adding 100 μL of β-mercaptoethanol to the aliquoated 900 μL of incomplete loading buffer when required.

7.2.5(f) SDS-PAGE running buffer

To prepare 10 × running buffer, accurately weighed 30 g of tris base, 10 g of SDS, and 144 g of glycine were dissolved in 700 mL of ddH₂O. Upon complete solubilisation of the mixture, more ddH₂O was added to reach a final volume of 1 L. Finally, the solution was kept in 4°C until used. Then, 100 mL of 10 × running
buffer was diluted with 900 mL of ddH₂O to freshly prepare 1 × buffer for further use. The solution was reused for up to 4 times and was kept at 4°C.

7.2.5(g) Coomassie Brilliant Blue, CBB R250, 0.05% (w/v)

One gram of Coomassie Brilliant Blue (CBB) R250 was completely dissolved in 300 mL of methanol. Then, ddH₂O was added until final volume of 500 mL was reached. The solution was kept at room temperature until further use.

7.2.5(h) Separating gel and stacking gel

Separating gel of 10% was prepared by mixing 1.5 mL of resolving buffer, 2 mL of 30% Bis-Acrylamide, 60 μL of 10% SDS, 60 μL of 10% APS, 6 μL of TEMED and finally appropriate deionised water was added to get a final volume of 6 mL. The mixture is immediately loaded in the gel casting glass frame and let to harden. Subsequently, 3% stacking gel was prepared by mixing 0.5 mL, 0.2 mL of 30% Bis-Acrylamide, 20 μL of 10% SDS, 20 μL of 10% APS, 2 μL of TEMED and finally deionised water was used to top up to a final volume of 2 mL. The volume mentioned is for the preparation of 1 gel.

7.2.5(i) SDS-PAGE

Sixteen microlitre of protein sample containing an amount of 30 μg proteins was mixed with 5 × sample buffer at a ratio of 4:1. The mixture was incubated in water bath at 100°C for 2 minutes. Meanwhile, a reservoir consisting of the preparative gels was submerged with 1× running buffer. The sample was cooled in a water bath at 25°C for a few seconds. Then, the samples were loaded into the wells.
One well was loaded with the pre-stained protein marker as the protein size reference. Proteins were separated according to molecular weights with 10% resolving gel as prepared using the recipe described in section 7.2.5(h). Electrophoresis was run at a constant voltage of 100 V until the dye reached approximately 1 cm from the gelend. After electrophoresis, the gel was stained with CBB stain for 30 minutes and de-stained in ddH₂O until the background was cleared. CBB stain was prepared as described in section 7.2.5 (g).

7.2.6 Buffer preparation and In-solution digestion

7.2.6(a) RapiGest

One milligram of RapiGest lyophilized powder was reconstituted with 1 mL of freshly prepared 50 mM ammonium bicarbonate. The solution was then stored in 100 μL aliquots at -80°C until further use. Working concentration of 0.05% RapiGest was then prepared by diluting 100 μL of 0.1% RapiGest with 100 μL of freshly prepared 50 mM ammonium bicarbonate.

7.2.6(b) 100 mM Dithiothreitol (DTT)

Accurately weighed 7.7 mg of Dithiothreitol (DTT) was dissolved in 500 μL freshly prepared 50 mM ammonium bicarbonate.

7.2.6(c) 200 mM Iodoacetamide (IAA)

Accurately weighed 18.5 mg Iodoacetamide (IAA) was dissolved in 500 μL freshly prepared 50 mM ammonium bicarbonate.
7.2.6(d) 1 μg/μL Trypsin

A vial containing 100 μg of lyophilized trypsin powder was reconstituted with 100μL of the supplied trypsin resuspension buffer containing 50 mM acetic acid. The mixture was then stored as 5 μL aliquots at -80°C until further use.

7.2.6(e) Trypsin Digestion

Based on the concentration of protein samples obtained by BCA assay, volume containing 100 μg of protein was aliquoted and appropriate volume of 50 mM ammonium bicarbonate was added to reach a final volume of 100 μL. This provides the sample concentration of 1 μg/μL. Then, 100 μL of 0.05% RapiGest was added to the mixture and mixed well by vortexing. The sample was then incubated at 80°C for 15 minutes. Subsequently, the sample was allowed to cool at room temperature for approximately 5 minutes before 5 μL of 100 mM DTT was added. The sample was mixed and incubated at 60°C for 15 minutes. Next, the sample was cooled to room temperature again for 5 minutes and 5 μL of 200 mM IAA was added. The sample was mixed well and then incubated at room temperature in the dark for 30 minutes, followed by an addition of 2 μL of 1 μg/μL trypsin. The mixture was incubated at 37°C for 16 hours. Subsequently, 2 μL TFA was added and the mixture was incubated at 37°C for 20 minutes to stop the digestion process and to remove the RapiGest. The aggregated RapiGest was then pelleted down at 14, 462 × g for 15 minutes. The supernatant containing the peptide mixtures was then collected and filtered with 0.45 μm minisart syringe filter. The peptide samples were then stored at -80°C until further analysis.
7.2.7 LC–ESI–MS/MS

The peptides from the four experimentally different HeLa cell samples: Untreated HeLa cells (Control), *P. longifolia* leaf extract treated HeLa cells, miR-221-5p mimics transfected *P. longifolia* leaf extract treated HeLa cells, and anti-miR-221-5p transfected *P. longifolia* leaf extract treated HeLa cells were sent to Proteomics Core Facility, Malaysia Genome Institute, National Institutes of Biotechnology Malaysia (NIBM) for mass spectrometry analysis. Briefly, the peptides separation was performed using Dionex UltiMate 3000 RSLCnano (Thermo Fisher Scientific, USA) nano-liquid chromatography system while mass spectrometry analysis was performed using Orbitrap Fusion (Thermo Fisher Scientific) mass spectrometer. For spatial discrimination of the peptides, they were packed into and eluted with EASY-Spray Column PepMap® RSLC, C18, 2 μm particle size, 50 μm id × 150 mm coupled with pre-column (μ-Precolumn PepMap 100, C18. 3 μm particle size, 300 μm id x 5mm) at a flow rate of 0.3 μL/min with mobile phase buffer A (deionised distilled water with 0.1 % formic acid) and B (ACN with 0.1 % formic acid). The sample was eluted for 101 min with a gradient of mobile phase B, 5-95% for 93 minutes, 95% for 2 minutes, and back to 5% in 2 minutes. For mass spectrometry analysis, the instrument was operated in the data-dependent mode. The parameters for the fullscan spectra were as follow: scan range 310-1800 m/z, resolving power of 120000, AGC target of 4.0 e5 (400 000), and maximum injection time of 50 ms. The method consisted of 3 second Top Speed Mode where precursors were selected for a maximum of 3 second cycle. Only precursors with an assigned monoisotopic mass to charge ratio (m/z) and a charge state of 2 – 7 were further analysed for MS/MS. All precursors were filtered using a 20 second dynamic exclusion window and intensity threshold of 5000. The MS/MS spectra were analysed using the following
parameters: rapid scan rate with solving power of 60000, AGC target of $1.0 \times 10^2$ (100), 1.6 m/z isolation window, and a maximum injection time of 250 ms. Precursors were fragmented by Collision-induced dissociation (CID) and Higher-energy collisional dissociation (HCD) at normalised collision energy of 30% and 28%, respectively. Data analysis and database matching against OS=Homo sapiens was performed using Proteome DiscovererTM Software Version 2.1 (Thermo Scientific). The parameters used in the analysis were fixed modifications: carbamidomethylation (C), variable modification: Oxidation (M), deamidation of asparagine (N) and glutamine (Q), maximum missed cleavage set at 2, false detection rate (FDR) <0.1% and parent mass and precursor mass tolerance at 10 ppm and 0.6 Da, respectively. Significant score (-10lgP) for protein acceptance were set at >20, whereas minimum unique peptide was set at 2.

### 7.2.8 Functional annotation of identified proteins

Proteins identified by the LC–ESI–MS/MS were further characterized with the incorporation of PANTHER (Protein Analysis THrough Evolutionary Relationships) Classification System version 14.0 (27 January 2019) at http://www.pantherdb.org/. Functional annotation based on protein classes and biological processes were analyzed by entering the Protein accession numbers for Homo sapiens.
7.3 Results

7.3.1 Determination of Protein Concentration

The total proteins extracted using RIPA lysis buffer from untreated HeLa cells, *P. longifolia* leaf treated HeLa cells, miR-221-5p mimics transfected *P. longifolia* leaf extract treated HeLa cells and anti-miR-221-5p transfected *P. longifolia* leaf extract treated HeLa cells were then evaluated for protein concentration with the incorporation of Protein Assay Bicinchoninate Kit (NacalaiTesque, Inc., Japan) using BSA as standard (Plate 7.1). The yield of total protein extracted from the untreated HeLa cells, *P. longifolia* leaf treated HeLa cells, miR-221-5p mimics transfected *P. longifolia* leaf extract treated HeLa cells and anti-miR-221-5p transfected *P. longifolia* leaf extract treated HeLa cells were 400 µg, 422 µg, 359.7 µg and 283.22 µg, respectively which were evaluated based on the standard curve obtained from the BCA assay (Figure 7.1).
Plate 7.1: The 96-well micro plate used in BSA assay to evaluate the protein concentration of (A) Untreated HeLa cells, (B) *P. longifolia* leaf extract treated HeLa cells, (C) miR-221-5p mimic tranfected, *P. longifolia* leaf extract treated HeLa cells and (D) anti-miR-221-5p transfected, *P. longifolia* leaf extract treated HeLa cells.
Figure 7.1: A standard curve of protein concentration using Bicinchoninate Protein assay.

BSA was used as a standard. Protein concentration determination of HeLa cells was interpolated from the curve. The data are expressed as mean ± SD (n=6).
7.3.2 SDS-PAGE

The total proteins extracted from the untreated HeLa cells, *P. longifolia* leaf treated HeLa cells, miR-221-5p mimic transfected *P. longifolia* leaf extract treated HeLa cells and anti-miR-221-5p transfected *P. longifolia* leaf extract treated HeLa cells were evaluated for protein band profiles by performing SDS-PAGE. As shown in Plate 7.2, protein band profiles on lane 1, 2, 3, and 4 indicated good reproducibility. The DMEM as a blank medium in Lane 5 showed that the medium was indeed protein free and did not contribute any artefact in the total protein band profiles. Lane M represents protein marker, Precision Plus Proteins Kaleidoscope Standards (Bio-rad, USA). In addition, visual observation showed distinct protein bands with no protein smearing. This suggested that the total proteins were suitable for downstream mass spectrometry analysis.
Plate 7.2: Protein profiles of total protein extracted from HeLa cells by SDS-PAGE (The proteins were electrophoresed in 10% polyacrylamide resolving gel)

Lane M: Marker [Precision Plus Proteins Kaleidoscope Standards (Bio-rad, USA)]; Lane 1: Untreated HeLa cells; Lane 2: *P. longifolia* leaf extract treated HeLa cells; Lane 3: miR-221-5p mimic transfected, *P. longifolia* leaf extract treated HeLa cells; Lane 4: anti-miR-221-5p transfected, *P. longifolia* leaf extract treated HeLa cells; Lane 5: Blank (DMEM)
Protein identification by LC-ESI-MS/MS

Proteins were identified with the incorporation of Liquid chromatography-Electrospray-ionisation quadrupole time-of-flight mass spectrometry (LC-ESI-MS/MS) from the digested protein samples extracted from untreated samples (A), *P. longifolia* leaf extract treated samples (B), miR-221-5p mimic transfected *P. longifolia* leaf extract treated samples (C) and anti-miR-221-5p transfected *P. longifolia* leaf extract treated samples (D). The LC-ESI-MS/MS identified a total of 1061, 668, 564 and 940 proteins from samples A, B, C, and D respectively (Supplementary 3). Among which proteins with high FDR confidence and consisted of at least more than 2 unique peptides which are found in all three replicates were filtered for the down-stream proteomics analysis. This accounted for 520, 342, 381 and 458 proteins from the samples A, B, C and D respectively. The summary of the selected identified proteins from the four different samples are shown in the Venn diagram (Figure 7.2) (Oliveros, 2007-2015). Representatives of the identified proteins from samples A, B, C, and D are shown in Table 7.1, Table 7.2, Table 7.3 and Table 7.4, respectively.

Among all the protein groups, 206 proteins (27.1%) were found to be common in all four experimental groups. Two hundred and thirty six proteins were found to be common in untreated and treated samples while 106 different proteins were found in the *P. longifolia* leaf extract treated cells. Greater number of proteins (458) was found to be in anti-miR-221-5p transfected cells compared to that of mimic transfected cells (381), suggesting knock down of a particular miRNA increases the protein targets.
Figure 7.2: The Venn diagram represents the number of proteins identified by LC-ESI-MS/MS from total proteins extracted from HeLa cells

(A) Untreated HeLa cells, (B) *P. longifolia* leaf extract treated HeLa cells, (C) miR-221-5p mimic transfected, *P. longifolia* leaf extract treated HeLa cells and (D) anti-miR-221-5p transfected, *P. longifolia* leaf extract treated HeLa cells
### Table 7.1: Examples of proteins identified from untreated HeLa cells by LC-ESI-MS/MS

<table>
<thead>
<tr>
<th>Accession</th>
<th>Description</th>
<th>Coverage</th>
<th>Unique Peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q09666-1</td>
<td>Neuroblast differentiation-associated protein AHNAK [OS=Homo sapiens]</td>
<td>48.3870968</td>
<td>129</td>
</tr>
<tr>
<td>P29401-2</td>
<td>Isoform 2 of Transketolase [OS=Homo sapiens]</td>
<td>74.0095087</td>
<td>37</td>
</tr>
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<td>glyceraldehyde-3-phosphate dehydrogenase [OS=Homo sapiens]</td>
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**Table 7.2:** Examples of proteins identified from *P. longifolia* leaf extract treated HeLa cells by LC-ESI-MS/MS

<table>
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<th>Accession</th>
<th>Description</th>
<th>Coverage</th>
<th>Unique Peptides</th>
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<td>P09429</td>
<td>High mobility group protein B1 [OS=Homo sapiens]</td>
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<td>P62158</td>
<td>Calmodulin [OS=Homo sapiens]</td>
<td>77.18121</td>
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<td>P51858</td>
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Table 7.3: Examples of proteins identified from miR-221-5p mimics transfected *P. longifolia* leaf extract treated HeLa cells by LC-ESI-MS/MS

<table>
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<th>Accession</th>
<th>Description</th>
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<th>Unique Peptides</th>
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<td>P29966</td>
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<td>hepatoma-derived growth factor [OS=Homo sapiens]</td>
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Table 7.4: Examples of proteins identified from anti-miR-221-5p transfected *P. longifolia* leaf extract treated HeLa cells by LC-ESI-MS/MS

<table>
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<th>Accession</th>
<th>Description</th>
<th>Coverage</th>
<th>Unique Peptides</th>
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<td>Q09666-1</td>
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<td>P80723</td>
<td>Brain acid soluble protein 1 [OS=Homo sapiens]</td>
<td>83.70044</td>
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<tr>
<td>P31948-2</td>
<td>Isoform 2 of Stress-induced-phosphoprotein 1 [OS=Homo sapiens]</td>
<td>56.94915</td>
<td>39</td>
</tr>
<tr>
<td>P67809</td>
<td>Nuclease-sensitive element-binding protein 1 [OS=Homo sapiens]</td>
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<td>15</td>
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<td>P51858</td>
<td>Hepatoma-derived growth factor [OS=Homo sapiens]</td>
<td>75.41667</td>
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<td>Myristoylated alanine-rich C-kinase substrate [OS=Homo sapiens]</td>
<td>50.3012</td>
<td>16</td>
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<td>Q8NC51-3</td>
<td>Isoform 3 of Plasminogen activator inhibitor 1 RNA-binding protein [OS=Homo sapiens]</td>
<td>49.36387</td>
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<td>P62158</td>
<td>Calmodulin [OS=Homo sapiens]</td>
<td>77.18121</td>
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In comparison with treated samples (B), more similar proteins are found to be in anti-miR-221-5p transfected samples (321) than in the mimic transfected samples (284), suggesting the role of *P. longifolia* treatment in highly down-regulating the miR-221-5p. Among the similar proteins, 20 proteins were independently common in the treated (B) and anti-miR-221-5p transfected samples (D) while only 2 were found to be independently common in treated (B) and mimic transfected samples (C). Individually 69 proteins were found in the anti-miR-221-5p transfected treated cells (D) while 40 proteins were found in the miR-221-5p mimic transfected samples (C).

### 7.3.4 Functional annotation of proteins

Proteins identified by the mass spectrometry for untreated HeLa cells, *P. longifolia* leaf extract treated HeLa cells, miR-221-5p mimic transfected *P. longifolia* leaf extract treated HeLa cells and anti-miR-221-5p transfected *P. longifolia* leaf extract treated HeLa cells were independently analysed for protein classification and gene ontology for biological process with the aid of web-based proteomics analysis tool called the PANTHER. Proteins are classified into various protein classes such as nucleic acid binding, transcription factors, cytoskeletal proteins, oxidoreductase, signalling molecules and so forth for the proteins identified from all four samples. Besides, PATHER also categorized the proteins based on their participation in various biological processes such as biological regulation, cellular component organization or biogenesis, growth, localization, metabolic process, signalling and so forth.

*Polyalthia longifolia* leaf extract treatment induces apoptosis in HeLa cells through the regulation of numerous miRNAs expression and thus, the protein classification and biological process were compared with untreated in order to
understand the action of differential proteins in *P. longifolia* treated cells. The number of proteins clustered into differential protein classes and biological processes were demonstrated to be lesser in *P. longifolia* treated cells as compared to that of untreated cells. On the other hand, in order to understand the role of miR-221-5p expression in the *P. longifolia* treated cells, protein classification and biological process for the proteins identified from the miR-221-5p over-expressed and knock down samples were compared. The number of proteins clustered into differential protein classes and biological processes were shown to be greater in miR-221-5p knock down HeLa cells as compared to that of miR-221-5p over-expressed HeLa cells. Protein classification chart generated by PANTHER bioinformatics tool for proteins identified from the Untreated HeLa cells, *P. longifolia* leaf extract treated HeLa cells, miR-221-5p mimic transfected *P. longifolia* leaf extract treated HeLa cells and anti-miR-221-5p transfected *P. longifolia* leaf extract treated HeLa cells are shown in Figure 7.3 (A), 7.3 (B), 7.4 (A) and 7.4 (B), respectively. Gene Ontology for biological process generated by PANTHER bioinformatics tool for proteins identified from the Untreated HeLa cells, *P. longifolia* leaf extract treated HeLa cells, miR-221-5p mimic transfected *P. longifolia* leaf extract treated HeLa cells and anti-miR-221-5p transfected *P. longifolia* leaf extract treated HeLa cells are shown in Figure 7.5 (A), 7.5 (B), 7.6 (A) and 7.6 (B), respectively.
Figure 7.3: PANTHER generated protein classification obtained for the proteins identified from the (A) Untreated HeLa cells and (B) *P. longifolia* leaf extract treated HeLa cells.
Figure 7.4: PANTHER generated protein classification obtained for the proteins identified from the (A) miR-221-5p mimic transfected *P. longifolia* leaf extract treated HeLa cells and (B) anti-miR-221-5p transfected *P. longifolia* leaf extract treated HeLa cells.
Figure 7.5: PANTHER generated gene ontology for biological process obtained for the proteins identified from the (A) Untreated HeLa cells and (B) *P. longifolia* leaf extract treated HeLa cells. Highlighted in red indicates proteins involved in growth biological process (GO: 0040007) which is absent in *P. longifolia* treated HeLa cells.
Figure 7.6: PANTHER generated gene ontology for biological process obtained for the proteins identified from the (A) miR-221-5p mimic transfected P. longifolia leaf extract treated HeLa cells and (B) anti-miR-221-5p transfected P. longifolia leaf extract treated HeLa cells.
7.4 Discussion

MiRNA targets mRNA by partial complementary pairing to silence the expression and subsequently inhibits the translational process which affects the protein expression. Earlier in this study, the imperative validation of miR-221-5p expression, functional analysis of miR-221-5p with its effects on morphological changes in HeLa cells through microscopic analysis and bioinformatics analysis of miR-221-5p were performed, which have been documented in our previous chapters. This chapter elucidates the identification and characterization of the protein targets of miR-221-5p alongside with *P. longifolia* leaf extract treatment in HeLa cells. *P. longifolia* treatment induced apoptosis in HeLa cells by dysregulation of miRNAs, in which miR-221-5p was found to be highly down-regulated. The down-regulation of miR-221-5p leads to over-expression of its mRNA targets and its subsequent protein expression. It is now highly important to further conduct the proteomic analysis of miR-221-5p with the advancement available currently in technology for proteomics using mass spectrometry.

7.4.1 Isolation of total protein

Total protein extraction is one of the most important steps in proteomics analysis (Simpson, 2003) which has to be an optimal method not just to extract out most of the proteins but also to uphold the integrity and morphology of the proteins (Ngoka, 2008). Proteins are usually present as complexes with other proteins, biological macromolecules or with other matrix molecules which are seen to be encompassed within cell membrane and organelles. They are connected by various bonds such as disulfide bond (reduction & alkylation), hydrogen bond (chaotropes), electrostatic interactions (salts, charged detergents, chaotropes), charge-dipole (chaotropes),
dipole-dipole (strong dipolar molecules), van der Waals (salt, dipolar molecules, chaotropes), and hydrophobic interactions (salts, dipolar molecules, chaotropes) depending on their physical and biological statement. Therefore, breaking these bonds to solubilise them without degrading them is the critical part of extraction which can be done conventionally with the use of buffers like chaotropes, detergents, reducing agents, buffers, salts, and ampholytes.

In our study, Radio-Immunoprecipitation Assay (RIPA) buffer (Harlow et al., 1988) was utilized to extract total proteins from untreated HeLa cells, *P. longifolia* leaf extract treated HeLa cells, miR-221-5p mimic transfected *P. longifolia* leaf extract treated HeLa cells and anti-miR-221-5p transfected *P. longifolia* leaf extract treated HeLa cells in order to conduct the down-stream proteomics analysis using mass spectrometry. RIPA buffer is a widely used lysis buffer for total protein extraction from human cell samples due to its expeditious and efficient lysis capability. RIPA buffer is also known for its competency in solubilising broad field of proteins such as cell, nuclear, mitochondrial, membrane receptors, cytoskeletal-associated, and soluble proteins as the solubilisation of proteins highly depends on their phytochemical properties like average charge and turn-forming residues content. In comparison with RIPA buffer and Urea buffer, proteins extracted using RIPA buffer were evidently shown to be more enriched with nuclear proteins, protein complexes, and mitochondrial proteins (Ngoka, 2008). RIPA buffer is also reported to be adaptable with protease and phosphatase inhibitors as well as for various down-stream analysis including western blot, protein assays, immunoassays and BCA protein assay. The compatibility of RIPA buffer allowed the addition of protease inhibitor cocktail and iodoacetamide in order to protect the proteins from degradation and to inhibit the cysteine protease activity, preventing proteins from
forming disulphide bonds (Broadwell et al., 1994). Apparently, protein isolation using RIPA buffer is highly suitable for down-stream proteomic analysis particularly for cancer proteomics because it recovers the most cellular proteins in greater yield, extraction consistency and reliability with minimized non-specific protein-binding interactions, maintaining a lower background (Kershaw et al., 2018; Sekhon et al., 2015; Zhang et al., 2015).

However, there are few drawbacks in using this buffer which has to be taken into consideration. For instance, the detergent formulation of RIPA buffer may cause sample contamination with salts, nucleic acids, lipids-polysaccharides, and phenols which may interfere with the down-stream experiments (Bodzon-Kulakowska et al., 2007; Antonioli et al., 2009). However, this can be resolved by performing protein precipitation methods using solvents like acetone, ethanol, chloroform, methanol, acetonitrile, trichloroacetic acid (Chen et al., 2005; Zellner et al., 2005). In our study, protein precipitation was conducted using acetone precipitation which removes all the undesirable components in the sample while the centrifugation of precipitated proteins also allows us to exchange the buffer to a specifically compatible buffer for down-stream analysis (Buxton et al., 1979; Wu et al., 2014). However, it requires greater volume of organic solvent, usually 4:1 of organic solvent to protein sample. Various comparison studies between the solvents used for precipitation, acetone have been reported to recover the highest protein concentration with better isoelectric focusing (IEF) by improving delipidation and also considered to be a time-efficient and cost-effective method (Gorg et al., 2007; Evans et al., 2009; Fic et al., 2010; Feist and Hummon, 2015). It is an imperative step to determine the concentration or protein yield obtained from the final product as well as to check the protein separation by running SDS-PAGE. The yields of total protein obtained from
the four samples were shown to be sufficiently high, accounting approximately 400 µg for each sample.

7.4.2 Protein identification by LC-ESI-MS/MS

Mass spectrometry-based proteomics analysis has attained an increasing popularity and widely used technology in proteomics research due to its increased sensitivity and accuracy as well as its capability to provide structural data of the identified proteins (Cao and Limbach, 2017). Basically, mass spectrometry separates proteins based on mass-to-charge ratio (m/z), involving ionization of protein molecules, followed by fragmentation of molecules into smaller fragment ions which will be directed to the detector through an analyzer. Electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) are the two commonly utilized techniques for vaporization and ionization of the protein molecules for mass spectrometry analysis (Karas and Hillenkamp, 1988; Fenn et al., 1989) each with its own advantages and disadvantages. In our study, Orbitrap-based LC-ESI-MS/MS has been incorporated to conduct the proteomics analysis due to its high sensitivity, efficiency and reliability. ESI mass spectrometry can be performed using a very little amount of sample as femto-mole quantities in micro-litre sample volumes is sufficient (Ho et al., 2003). ESI uses soft ionization technique with the development of nanospray technology (Gale et al., 1993; Emmett et al., 1994) and incorporates the highly efficient quadrupole and time of flight (QToF) mass analyzers (Ens et al., 2005; Glish et al., 2008; Krutchinsky et al., 1998).

The high-resolution LC-ESI-MS/MS mass spectrometry analysis successfully identified 520, 342, 381 and 458 proteins from untreated samples, *P. longifolia* leaf extract treated samples, miR-221-5p mimic transfected *P. longifolia* leaf extract
treated samples and anti-miR-221-5p transfected *P. longifolia* leaf extract treated samples. More proteins (458) were found to be in anti-miR-221-5p transfected cells compared to that of mimic transfected cells (381). This supports our findings in which *P. longifolia* treatment promotes down-regulation of miR-221-5p. In comparison with treated samples, more similar proteins are found to be in anti-miR-221-5p transfected samples (321) than in the mimic transfected samples (284), suggesting the role of *P. longifolia* treatment in down-regulating the miR-221-5p.

One of proteins identified from the *P. longifolia* treated HeLa cells which is not found in untreated HeLa cells with high confidence is tropomyosin alpha chains (Accession number: P09493), indicating knock-down of miR-221-5p may lead to over-expression of this protein. In previous studies, tropomyosin alpha chains were found to be significantly lower in cancer tissues as compared to that of adjacent normal tissues (Amiri-Shoar et al., 2017), suggesting over-expression of this protein may inhibit cell proliferation. Other studies have also reported the importance of tropomyosin protein in the regulation of anoikis. Anoikis is a type of apoptosis which is initiated when there is an absence in anchorage-environment (Frisch and Screato, 2001). Down-regulation of tropomyosin-1 was shown to promote resistance to anoikis in breast cancer cells facilitating the malignant cell growth (Raval et al., 2003), meanwhile over-expression of the protein was shown to increase sensitivity towards anoikis leading to detachment induced apoptosis in breast cancer cells (Bharadwaj et al., 2005).

The tropomyosin, actin binding protein is found to be highly down-regulated in various tumors which in turn the over-expression of this protein was evidently demonstrated to induce apoptosis in renal cell carcinoma (Tang et al., 2018). The study was further extended to investigate the role of tromomysin protein and its
underlying mechanism involved in the apoptosis, which revealed that the over-expression of tropomyosin protein not only inhibited the cell proliferation of cancer cells but also demonstrated an intense DNA damage through comet assay (Tang et al., 2018). The over-expression of tropomyosin was also resulted in the down-regulation of anti-apoptotic factor BCL-2 with an increased expression of pro-apoptotic proteins like Bax, caspase 3 and p53 which has been demonstrated through western blot analysis, thus concluding that tropomyosin promotes apoptosis via p-53 mediated mitochondrial pathway in renal cell carcinoma (Tang et al., 2018). The present mass spectrometry finding is interestingly consistent with the previous study conducted by Vijayarathna et al. (2017) reporting the induction of apoptosis in HeLa cells by *P. longifolia* leaf extract treatment which was well documented to be through the mitochondrial potential depolarization. It was evidently shown that the *P. longifolia* leaf extract treatment increased the expression of pro-apoptotic proteins like BAX, BAD, cytochrome c, caspase-3, p21, p27 and p53 while the anti-apoptotic proteins like BCL-2 and BCL-w was down-regulated, which was revealed by proteomic profiling array (Vijayarathna et al., 2017a). DNA damage was also reported through comet assay in the previous study. The interesting correlation between over-expression of tropomyosin protein and the regulation of caspase-dependent intrinsic apoptotic pathway by *P. longifolia* leaf extract treatment clearly supports our hypothesis.

Another protein identified from the *P. longifolia* treated HeLa cells is cyclin-dependent kinases regulatory subunit 1 (Accession number: P61024). Various studies have reported the activation of cyclin-dependent kinases (CDKs) downstream the caspase activation is also involved in the apoptotic mechanism. CDK activity was demonstrated to be involved in cell cycle arrest to inhibit cancer cell
growth. Recent study reported the inhibition of CDK to result in increased resistance to doxorubicin-induced apoptosis whereas the over-expression of CDK activity markedly increased the apoptotic effect of doxorubicin through up-regulation of Bim, leading to Bax/Bak-dependent mitochondrial membrane depolarization (Xia et al., 2018). The findings is highly corresponding to the previous study conducted by Vijayarathna et al. (2017) reporting the apoptotic effect of P. longifolia leaf extract treatment in HeLa cancer cells through cell cycle arrest at G1 phase of cell cycle, eventually inhibiting the cell proliferation. The accumulation of cells at G1 phase due to cell cycle arrest also allows cells to undergo DNA repair, but in case the damage is beyond repair, it was reported to undergo p53 dependent apoptosis (Zhou and Elledge, 2000). The evidence showing the up-regulation of pro-apoptotic proteins including BAX, BAD, caspase-3, and cytochrome c, as well as the increased expression of tumor suppressor genes like p53, p21 and p27 through P. longifolia leaf extract treatment in HeLa cells (Vijayarathna et al., 2017) can also be correlated with the expression of CDK shown in our current study through mass spectrometry. This is because the participation of CDK has been well documented not only in the induction of cell cycle arrest but also in the regulation of p27 (Gil-Gomez et al., 1998), p21 (El-Deiry et al., 1993; Harper et al., 1993) and p53 (Kastan et al., 1991; Lin et al., 1992) during the induction of apoptotic mechanism.

On the other hand, one of the proteins found in the exclusive subset of proteins identified from treated HeLa cells and anti-miR-221-5p transfected HeLa cells is Proline-rich protein (Accession number: Q92733). Proline-rich protein has been reported to function as a pro-apoptotic factor, leading to Fas-mediated programmed cell death in neuronal cells (Gomes et al., 1999). Fas-induced apoptosis can be due to reduced NF-kappaB activity and increased expression of caspase
inhibitors cIAP-1/2 which consequently increases caspase-induced apoptosis (Carlisle et al., 2003). Another such protein is Geminin (Accession number: O75496) which is involved in inhibiting DNA replication and to control cell cycle. This protein has been previously reported to promote apoptosis by suppressing the replication licensing via a cell type-dependent mechanism where it causes S-phase cell cycle arrest (Shreeram et al., 2002). Other studies have also evidently shown the pro-apoptotic effect of geminin through its interaction with polo-like kinase 1 (Plk1), leading to disruption of DNA pre-replication complex and activation of DNA damage-sensing kinases (Yim and Erikson, 2009). Crk-like protein (Accession number: P46109) which is actively associated with numerous important biological process, including apoptosis. Although there are reports showing the oncogenic property of this protein, previous study has evidently demonstrated the pro-apoptotic function of crk proteins, namely nuclear crk II in which over-expressing crk II protein induced apoptosis through cell cycle arrest in HeLa and MCF-7 cancer cells (Kar et al., 2007). Participation of crk proteins in the induction of caspase-dependent intrinsic apoptosis is also well documented (Evans et al., 1997; Kornbluth et al., 1997). The data obtained from mass spectrometry also provides the rationale behind the results obtained in our previous study conducted by Vijayarathna et al. (2017) demonstrating the differential expression of apoptotic proteins upon *P. longifolia* leaf extract treatment, leading to cell cycle arrest at G0/G1 phase and G2/M phase. For instance, the expression of p21 was shown to inhibit the action of Cdk1 and Cdk2 from regulating the cell cycle while the expression of p21 is known to be regulated by p53 (El-Deiry et al., 1993; Harper et al., 1993).

Although there are proteins identified by the ESI-MS/MS which appear to be counterintuitive with the current findings, it is important to consider that there is
large number of proteins which have been evidently reported to play dual roles, depending on the cellular condition. For example, the proteins myc (Hoffman and Liebermann, 1998) and cyclin D1 (Sofer-Levi and Resnitzky, 1996) were evidently shown to perform both pro-apoptotic and anti-apoptotic roles.

7.4.3 Functional annotation of proteins identified by LC-ESI-MS/MS

PANTHER (Protein Analysis THrough Evolutionary Relationships) is one of the popularly employed online resource (http://pantherdb.org) to investigate the functional classification, and to perform biological data analysis. PANTHER collaborates with GO Consortium in order to ascertain the GO annotation with the up to date information generated by GO Consortium (Mi et al., 2016).

Gene ontology of biological process obtained for the proteins identified by untreated HeLa cells has revealed a gene ontology for growth (GO: 0040007) which is absent in the gene ontology data obtained for the proteins identified by P. longifolia leaf extract treated HeLa cells. The protein responsible for growth namely Annexin A1 which was only found in the untreated cells has been previously reported to be present or up-regulated in various cancer types including leukemia (Falini et al., 2004) and breast cancer (Tu et al., 2017). The up-regulation of Annexin A1 expression may lead to negative regulation of NF-κB signal transduction pathway by binding to p65 subunit which eventually induces cell proliferation and inhibits apoptosis (Zhang et al., 2010). P. longifolia leaf extract treatment has been successfully demonstrated to inhibit Annexin A1. This supports our finding that P. longifolia leaf extract treatment induces apoptosis in HeLa cervical cancer cells.
Other than that, number of proteins involved in the important biological processes such as metabolic process (GO: 0008152), biological regulation (GO: 0065007), cellular component organization or biogenesis (GO: 0071840) and cellular process (GO: 0009987) were shown to be significantly decreased in *P. longifolia* treated HeLa cells compared to that of untreated HeLa cells. One such protein responsible for metabolic process found in untreated HeLa cells which is inhibited by *P. longifolia* leaf extract treatment is Heterogeneous nuclear ribonucleoprotein Q (HnrQ) (Accession number: O60506). Previous study evidently reported that over-expression of HnrQ protein increases cell proliferation in human colon cancer cells (Yoo *et al.*, 2009). Another protein which is found to be inhibited by *P. longifolia* treatment includes Cathepsin D (Accession number: P07339). As cathepsin D was previously reported to play an important role in breast cancer progression (Johnson *et al.*, 1993; Dian *et al.*, 2012), inhibition of this protein by *P. longifolia* treatment is a promising strategy to inhibit cancer progression. Adenylate kinase isoenzyme 1 (Accession number: P00568) was also shown to be inhibited by *P. longifolia* treatment as it was only found in the metabolic process of untreated HeLa cells. Targeting this protein was proven to be an effective anti-cancer approach as it was evidently shown to be involved in stress, drug resistance, malignant transformation in cancer, and ATP regulation in HeLa cells (Fujisawa *et al.*, 2016).

The number of proteins identified in miR-221-5p knock down HeLa cells is comparatively greater than that of miR-221-5p enhanced HeLa cells, indicating the basic concept behind miRNA functional mechanism where the down-regulation miRNA increases the expression of its gene targets and subsequently the protein expression. The investigation of protein classification and gene ontology obtained for the over-expressed and knock down of miR-221-5p allows understanding the
Insight of miR-221-5p in HeLa cells. Protein classification and biological processes are inter-related as various types of proteins are involved and interact with one another in order to perform a biological process. The number of proteins found in each class of protein and the number of proteins found to be involved in most of the biological processes are greater in the miR-221-5p knock down cells compared to that of miR-221-5p over-expressed cells.

For instance, 103 proteins were documented to be involved in metabolic activity in miR-221-5p knock down cells while only 92 proteins were observed in metabolic activity in miR-221-5p over-expressed cells. Among the 103 proteins, 21 proteins were found to be unique in the miR-221-5p knock down cells. Several zinc finger proteins was found be unique in the miR-221-5p knock down cells which has been previously reported to be involved in cellular processes especially in cell proliferation and apoptosis in cancer (Bandrés et al., 2007; Siavoshian et al., 2000). Zinc finger proteins were demonstrated to act as tumor suppressor gene in colorectal cancer by inducing apoptosis.

Another protein which is found to be present only in the anti-miR-221-5p transfected HeLa cells is Intracellular hyaluronan-binding protein 4 (Accession number: Q5JVS0). The up-regulation of this protein has been previously reported to be important in cisplatin induced apoptosis in HeLa cells, eventually leading to nuclear fragmentation, rise in subdiploid population and the elevated activation of ERK and over-expression of p53 while the knock down of hyaluronan-binding protein in HeLa cells significantly found to be resistant to cisplatin induced apoptosis (Kamal and Dutta, 2006). Besides, other study conferred the role of hyaluronan binding proteins in inhibiting the tumor growth and metastasis in MDA-435 melanoma cells and was demonstrated to induce apoptosis by elevating the
activation of caspase-3 and caspase-8 (Xu et al., 2003). The anti-tumor effect of hyaluronan binding proteins was also shown in HeLa cells through the inhibition of anchorage-independent tumor growth (Ramakrishna et al., 2012).
7.5 Conclusion

The mass spectrometry-based proteomics analysis for the untreated HeLa samples and *P. longifolia* leaf extract treated HeLa samples allowed the identification of proteins regulated by the *P. longifolia* leaf extract treatment in comparison with the control untreated HeLa cells. This chapter successfully revealed that the protein responsible for cell growth in HeLa cancer cell to be inhibited by the *P. longifolia* leaf extract treatment in the process of inducing apoptosis. Besides, the use of mass spectrometry has also emerged as an important advanced technique for experimental identification of miRNA target proteins with the incorporation of miR-221-5p mimic and anti-miR-221-5p transfection to conduct the gain-of-function and loss-of-function approach. Hence, this chapter further demonstrated the differential protein identification from the miR-221-5p over-expressed and knock down HeLa cells alongside with *P. longifolia leaf* extract treatment. It has been revealed that the number of proteins identified in the miR-221-5p knock down cells to be higher than the number of proteins present in the miR-221-5p over-expressed HeLa cells, indicating the down-regulation of miRNA enhances its protein targets. The knock down of miR-221-5p was demonstrated to enhance a number of proteins to be involved in the induction of apoptosis as well as in the inhibition of cell proliferation, suggesting the induction of apoptosis by *P. longifolia* through the down-regulation of miR-221-5p may be a promising strategy to inhibit cancer progression.
CHAPTER 8: GENERAL CONCLUSION AND FUTURE RECOMMENDATIONS

8.1 General conclusion

Cancer being one of deadly diseases in present has urged the search for chemotherapeutic agents. The traditional medicinal plants is being widely used as adjunctive therapies for cancer as plants rich in phytochemicals are evidently shown to exhibit anti-cancer property by inducing apoptosis. The rapid advancement in Human Genome Project had lead to the discovery of the small non-coding RNA molecules, known as the miRNAs. Ever since the participation of miRNAs in various biological processes including cell proliferation and apoptosis have been widely proven, miRNA targeting has been found to be a novel therapeutic approach in cancer treatment. The abundantly available pytochemical-rich plants are widely investigated and evidently reported to regulate miRNA expressions. Therefore, targeting miRNA through the plant extract treatment can be a promising novel therapeutic approach for cancer treatment as well as to enhance the overall response.

The finding of our previous study reporting the induction of apoptosis in HeLa cells by methanolic *P. longifolia* leaf extract treatment through the regulation of miRNA expressions has led to our current study, aimed to validate the miRNA expression and to perform functional and proteomics analysis of a specific miRNA, namely miR-221-5p which was found to be highly down-regulated in HeLa cells upon *P. longifolia* treatment. As the initial phase of the research freshly extracted *P. longifolia* leaves using maceration method consistently upheld the cytotoxicity of *P. longifolia* leaf extract on HeLa cells via MTT assay with an IC$_{50}$ value of 26.67 µg/mL. The IC$_{50}$ falls in the safe confines set by the American National Cancer
Institute to be utilized as a chemotherapeutic agent. Subsequently, this study successfully validated the expression of miR-221-5p in HeLa cells upon methanolic *P. longifolia* leaf extract, accomplishing the first objective of this research. The down-regulation of miR-221-5p was demonstrated in *P. longifolia* treated HeLa cells as compared to the control untreated HeLa cells by taqman real time RTqPCR. The transfection of miR-221-5p mimics and anti-miR-221-5p into HeLa was also quantified prior to the down-stream functional analysis.

The second objective of the research was successfully achieved by demonstrating the role of miR-221-5p expression in the induction of apoptosis alongside the *P. longifolia* leaf extract treatment. This was done using gain-of-function and loss-of-function as the miR-221-5p is over-expressed by transfecting miR-221-5p mimics and silenced by transfecting anti-miR-221-5p, respectively. Findings obtained through functional analysis such as MTT cell viability analysis, flow cytometric Annexin V/ PI analysis and Caspase-3 analysis evidently revealed the induction of apoptosis increased when the expression of miR-221-5p is silenced while the apoptotic effect of *P. longifolia* treatment was clearly shown to be inhibited in the miR-221-5p over-expressed HeLa cells, suggesting the oncogenic effect of miR-221-5p in HeLa cervical cancer cells.

Furthermore, the ultra-morphological analysis of HeLa cells was also conducted to elucidate the role of miR-221-5p expression. The investigation of cell morphology through scanning electron microscope, transmission electron microscopy and Acridine Orange / Propidium Iodide (AO/PI) Staining successfully demonstrated the typical apoptotic morphological changes such as cell shrinkage, cell blebbing, chromatin condensation and fragmentation in *P. longifolia* treated HeLa cells while these apoptotic hall marks have been clearly absent or inhibited in
the miR-221-5p over-expressed HeLa cells, which tends to show similar structure like the untreated HeLa cells. Therefore, the third objective of this research was achieved, revealing that the over-expression of miR-221-5p promotes cell proliferation and inhibits the apoptotic effect of \textit{P. longifolia} leaf extract treatment.

In addition, bioinformatics analysis which was conducted by utilizing the various web-based bioinformatics tools revealed the mRNA targets of miR-221-5p based on the different types of computational algorithms. The mRNA targets of miR-221-5p which was used to identify the functional annotation using DAVID also revealed the active participation of miR-221-5p in the regulation of intrinsic apoptotic pathway. The analysis protein-protein interaction networking using STRING revealed the various binary interactions of target proteins in the regulation of apoptosis.

Finally, the proteomics analysis of miR-221-5p using mass spectrometry experimentally identified the miRNA target proteins (Supplementary 3) with the incorporation of miR-221-5p mimic and anti-miR-221-5p transfection alongside with \textit{P. longifolia} leaf extract treatment. The proteomics analysis also experimentally identified the proteins regulated by the \textit{P. longifolia} leaf extract treatment. The results obtained revealed the presence of apoptotic proteins in \textit{P. longifolia} treated cells which are suggested to be involved in the induction of apoptosis. The functional annotation analysis through PANTHER bioinformatics tool also comparatively analysed the protein classification and number of proteins involved in various biological processes in order to understand the role of miR-221-5p upon \textit{P. longifolia} treatment in HeLa cells in the induction of apoptosis. The functional analysis revealed the protein classes as well as the participation of proteins identified by LC-ESI-MS/MS in various biological processes such as biological regulation,
cellular component organization or biogenesis, growth, localization, metabolic process, and signalling. The role of miR-221-5p expression upon *P. longifolia* treatment was successfully elucidated through the presence of several proteins involved in apoptosis such as Tropomyosin alpha chains, cyclin-dependent kinases regulatory subunit, proline-rich protein and so forth in anti-miR-221-5p transfected HeLa cells which are absent in the miR-221-5p mimic transfected cells. This finding coincide with the rationale of this research suggesting the down-regulation of miR-221-5p by *P. longifolia* leaf extract treatment induces apoptosis in HeLa cervical cancer cells by regulating its target proteins involved in apoptosis.

Comprehensively, the overall research provides the first insight of molecular mechanism underlying the apoptosis induced by *P. longifolia* leaf extract with an elucidation of functional role of miR-221-5p expression as well as demonstrating the protein targets of miR-221-5p involved in the caspase-dependent apoptosis in HeLa cells. In conclusion, targeting miRNAs would provide a novel therapeutic approach in cancer therapy by improving overall response and survival outcomes in cancer treatment, especially with the utilization of abundantly available natural product such as the pytochemical-rich *P. longifolia* leaf extract. Furthermore, this study also contributes to achieve the sustainable development goals (SDG) set by the United Nations Development Programme, especially in achieving goal number 3, namely Good health and well-being.
8.2 Suggestions for future studies

The present study has provided valuable findings on the molecular mechanism that comprehend the miRNA expression in the induction of apoptosis by *P. longifolia* leaf extract treatment in HeLa cells through functional and proteomic analysis. This can highly be beneficial for future studies which can be further extended to investigate the *in vivo* anti-tumor activity of *P. longifolia* leaf extract by using tumor-carrying mouse xenograft models. The *in vivo* study can provide further understanding of the *P. longifolia* effect on a huge variety of physiological and pathological conditions in animals, predicting the clinical effects as compared to *in vitro* experimentations. Pre-clinical and clinical studies of this extract and anti-miR-221-5p can be further investigated with an insight of miRNA therapeutic approach for cancer treatment. Further toxicity investigations through safety profiling namely clastogenicity, chronic oral toxicity as well as the effects on animal fetus and pregnant mice may lead to the development of an affordable functional food with anti-cancer property. As the participation of miRNA in biological processes is highly acknowledged at present, miRNA targeting is the latest clinical application and approach in the cancer treatment.
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### APPENDIX A

![Image of plant specimen]

<table>
<thead>
<tr>
<th>Family</th>
<th>Annonaceae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name</td>
<td>Polyalthia longifolia</td>
</tr>
<tr>
<td>Date</td>
<td>24/ 3/ 2013</td>
</tr>
<tr>
<td>Locality &amp; Habitat</td>
<td>Dato Haji, University of Malaya, Sempil, Malaya</td>
</tr>
<tr>
<td>Notes</td>
<td>Leaf blade 6-8 x 7-1.5 cm, very narrow; dark green, edges stripped and wavy; tip often incurved upward. Fruits 5-7 cm, 9 - 10 mm wide on short stalk. Flowers 3-4, 1.5 cm long, greenish white.</td>
</tr>
<tr>
<td>Collector</td>
<td>Mr. Khairy Latonoy Subramanian</td>
</tr>
<tr>
<td>Identified</td>
<td>Ms. Hien</td>
</tr>
</tbody>
</table>

*FLORA OF MALAYA*

University of Malaya Herbarium.
APPENDIX B

SEM TISSUE PREPARATION SCHEDULE
(HMDS Technique)

1. Fix in McDowell-Trump fixative (alternatives: Karnovsky’s fixative or 5% Glutaraldehyde) prepared in 0.1M phosphate buffer or cacodylate buffer (pH 7.2) at 4°C for 2 – 24 hours.

2. Wash in buffer. (Use the same buffer as in step 1) (3 x 10 minutes)

3. Postfix in 1% Osmium tetroxide prepared in the same buffer as above at room temperature. (1 – 2 hours)

4. Wash in distilled water. (2 x 10 minutes)

5. Dehydrate as follows:
   - 50% ethanol ........................................15 minutes
   - 75% ethanol ........................................15 minutes
   - 95% ethanol .......................................15 minutes \( \times 2 \)
   - 100% ethanol ......................................20 minutes \( \times 3 \)

6. Immerse the dehydrated tissues in 1 – 2 ml of hexamethyldisilazane (HMDS) for 10 minutes.

7. Decant the HMDS from the specimen vial. Leave the specimen vial with the tissues in the dessicator to air-dry at room temperature.

8. The dried specimen are then mounted on to a SEM specimen stub with a double-sided sticky tape (or silver paint). 

9. Coat the specimen with gold, gold/palladium, chromium or carbon.

10. View in the SEM

*****************************************************************************

NOTES:
# Times recommended are for small pieces of tissues (smaller than 1-2 mm²). For larger pieces times may have to be varied accordingly.
# Buffer: normally 0.1M of pH 7.2, but requirements may vary for different tissues. Refer to published papers.
# Never let the tissues to dry at any stage (till the samples are immersed in the HMDS).
# The tissues may be dehydrated in acetone series instead of the alcohol series stated above.

---- EM Unit, P.P.Sains Kajihayat, USM
APPENDIX C

TEM PREPARATION SCHEDULE
for micro-organisms grown in liquid/solid culture

1. Centrifuge the sample. (Pellet formation)
   [Note: All centrifugation in this technique should be at 1,000g -2,000g for 15 minutes.]

2. Discard the supernatant and resuspend the pellet with McDowell-Trump fixative prepared in 0.1M phosphate buffer (pH 7.2) for at least 2 hours. (Fixation)

3. Centrifuge the resuspended sample. Discard the supernatant.
   {you may transfer the pellet to an Eppendorf tube at this stage, if needed}
   Resuspend the pellet in 0.1M phosphate buffer. (Buffer wash 1)

4. Centrifuge the resuspended sample. Discard the supernatant and resuspend the pellet in 0.1M phosphate buffer. (Buffer wash 2)

5. Centrifuge the resuspended sample. Discard the supernatant and resuspend the pellet in 1% Osmium tetroxide prepared in the phosphate buffer above for 1 hour. (Post-fixation)
   [Note: Osmium tetroxide changes should be done in a fume-hood.]

6. Centrifuge the resuspended sample. Discard the supernatant and resuspend the pellet in distilled water. (Post-fix wash 1)
   [Note: Osmium tetroxide waste should be discarded with care. Please consult EM Unit staff]

7. Centrifuge the resuspended sample. Discard the supernatant and resuspend the pellet in distilled water. (Post-fix wash 2)

8. Centrifuge the resuspended sample, discard the supernatant and place this tube containing the pellet of fixed cells in a waterbath at 45°C for about 15 – 30 minutes depending on the amount of sample.

9. Prepare a 2% solution of agar by dissolving the agar in boiling distilled water. Pour the solution into a test tube while it is still molten, and place the tube in the water bath at 45°C. At this temperature the agar just remains liquid.
10. After the temperature of both the agar and the pellet have equilibrated to 45°C, transfer a small drop of the agar to the tube containing the pellet of cells (with a warm pipette), and stir up the pelette, just enough to break the pellet into small blocks, and to suspend these small blocks in the agar.

11. Immediately pour the agar with the suspended pellet blocks on to a glass microscope slide.

12. After the agar has set (1-2 minutes), cut the solidified agar containing the cells into small cubes, about 1 mm³, with a sharp razor blade and place them in a vial containing 50% ethanol.

13. These cubes are then processed in the same way as pieces of a cohesive pellet or tissue as follows:
   Dehydration:
   - 50% ethanol ........................................15 minutes
   - 75% ethanol ........................................15 minutes
   - 95% ethanol .......................................15 minutes (x2)
   - 100% ethanol .....................................30 minutes (x2)
   - 100% acetone ....................................10 minutes (x2)

14. Infiltrate resin using the mixture of Acetone: Spurr's resin mix (1:1) in a rotator. (15 - 30 minutes)

15. Infiltrate in Spurr's mix overnight in the rotator.

16. Infiltrate in a new change of Spurr's mix for another 5 hours in the rotator. (2-3 days for difficult samples)

17. Embed. Cure at 60°C for 12-48 hours.

********************************************************************************************

NOTES:
# Times recommended are for small pieces of tissues (smaller than 1-2 mm³). Never let the tissues dry at any stage of the processing steps.
# Buffer: normally 0.1 M of pH 7.2, but requirements may vary for different tissues. Refer to published papers.
# Preparation of the resin must be done in a fume hood. Keep all the components of the resin mixture tightly capped. Do not leave the prepared resin mixture uncovered. Paraffin may be used for this purpose.
# For difficult specimens, the specimens may need to be rotated in the resin mixture for as long as 3 days but fresh changes of resin must done at least once a day.

--- E.M Unit, P.P.Salas Kajihayat, USM
LIST OF PUBLICATIONS

Journal article


Conference proceedings


Chapter in books


Award

WRFER Excellent Paper Award for the paper entitled “Induction of Apoptosis in Hela Cervical Cancer Cells by *P. longifolia* Leaf Extract through Down-Regulation of miR-221-5p” for the category of Best presentation/ Best content at the WRFER International Conference held at Chennai, India on 24th June, 2018.
Prediction of Genes and Protein-Protein Interaction Networking For mir-221-5p Using Bioinformatics Analysis

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Abstract Background: The role miR-221-5p through the regulation of its target genes and the subsequent protein-protein interaction networking is important in various cellular processes, especially in cancer pathogenesis.

Objective: Therefore, the prediction of miR-221-5p target genes and the subsequent post-translational analysis is beneficial for exposure to understand the cellular mechanisms and therapeutic approaches regulated by miR-221-5p. Since the experimental approaches are laborious, time-consuming and pricey, in silico approach is utilized to predict the target genes and protein-protein interaction networking related to miR-221-5p. Method: In this study, computational workflow was proposed to identify the predicted target genes of miR-221-5p using miRGate (http://mirgate.bioinfo.cnio.es) database, followed by gene enrichment analysis by DAVID (https://david.ncifcrf.gov/) and Enrichr bioinformatics (http://amp.pharm.mssm.edu/Enrichr/), and finally protein-protein interaction networking analysis using STRING resource (http://string-db.org/). Down-stream feature selection method related to cancer and apoptosis as well as the reliable settings with increased confidence level and low false discovery rate were adopted to analyze the target genes and to build the protein-protein interaction networking for miR-221-5p. Results: miR-Gate bioinformatics tool predicted a total of 4910 protein coding gene targets for miR-221-5p, which includes 326 apoptotic genes based on 5 different computation approaches and 4 different validated prediction methods. The gene enrichment analysis through DAVID and Enrichr bioinformatics tools revealed that the predicted genes are involved in the regulation apoptotic pathway based on the gene ontology analysis accounting an enrichment score as high as 7.41 and a false discovery rate as low as 0.12. The protein-protein interaction networking analysis through STRING bioinformatics tool revealed the functional enrichment of the protein network of the post-transcriptional of the predicted gene targets of miR-221-5p are highly related to the regulation of apoptosis with an interaction confidence score of 0.99. Conclusion: The bioinformatics analysis of miR-221-5p revealed the essential role of miR-221-5p in various biological processes, especially in apoptosis regulation, suggesting the regulation of miR-221-5p to be an efficient gene therapeutic target for cancer as a clinically admissible treatment approach.

Keywords: microRNA, gene targets prediction, gene ontology, apoptosis, protein-protein interaction networking.
INDUCTION OF APOPTOSIS IN HELA CERVICAL CANCER CELLS
BY P. LONGIFOLIA LEAF EXTRACT THROUGH DOWN-
REGULATION OF MIR-221-5P

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Abstract -
Background: Cervical cancer is one of the most diagnosed cancers and leading cause of death among women. The evolution of microRNA and its role in the regulation of various biological activities lead to the break thorough of miRNA-based therapeutic approaches. Our recent study showed that Polyalthia longifolia methanolic leaf extract (PMLE) induced apoptosis through microRNA dysregulation in HeLa cervical cancer cells. This study would contribute to illustrate the functional analysis of miR-221-5p in relation to PMLE treatment in HeLa cells with scientific evidence.

Methods: The PMLE was prepared by maceration method and MTT assay was incorporated to determine the IC50 value of the plant extract in HeLa cells. Over-expression and knock down of miR-221-5p in HeLa cells were performed by transfection of miR-221-5p mimic and anti-miR-221-5p respectively. Annexin V/PI analysis and Caspase 3 analysis was performed to study the functional analysis of miR-221-5p in HeLa cells. Ultra-morphological study was performed through SEM and TEM.

Results: Annexin V/PI analysis and Caspase 3 analysis revealed the induction of apoptosis in anti-miR-221-5p transfected cells in relation to P. longifolia treatment. Besides, ultra-morphological studies through SEM and TEM showed significant apoptotic hallmarks in anti-miR-221-5p transfected HeLa cells as compared to that of in untreated and miR-221-5p over-expressed cells.

Conclusion: Based on this study, it can be deduced that PMLE exert an excellent cytotoxicity on cancerous cells through the down-regulation of miR-221-5p, thus indicating the leaf extract of P. longifolia is a promising anti-cancer drug candidate.

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Keywords - Polyalthia longifolia, microRNA, Cervical Cancer, Apoptosis, Anti-Cancer Drug
This is to certify that Shanmugapriya has presented a paper entitled "Induction of Apoptosis in Hela Cervical Cancer Cells by P. Longifolia Leaf Extract through Downregulation of MIR-221-5P" at the International Conference on Medical and Health Science (ICMHS) held in Chennai, India on 24th June, 2018.
Functional Analysis of Circular RNAs

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Abstract
Circular RNAs characterize a class of widespread and diverse endogenous RNAs which are non-coding RNAs that are made by back-splicing events and have covalently closed loops with no polyadenylated tails. Various indications specify that circular RNAs (circRNAs) are plentiful in the human transcriptome. However, their participation in biological processes remains mostly undescribed. To date, thousands of circRNAs have been revealed in organisms ranging from Drosophila melanogaster to Homo sapiens. Functional studies specify that these transcripts control expression of protein-coding linear transcripts and thus encompass a key component of gene expression regulation. This chapter provides a comprehensive overview on functional validation of circRNAs. Furthermore, we discuss the recent modern methodologies for the functional validation of circRNAs such as RNA interference (RNAi) gene silencing assay, luciferase reporter assays, circRNA gain-of-function investigation via overexpression of circRNA transcript assay, RT-q-PCR quantification, and other latest applicable assays. The methods described in this chapter are demonstrated on the cellular model.

Keywords
CircRNAs - Functional validation - Cellular model

1 Introduction
Circular RNAs (circRNAs) are closed RNA transcripts made by back-splicing of a single pre-mRNA that is found in all higher eukaryotes including mammals. The first circRNA was discovered in the early 1990s [1] as an obviously befalling family of non-coding RNAs that is
Conventional and Non-conventional Approach towards the Extraction of Bioorganic Phase

Abstract

Natural products such as natural food are the richest bio-resource of bioorganic compounds for modern medicines, nutraceuticals, food supplements and pharmaceutical applications. The research and application on natural food started with the extraction techniques that play an important role to the extraction quantity (Yield), quality (extracted *phytochemicals*) and also to the subsequent analyses accomplished to evaluate the biological and chemicals activities. Various types of technologies with different principles of extraction of bioorganic compounds are available today. Based on the literature the conventional extraction methods show better recoveries of bioorganic substances of natural food. Also, conventional extraction methods facilitate the extraction of high concentration of bioorganic substances with the safe solvents system such as pure ethanol. Moreover, conventional extraction methods is still widely used due to its simplicity. However, the conventional extraction methods is not always suitable for industrial uses due to long extraction time and large consumption of harmful solvents systems such as methanol. Therefore, modern non-conventional extraction methods could be an alternative extraction method. Hence, in spite of good results achieved with the conventional extraction methods, modern non-conventional extraction methods was established to search for a faster and better extraction method consuming less solvent, especially those that are unattractive in food industry. This chapter is intended to provide insights on conventional and non-conventional extraction methods with their advantages and disadvantage or limitation.

Keywords

Extraction  Natural products  Solvent  Quantity
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