CYTOTOXICITY STUDY OF MCF-7 BREAST CANCER CELL LINES TREATED WITH *Physalis minima* L. EXTRACT

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by

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DNA	Deoxyribonucleic acid
ITIS	Integrated Taxonomic Information System
ATP	Adenosine triphosphate
TNF	Tumor necrosis factor
FADD	Fas-associated death domain protein
MCF-7	Michigan Cancer Foundation-7
IC_{50}	50% inhibitory concentration
$AlCl_3$	Aluminium chloride
APS	Ammonium persulphate solution
β-ME	Beta-mercaptoethanol
$CHCl_3$	Chloroform
DCM	Dichloromethane
DMSO	Dimethyl sulphoxide
DMEM	Dulbecco Modified Eagle's Medium
EGF	Epidermal Growth Factor
GAE	Gallic acid equivalent
FBS	Heat-inactivated fetal bovine serum
HCl	Hydrochloric acid
HeLa	Cervical cancer cell line
HL-60	Human leukemia cell line
HT-29	Human colon cancer cell line
K562	Human myeloid leukemia cell line
MeOH	Methanol
PenStrap	Penicilin/streptomycin
PBS	Phosphate-buffered saline
KCl	Pottasium chloride
$K_2S_2O_8$	Potassium persulfate

QE	Quercetin equivalent
RPMI	Roselle's Park Memorial Institute Media
Na_2CO_3	Sodium carbonate
NaCl	Sodium chloride
SDS	Sodium dodecyl sulphate
ABTS	2'-Azinobis(3-ethylbenzothiazoline-6-sulphonic Acid)
DPPH	2,2-Diphenyl-1-picrylhydrazyl
Hex	Hexane
nBuOH	n-butanol
ATCC	American Type Culture Collection
CO_2	Carbon dioxide
TFC	Total flavonoid content
TPC	Total phenolic content
PS	Phosphatidylserine
PI	Propidium iodide
TEMED	N,N,N',N'-tetramethylethylenediamine
TBS	Tris-buffered saline
TBST	TBS -Tween 20
PVDF	Polyvinylidene fluoride
ND	Not determine
SD	Standard deviation

KAJIAN SITOTOKSIK KE ATAS SEL KANSER PAYUDARA MCF-7 YANG DIRAWAT DENGAN EKSTRAK *Physalis minima* L.

ABSTRAK

Physalis minima L. dipercayai mempunyai pelbagai aktiviti biologi seperti anti-kanser dan anti-oksida. Dalam kajian ini, kesan ke atas pelarut yang mempunyai polariti berbeza; heksana (P-Hex), diklorometana (P-DCM), kloroform ($CHCl_3$), n-butana (P-nBuOH), akues (P-Aq) dan metana mentah (C-MeOH) diuji untuk aktiviti anti-kanser ke atas sel MCF-7. Ekstrak P-DCM menunjukkan sebagai agen anti-kanser yang berpotensi dengan perencatan 50% pada 24 μg/ml kepekatan dos dan ketidaktoksikan ke atas sel normal MCF-10A. Analisis mekanisma kematian sel menyifatkan bahawa ekstrak ini telah menginduksi kematian sel berprogram apoptosis ke atas sel MCF-7 dengan pembentukan kromatin dan badan apoptotik secara tipikal, dimana hal ini merupakan ciri biokimia apoptotis. Peringkat tahap kematian sel berprogram apoptosis bersama dengan eksternalisasi phosphatidylserine telah dijalankan dengan menggunakan annexin V dan pewarnaan propidium iodide. Selain itu, pendedahan akut ekstrak ini terhadap sel MCF-7 telah menghasilkan regulasi peningkatan yang ketara kepada ekpresi p53 dan Caspase 8, jadi hal ini mencadangkan bahawa laluan ekstrinsik telah terlibat. Selain itu, ekstrak P-DCM telah disemak sifat anti-oksidanya. Jumlah kandungan fenolik yang ditemui dalam ekstrak ini telah direkod sebanyak 75390 μg gallic asid /g manakala jumlah flavanoid adalah 2480 μg kuarcetin /g. Di samping itu, ekstrak ini merupakan pengurai radikal bebas dengan nilai IC_{50} sebanyak 2.2 μg/mL bagi DPPH dan 8.64 μg/mL untuk ABTS. Toleransi positif yang kuat wujud dalam kandungan fenolik dengan aktiviti-aktiviti anti-oksida ekstrak P-DCM. Kompoun analisis oleh GC-MS mengesan beberapa potensi sebatian

dinamakan; 2-asid propenoik, isomer phytol, 4-undekana, 9-metil-, (Z)-, asid heksadekanoik, 2-hidroksi-1-(hidrometil) etil ester, siklododekana, 9,12,15-asid oktadekatrinoik, (Z,Z,Z)-, 9,12,15-Oktadekatrinal, n-asid Heksadekanoik, Fenol, 2,5-bis(1,1-dimetietil)-, asid Phthalic, isobutyl nonil ester and 9,12,15-asid Oktadekatrinoik, metil ester, (Z,Z,Z)- manakala dalam analisis LC-TOF-MS, terdapat tujuh sebatian telah ditemui iaitu (24E)-15alfa-acetoksi-3alfa-hidroksi-23-oxo-7,9(11),24-lanostatrien-26-oic asid, 2,6-dikloro-para-fenilenediamin, Natrium thiosalicylate, I-Urobilin, Cerberin, Linoleoyl ethanolamide and 5S-HETE-d8. Memandangkan terdapat kesan apoptosis ke atas sel MCF-7 and bersifat anti-oksida, kajian mencadangkan secara kukuhnya bahawa ekstrak ini disyorkan sebagai salah satu alternatif untuk merawat kanser payudara.

CYTOTOXICITY STUDY OF MCF-7 BREAST CANCER CELL LINES TREATED WITH *Physalis minima* L. EXTRACT

ABSTRACT

Physalis minima L. is believed for having a wide range of biological activities such as anti-cancer and antioxidant properties. In this study, the effect different solvents of several degree of polarities; hexane (P-Hex), dichloromethane (P-DCM), chloroform ($CHCl_3$), n-butanol (P-nBuOH), aqueous (P-Aq) and crude methanolic (C-MeOH) on the anti-cancer activity of MCF-7 cells were studied. P-DCM extract exhibited as a potent anti-cancer agent with inhibition of 50% at 24 μg/ml on MCF-7 cells and non-toxic towards MCF-10A normal cells. Analysis of cell death mechanism revealed that the extract induced apoptotic programmed cell death in MCF-7 cells with typical chromatin condensation and apoptotic body formation, which are the biochemical hallmark of apoptosis. Different stage of apoptotic programmed cell death together with phosphatidylserine externalization were established using annexin V and propidium iodide staining. Besides that, acute exposure of the extract on MCF-7 cells produced a significant up-regulation of p53 and Caspase 8 expression, suggested that extrinsic pathway was involved. Besides that, P-DCM extract was examined for the antioxidant properties. The total phenolic that contained in the extract was recorded as 75390 μg Gallic acid equivalents/g while the flavanoids was 2480 μg of Quercetin equivalents/g. Moreover, the extract showed as strong scavenger with IC_{50} value of 2.2 μg/mL in DPPH and 8.64 μg/mL in ABTS. A strong positive correlation existed in respects to the phenolic content with the antioxidant activities of P-DCM extract. Compound analysis by GC-MS discovered some possible compounds in P-DCM extract named; 2-Propenoic acid, Phytol isomer, 4-Undecene, 9-methyl-, (Z)-,

Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester, Cyclododecane, 9,12,15-Octadecatrienoic acid, (Z,Z,Z)-9,12,15-Octadecatrienal, n-Hexadecanoic acid, Phenol, 2,5-bis(1,1-dimethylethyl)-, Phthalic acid, isobutyl nonyl ester and 9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)- whereas in LC-TOF-MS analysis, there were seven compounds detected which were (24E)-15alpha-Acetoxy-3alpha-hydroxy-23-oxo-7,9(11),24-lanostatrien-26-oic acid, 2,6-Dichloro-para-phenylenediamine, Sodium thiosalicylate, I-Urobilin, Cerberin, Linoleoyl ethanolamide and 5S-HETE-d8. Due to apoptotic effect on MCF-7 cells and antioxidant properties, it is strongly proposed that the extract as one of alternative form to cure breast cancer.

CHAPTER 1

INTRODUCTION

Cancer have become a serious health issue in both developing and developed countries (Ma & Yu, 2006; Graidist et al., 2015). According to World Health Organization (2018), estimated 9.6 million deaths in 2018 was due to cancer. The most common cancers are breast (2.09 million cases), lung (2.09 million cases), colorectal (1.80 million cases), prostate (1.28 million cases), skin cancer non-melanoma (1.04 million cases) and stomach (1.03 million cases) (World Health Organization, 2018). In men, the major cancer that attacked them were lung, prostate, colorectal, stomach and liver. While in women, breast cancer was the most often cancer followed by colorectal, lung, cervix and stomach cancer (American Cancer Society, 2018).

In Malaysia, 103,507 new cancer cases were diagnosed for the period of 2007-2011 according to Malaysia National Cancer Registry Report (Azizah et al., 2016). A 64.3% was medically certified cancer deaths and non-medically certified cancer death was 35.7% (Azizah et al., 2016). The five most common cancers among Malaysian residents were breast (17.7%), colorectal (13.2%), lung (10.2%), lymphoma (5.2%) and nasopharynx (4.9%). The breast cancer incidence is higher in Chinese and Indian women compared to Malay and this differences could be due to reproductive, environmental and dietary factors (Yip et al., 2006).

Breast cancer is mainly treated with surgery, chemotherapy, radiation therapy, hormone therapy and targeted therapy (American Cancer Society, 2016). Even though

the chemotherapeutic drugs were widely used in treatment of breast cancer and gave a positive result in some cases, they have various kind of side effects. These powerful agents are highly cytotoxic to almost cells in the body, thus they can destroy some of the healthy cells that have a function in dividing and growing for a period of life. The post-effect experienced by patients after the chemotherapy session was fatigue, infection, anemia, hair loss, bleeding problems, heart problems and lung tissue damage.

The intention and increase in demand of the natural products, specifically the usage of medicinal plants was started when bad side effects after chemotherapy process had been spread. Thus, the alternative ways to treat the cancer patients is needed. The main reason is because the modern treatment has enormously expensive and the drugs used had a serious bad side effects and lead to morbidity (Sahdeo et al., 2012). Estimated 75-80% of the world populations still depend to the herbal medicine as the primary health care, especially in developing countries (Ekor, 2013). The benefits of alternative treatment that using medicinal plants were more compatible with human body, having therapeutic efficacy, not costly and lesser side effects (Daniel et al., 2012).

Malay folk community in Malaysia was practiced the medicinal plants such as *Goniothalamus umbrosus, Typhonium flagelliforme, Myrmecodia pendens, Strobilanthes crispus* and *Clinacanthus nutans* in cancer treatment (Ali et al., 2014; Wan Afiqah Syahirah et al., 2016). Based on the scientific studies, Malay traditional vegetables or locally called ‘ulam’ had reported to kill various types of cancer cells such as HeLa, HL-60, MCF-7, K562 and HT-29 (Yih et al., 2012; Tayebeh et al., 2014)

as they contained a huge amount of bioactive compounds (Srikanth & Chen, 2016). Besides, the decoction of *Physalis minima* was used for cancer treatment (Zakaria & Mohamad, 1994).

P. minima L. is one of the plant that listed as a medicinal plant, believed to have an anti-cancer properties (Zakaria & Mohamad, 1994). A few studies were carried out abroad have proven the effectiveness of *P. minima* as an antitumor agent. Chloroform extract of *P. minima* exhibited cytotoxic effect on primary ovarian cancer cell line (CaOv-3), human breast carcinoma cell line (T-47D) and human lung adenocarcinoma cell line (NCI-H23) (Ooi et al., 2010(a); Ooi et al., 2010(b); Ooi et al., 2011). Besides that, *P. minima* methanolic extract also showed anti-cancer activity towards cervical cancer cell line (HeLa) and epithelial cell line (HEp-2) (Krishnakumar & Chauhan, 2016). However, there is still lack of information on the activity of this plant towards the MCF-7 breast cancer cells. Thus, the focus of present study is to access the anti-cancer activity of various solvent extract of *P. minima* together with its phytochemical contents. This approach was chosen in order to produce the quality standardization data and reference guidelines towards the development of *P. minima* as potential anti-cancer agent in the future.

1.1 RESEARCH QUESTION

1. Which *P. minima* extract give cytotoxic effect on MCF-7 breast cancer cell line?
2. Which apoptosis pathway does MCF-7 breast cancer cell line follow after treated with *P. minima* extract?

1.2 HYPOTHESIS

H_o: *P. minima* extract has no cytotoxicity effect on MCF-7 breast cancer cell line

H_1: *P. minima* extract possess cytotoxicity activity on MCF-7 breast cancer cell line and express apoptotic related protein

1.3 OBJECTIVES

The objectives of this study are:

1. to compare the cytotoxicity effect of hexane (P-Hex), dichloromethane (P-DCM), chloroform (P-$CHCl_3$), n-butanol (P-nBuOH) , aqueous (P-Aq) residue and crude methanolic (C-MeOH) extract of *P. minima* on MCF-7 breast cancer cell
2. to evaluate the anti-cancer properties of *P. minima* extract on MCF-7 breast cancer cell line through apoptosis and Western blot analysis
3. to determine the phytochemical profiling of *P. minima* extract using antioxidant assay, GC-MS analysis and LC-TOF-MS analysis

CHAPTER 2

LITERATURE REVIEW

2.1 An overview of breast cancer

The non-stop cells dividing will lead to cancer (Rajeswari et al., 2012). Cancer that develops from the breast tissues is called as breast cancer. It was reported as the most common cancer occurred in women worldwide (Lim & Halimah, 2008; Maznah et al., 2011). There are estimated 1.38 million new breast cancer cases were diagnosed in 2008 (Curado, 2011). In most of the Asian countries i11ncluding Malaysia, the occurrence of breast cancer was reported to be increasing from year 2006 and above (Medina et al., 2010; Park et al., 2011). It is still conquered as the top health problem in all ethnics in Malaysia as reported by Abdullah et al., (2013).

2.1.1 The biology of breast cancer

From the distinct features of breast cancer, the lifetime risk can be determine. Besides, the overall prognosis after a diagnosis of breast cancer and the possibility of response to specific therapy also can be analyze. In addition, the deep understanding of breast cancer pathways may help peoples to plan their targeted approaches. Various factors that caused the growth of the breast cancer and its progression including certain steroid receptors (estrogen receptor [ER], progesterone receptor [PR] and retinoic acid receptor-ß), members of the HER/erbB family and selected tumor suppressor or susceptibility genes (p53, BRCA1, and BRCA2) (Judith & Nancy, 2003). Thus, the biology of breast cancer can contribute vital information regarding many aspects of the disease.

Breast cancer can be divided into two types based on the cell's formation under the microscope which are carcinomas and sarcomas (Rodney & John, 2003). Carcinomas breast cancer started in epithelial cells of breast where sarcomas are started in the cells of muscle, fat or connective tissues of the breast. But, there is a rare type of breast cancer where the breast was inflamed and looked red, swollen and feel warmed. There are four stages reported in breast cancer (Sepideh et al., 2015). Stage one is the earliest detection of breast cancer development. At this stage, the production of the cancer cells are very limited. In stage two, the cancer cells have a tendency to grow or metastasize. Stage three considered as advanced cancer, where it had invaded the neighboring tissues. In stage four, the cancer cells had spread throughout other parts of the body.

2.1.2 Modern treatment of breast cancer

Breast cancer can be treated with local and systemic treatments depending on the type of the breast cancer (American Cancer Society, 2016). Local treatment can be divided into surgery and radiation therapy. There are two types of surgery, which are breast-conserving surgery and total mastectomy. Breast-conserving surgery is a process of removing the tumor and nearby margin only from the patient's body while, total mastectomy is the process removing the whole breast that was confirmed having a cancer. Radiation therapy usually used x-rays or particles radiation. For systemic treatments, they consist of chemotherapy, hormone therapy and targeted therapy. Chemotherapy used the anti-cancer drug to destroy the cancer cells (Henry et al., 2013) while, hormone therapy involved hormone to slow or stop the cancer growth. Meanwhile, targeted therapy used anti-cancer drug but it focused on specific gene or protein to stop the cancer.

However, there are more than 500 antagonistic effects that related with modern cancer treatments have been compiled including minor effect up to life threatening injuries (Wang et al., 2006). Thus, alternative therapies using medicinal plants have been developed for centuries to treat the cancer (Schröder et al., 2013). Approximately 6 out of 10 peoples with cancer in United Kingdom used natural remedies together with alternative cancer therapies as it is effective, affordable, easy and simple to prepare (Ling et al., 2014).

2.2 Medicinal plants

Medicinal plants had been recognized for centuries in having anti-cancer properties. Based on the data obtained from The National Cancer Institute (NCI), nearly 35,000 plant species from 20 countries had been screened for potential anti-cancer activities (Desai et al., 2008). The demand of the medicinal plants was increased from time to time as it showed non-toxic effects towards normal cells and had cytotoxic effects on cancer cells. Many plant species that investigated as herbal therapy were selected from developing countries in Asia, where peoples there relied on medicinal plants as a primary treatment (Ochwang'i et al., 2014). Some of the herbal plants that have been used in cancer treatment were listed in **Table 2.1**.

Table 2.1. Example of herbal plant used in cancer study

Plant materials	Solvent extraction	Cell line	Region	Phytochemicals content	Reference
Physalis minima	Chloroform Methanol	CaOv-3 T-47D NCI-H23 HeLa HEp-2	Malaysia	Physalin F	Ooi et al., 2010(a); Ooi et al., 2010(b); Ooi et al., 2011 Krishnakumar & Chauhan (2016)
Echinacea purpurea	Aqueous ethanol	Caco-2 HCT-116	Taiwan	Cichoric acid	Tsai et al., (2012)
Synsepalum dulcificum	Methanol Ethanol	HCT-116 HT-29	Philippines	Phenolics, carotenoids	Seong et al., (2018)
Curcuma longa	Methanol	HeLa	India	Alkaloid, flavanoids, phenolics	Shukla et al., (2016)
Azadirachta indica	Methanol	HeLa	India	Alkaloid,flavanoid, tannins, saponins, phenolic, glycoside	Shukla et al., (2016)
Piper cubeba	Methanol	MDA-MB-468	Thailand	Phenolic	Graidist et al., (2015)

2.3 *Physalis minima*

This study is focusing on *P. minima*. It belongs to *Physalis* genus and Solanaceae family (Burkill et al., 1966; Ganapathi et al., 1991). **Figure 2.1** shows the taxonomic rank of *P. minima*. The genus *Physalis* L. consisted of 120 species scattered all over the world (Navdeep et al., 2015). *P. minima* is one of the species that became famous among researchers nowadays as it was believed to have medicinal values. Cape gooseberry, bladder cherry, pygmy ground cherry and 'letup-letup' is a common name for the plant. It is also known as *P. eggersii* O.E. Schulz, *P. lagascae* Roem. & Schult, and *P. lagascae* var. *glabrescens* O.E. Schulz (Navdeep et al., 2015). According to Chotani and Vaghasiya (2012) and Integrated Taxonomic Information System (ITIS) (2015) report, it is widely distributed in tropics regions such as India, Baluchistan, Afghanistan, Tropical Africa, Singapore, Australia and Malaysia, yet it was probably originated from neotropics.

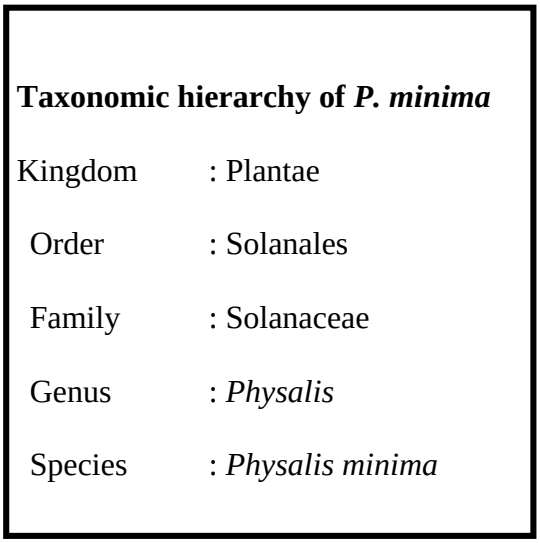

Figure 2.1. Taxonomic rank of *P. minima*

2.3.1 Botanic description of *Physalis minima*

P. minima is annual plant having a weedy stem and usually grew at disturbing sites especially on sandy to gravelly soil (Azlan et al., 2002). It has been classified in dicotyledonous group. It can reach up to 0.5-1.5 m height with a purple-tinged stem (**Figure 2.2**). It has ovate leaves with 9.7 m long and 8.1 m broad. Its corolla is yellowish with no coloration inside and less than 6 mm in diameter (**Figure 2.3**). It has a yellow berry-like fruit with oblong-ovoid in shape, 0.8-1 cm in diameter and enclosed in calyx (**Figure 2.4**) (Reddy et al., 1999; Nathiya & Dorcus, 2012). The shape of the seeds is suborbicular-oblong and 2 x 1.8 mm diameter (Reddy et al., 1999).

Figure 2.2. The *P. minima* plant

Figure 2.3. Corolla of *P. minima*

Figure 2.4. Fruits of *P. minima*

2.3.2 Traditional uses of *Physalis minima*

P. minima has been used since long time ago as a remedy for headache and fever (Panda, 2000; Agbor & Ngogang, 2005; Pullaiah, 2006). Spleen disorders, wound pustules, intestinal pains, purgative, diuretic and gonorrhea also have been treated with this plant (Chopra et al., 1969; Parmar & Kaushal, 1982; Karthikeyani & Janardhanan, 2003; Raju et al., 2007; Anisuzzaman et al., 2007; Khare, 2007; Dilip et al., 2008). Besides that, it has been used to restore the flaccid breasts, treatment of colic and gastrophy (Burkill et al., 1966; Sethuraman & Sulochana, 1988; Gupta et al., 2010). This plant appetizing bitter, thus peoples used it as a tonic for inflammation, spleen enlargement and abdominal problems (Vipin & Ashok, 2010). The fruits and flowers of *P. minima* was believed to cure stomach pain, constipation and ear troubles, while the root part was used for treatment of backache and odeama (Vipin & Ashok, 2010). Interestingly, the whole plant was practiced by the Malay community in Peninsular Malaysia for cancer treatment (Zakaria & Mohamad, 1994).

2.3.3. Pharmacological effects of *Physalis minima*

P. minima possessed beneficial health properties as reported scientifically. The current research towards *P. minima* is extremely being carried worldwide in order to assess the importance of the plant in terms of therapeutic value. A research on methanolic extract of *P. minima* leaves was reported to possess the diuretic effect in albino Wister rats (Jyothibasu, 2012). The doses of the methanolic extract of *P. minima* (MEPM) was set at 100 and 200 mg/kg, p.o. The quantity of urine was significantly increased with both doses. The sodium and potassium excretion in Wister rats also were increased by the MEPM and it was suggested that the extract produce significant effect in diuretic activity.

In addition, the gastric ulcer treated with *P. minima* methanolic extract has been investigated. The anti-ulcer activity was assessed on ethanol induced ulcer models and pylorus ligation in Wister rats by using MEPM. The acute gastric ulcer was prevented with the dosage of 100 and 200 mg *P. minima* per kg body weight, in two times per day for five days. The gastric volume, free acidity, total acidity and ulcer index were significantly reduced by MEPM, indicated that it had the healing action of chronic ulcer (Jyothibasu et al., 2013).

Another study on *P. minima* reported that its ethanolic extract possessed hepatoprotective activity on paracetamol induced hepatic injury rats (Pratheeba et al., 2014). There are significantly increased in aspartate aminotransferase (SGOT) at 212U/L, alanine aminotransferase (SGPT) at 320 U/L and Serum Bilirubin at 0.8mg/dl on paracetamol treated rats when compared to silymarin, a standard hepatoprotective drug. Besides that, aqueous soxhlet extraction of *P. minima* was tested *in-vitro* on

alloxan-induced diabetic albino rats to determine the hypoglycemic effects (Sucharitha & Estari, 2013). The root and stem parts of *P. minima* exhibited mild reduction in fasting blood glucose level. While, its leaves and flowers revealed a significant decreased in blood glucose level of fasting rats. Thus, the study indicated that both leaves and flowers extract of *P. minima* had higher efficiency and potent anti-diabetic agent than root and stem extract.

Anti-microbial activity treated with *P. minima* has been examined before. Nathiya and Dorcus (2012) worked on various bacterial strains which are *Bacillus cereus, B. subtilis, Citrobacter* sp., *Enterobacter aerogene, Escherichia coli, Klebsiella pneumonia, Pseudomonas aeruginosa, P. fluorescens* and *Staphylococcus aureus* that treated with chloroform, diethyl ether, ethanol, ethyl acetate and methanol extract of stem, leaf and unripe fruits of *P. minima*. *B.cereus, E. aerogene* and *S. aureus* had greater antibacterial activity with maximum inhibition zone (10.0 mm ± 0.5) in ethanol extract, suggested that ethanol was found to be more effective compared to other solvents used.

Moreover, *P. minima* has been used in the prevention of cancer disease. The chloroform extract of *P. minima* plant was treated on human lung adenocarcinoma NCI-H23 cell lines. The extract showed the time- and dose-dependent manner and induced apoptosis with typical DNA fragmentation. The extract also significantly regulated c-myc, caspase-3 and p53 mRNA expression in NCI-H23 (Ooi et al., 2011). In T-47D breast carcinoma cell lines, the chloroform extract of *P. minima* exerted programmed cell death via p53-, caspase-3-, and c-myc-dependent pathways (Ooi et al., 2010(b)). The extract also exhibited cytotoxic on CaOv-3 human ovarian

carcinoma. The cells experienced the combination of apoptosis and autophagic mechanism (Ooi et al., 2010(a)). Besides that, MEPM showed the increment of growth inhibition percentage in both HeLa and HEp-2 cell line through SRB and MTT assay.

2.4 Phytochemistry study of natural product

Chemotaxonomy is an application of chemical data to systematics. It has become a great attention among biochemists and botanists in development of natural products. To detect the various group of naturally occurring phytochemicals, some phytochemical investigations have been carried out. This approach considered effective in determining the bioactive profile of plants for beneficial value (Masih & Singh, 2012).

2.4.1 Gas Chromatography-Mass Spectrometry (GC-MS)

The discovery of the electron has been started since 1897, which was happened after the information on electrical discharges in gases was developed by Sir J. J. Thomson. After that, the innovation of first mass spectrometer (MS) was started in order to quantify the mass-to-charge ratios of ions. Before this, it was known as parabola spectrograph. The potential actions by MS were reported throughout 100 years, which involved the isotopes discovery, actual atomic weight determination, classification of new element, quantitative gas analysis, stable isotopes identification and the characterization of molecular structure.

GC-MS approach in herbal medical plant research is unable to be challenged. A study on identification and quantification of active natural compound in methanol extract of tea was made possible by GC-MS. The major biologically active constituents

identified include caffeine, ß-sitosterol, ß-amyrin, lupeol, linoleic acid and vitamin E (Novotny et al., 2015).

2.4.2 Liquid chromatography/time-of-flight mass spectrometry (LC-TOF-MS)

Liquid chromatography paired to mass spectrometry is a powerful technique to analyze various components in complex herbal matrices especially for qualitative applications (Zhou et al., 2009). LC-TOF-MS produced good analysis through its high resolution, specific molecular mass information and offered good linearity over a large dynamic range (Macherone et. al., 2018).

LC-TOF-MS analysis of extract from roots of *Gentiana macrophylla* was done by Qi et al., (2012). There were eight major peaks detected, including four secoiridoid glucosides group and four unknown compounds. The secoiridoid glucosides group consisted of loganic acid (mass = 375.3), 6′-O-β-D-glu-gentiopicroside (mass =553.4), swertiamarin (mass = 409.3) and gentiopicroside (mass = 391.3). The unknown compounds obtained possibly due to its extremely low content.

2.5 Cell death mechanism

The greatest phenomenon during the development of multicellular organism is a programmed cell death or called apoptosis (Kroemer et al., 2009). Apoptosis removed the unnecessary or potentially harmful cells in multicellular organism in normal physiology (Castro-Obregon et al., 2004).

Apoptosis can be seen with typical morphological structure changes where the cells will lost their integrity, shrinkage of cell, nuclear condensation or karyorrhexis,

forming a nucleosomal fragments from accumulation of chromatin, cell fragmentation, leaking in mitochondria, membrane blebbing and formation of apoptotic bodies (Kroemer et al., 2009). Once the apoptotic bodies formed, the neighboring cells engulfed it rapidly without any inflammatory response (Wiegand et al., 2001). Apoptosis has two main pathways which are mitochondria pathway (intrinsic) and death receptor pathway (extrinsic) (David et al., 2013). **Figure 2.5** shows an illustration of mitochondria pathway and death receptor pathway.

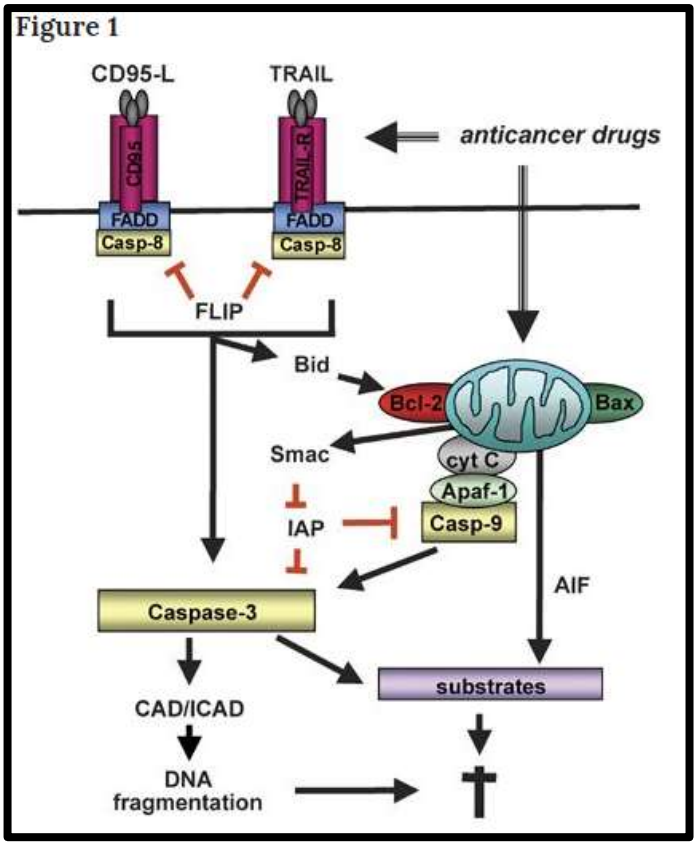

Figure 2.5. Illustration of mitochondria and death receptor pathway (Hengartner, 2000)

2.5.1 Mitochondria pathway (intrinsic)

The cellular stress like DNA damage, cell exposed to heat and radiation, viral infection, free radicals that caused oxidative stress to cells were initiated the

mitochondria pathway. The cellular stress resulted the pro-apoptotic protein of Bcl-2 family bound to the outer membrane of the mitochondria. A pro-apoptotic factor, Bax translocated from cytosol to outer mitochondria membrane and protein-lined channel was created. The released of intracellular content and cytochrome C from the mitochondria were promoted by the pro-apoptotic proteins through the protein-lined channel (Vladimir et al., 2006).

The cytochrome C is a main regulator in this pathway. Once it was released out from the mitochondria, the cells undergo irreversible to death. Then, it combined with adenosine triphosphate (ATP), enzyme called Apaf-1, and pro-caspase-9 to produce apoptosome in the cytoplasm. Apaf-1 induced the conformational change in pro-caspase-9 and became activated caspase-9. The caspase-9 activated the caspase-3, which is the effector protein, thus causing caspase cascade and degradation of cells (Peng et al., 2011).

Generally, Bcl-2 proteins family are the important regulator of apoptosis incidence in this pathway. The caspase cascade activation and process of cytochrome C discharging out from the mitochondria body was regulated by the anti-apoptotic members of Bcl-2 family proteins such as Bcl-X, Mcl-1 and Bcl-w and also pro-apoptotic members like Bax, Bak, Bad, Bid and Bok.

2.5.2 Death receptor pathway (extrinsic)

The death receptor pathway was initiated by extracellular ligands or sometimes by the removal of growth factor. Ligand like tumor necrosis factor (TNF) bound with its receptor, TNFR-1 caused conformational change in death domain. The death domain

was activated when two cytosolic adaptor protein (FADD and TRADD) and procaspase-8 bound together. The interaction between both procaspase-8 and FADD was generated an active caspase-8, initiator protein. The role of caspase-8 is similar to the caspase-9 that involved in intrinsic pathway, where it activated caspase-3 to start the degradation process (David et al., 2013).

2.6 Molecular analysis of herb extract on cancer cell

The mechanisms of herb extract on cancer cell can be understand by molecular analysis such as genomic, transcriptomics and proteomic approach.

2.6.1 Genomic analysis

DNA level is very basic cellular level that is common to many cancers. Cancer caused by DNA alteration of nucleotide sequence of the genome to enable the cell to proliferate in an unregulated manner (Hyndman, 2016; Ruth, 1997). It also called as somatic mutation (Nik-Zainal & Morganella, 2017). Stratton et al., (2009) stated that the mutation usually occurred during the cell division stage where the gene has been damaged or lost or replicated. DNA replication involved insertion and deletion of nucleotides (Stratton et al., 2009). This error caused the cell to stop its normal function and started to grow out of control (Hyndman, 2016).

2.6.2 Transcriptomics analysis

The study at RNA level is called transcriptomics analysis. Researchers have been used transcriptomics analysis for their understanding in cancer research. Jing et al., (2005) used DNA microarray to investigate the influence of the *Coptidis rhizoma* extract on appearance of the mutual cancer genes involved in human breast cancer cell lines, the

ER-positive MCF-7 and ER-negative MDA-MB-231 cells. From this assay, they revealed that MCF-7 treated with *C. rhizoma* extract dramatically upregulated the mRNA expression of interferon- β (IFN- β) and tumor necrosis factor-α (TNF- α). The changes in mRNA expression was confirmed by real time PCR (RT-PCR) analysis, showed that the extract increased IFN- β and TNF-α expression for ~200-fold and 17-fold, respectively. Since IFN- β as an important anticancer cytokines, it played the responsible in antiproliferative effect in MCF-7 treated with *C. rhizoma* extract (Lee & Margolin, 2011).

Kuo-Hua et al., (2018) also investigated the effect of shikonin extracted from dried root of *Lithospermum erythrorhizon* on different types of breast cancer cells lines, MCF-7, SKBR-3 and MDA-MB-231 through transcriptome analysis using RNA-seq. From RNA-seq transcriptome analysis, there are 38 common genes were expressed in different types of breast cancer. Among them, 36 common genes were consistently upregulated, one gene was downregulated and the only one gene RN7SL1 was inconsistently expressed. A qRT-PCR was done using five randomly selected genes (DUSP1, DUSP2, CDKN1A, SESN2, PGF) to validate the result of RNA-seq analysis. There are high correlation between expression ratio of RNA-seq and qRT-PCR. After the shikonin treatment, the expression of DUSP1 and DUSP2 was increased in all types of cancer cell lines. Therefore, the extract might be a therapeutic alternative medicine for treating cancer as there are induction in DUSP1 and DUSP2, the upstream regulator of MAPK signaling pathway.

2.6.3 Proteomics analysis

Proteins work as a main functional unit in the cell and the major target of most drugs. Unfortunately, the expression levels, modifications and functions of protein were poorly attributed by genomic and transcriptomics analysis (Zhao et al., 2017). Proteomics analysis is a study that involved proteins. Zejun et al., (2014) was studied the proteomic analysis of lung cancer cells treated with periplocin. To analyze the protein profile in human lung cancer cell lines A549, they had performed the 2-DE combined with MS/MS and validation was done through Western blotting analysis. From 2-DE profiling, 53 protein spots were found to express differently in periplocin- and NS-treated A549 cells. There were 39 proteins were recognized by MS/MS, where 29 of them were downregulated and the rest were upregulated by periplocin activity. Based on Western blotting analysis, the proteins EIF5A, PSMB6, ATP5A1 and ALDH1 were downregulated when treated with periplocin, which is correlated with 2-DE analysis.

Another study on proteomics analysis of human colon cancer cell line, HT-29 treated with 20S-Ginsenoside-Rg_3 was done by Lee et al., (2009). In 2DE, a 17 cm pH 4-7 linear IPG strips were used to examine the protein expressions in HT-29 cell line. There were 20 spots with different expression in treated and untreated 2D image by PDQuest software. Eight out of 20 spots identified were significantly different ($p < 0.05$), as they exhibited increased or decreased the spots intensities more than 2-folds when compared with control. By using MALDI-TOF/TOF-MS and NCBI database, five proteins (retinoblastoma binding protein 4, clathirin, tropomyosin 1, annexin 5, glutathione S-transferase-P1c) were upregulated and three proteins (serine, PCNA, rho GDP dissociation inhibitor alpha) were down-regulated. Retinoblastoma binding

protein 4 and clathirin were proteins related to mitosis inhibition, while rho GDP dissociation inhibitor alpha, tropomyosin 1 and annexin 5 were proteins that involved in apoptosis. The proteome changes correlated with mitosis inhibition, DNA replication and repair and growth factor signaling in HT-29. They concluded from the proteomics data obtained, there were various mechanisms for anti-cancer effect in HT-29 when treated with 20S-Ginsenoside-Rg_3 (Lee et al., 2009).

In addition, Nagappan et al., (2016) have been studied the anti-cancer effect of A549 human lung cancer cells treated with flavanoids isolated from *Citrus platymamma* (FCP) using a proteomics approach. The flavanoids exhibited cytotoxic effect on A549 cell line and no cytotoxic activity was determined in WI-38 human fetal lung fibroblasts. From the proteome analysis of 2DE, 15 protein spots were expressed with intensities $\geq$ 2-fold change at $p < 0.05$ compared to control. The matrix-assisted laser desorption/ionization time-of-flight/time-of-flight tandem mass spectrometry and peptide mass fingerprinting analysis has been identified eight differently expressed protein, where one of which was upregulated and the rest were downregulated. The annexin A1 and annexin A4 proteins were downregulated whereas the 14-3-3ε protein was upregulated. These proteins were involved in signal transduction. There were downregulated in cytoskeletal proteins (cofilin-1, cytokeratin 8 and cytokeratin 79) and molecular chaperones proteins (endoplasmin) together with elongation factor Ts, which is the protein involved in protein metabolism. Most of the proteins were involved in tumor growth, cell cycle, apoptosis, migration and signal transductions. Thus, they suggested that the proteomic findings contributed the information of molecular mechanism underlying the anti-cancer effects of A549 cells treated with FCP.

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Introduction

This chapter will be discussed about the materials, chemicals, reagents and methodology used in this study. This research has been divided into two major features to validate the potential of *P. minima* extracts. The methodology have been divided into two part; **Part I**: Anticancer study and **Part II**: Phytochemical screening. This study was conducted in Molecular Biology Laboratory, Integrative Medical Cluster, Advanced Medical and Dental Institute, Universiti Sains Malaysia. **Table 3.1** shows the chemicals and reagents, while the tools and apparatus used in this study are listed in **Table 3.2.**

Table 3.1 List of chemicals and reagents

Study	**Chemicals and reagents**	**Supplier**
Anti-cancer	Acrylamide (30% w/v)	Bio-Rad, USA
	Ammonium persulphate (AP)	Bio-Rad, USA
	Annexin V-FITC Kit	Miltenyi Biotec, Germany
	Beta-mercaptoethanol / 2-Mercaptoethanol (β-ME)	Sigma-Aldrich, USA
	Bradford 1X Dye	Bio-Rad, USA
	Bromophenol blue	Bio-Rad, USA
	CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS)	Promega, USA
	Chloroform	Merk, Germany
	Dichloromethane	Merk, Germany
	Dimethyl sulphoxide (DMSO)	Merk, Germany
	Dulbecco Modified Eagle's Medium (DMEM)	GIBCO, BRL, UK
	ECL™ Prime Western Blotting Detection Reagent	Bio-Rad, USA
	Epidermal Growth Factor (EGF)	GIBCO, BRL, UK

Anti-cancer	Ethanol	Merk, Germany
	Glycerol	Amresco, USA
	Glycine	Vivantis, USA
	Goat polyclonal Secondary Antibody to Rabbit IgG – H&L (HRP)	Cell Signaling Technology, Inc.
	Heat-inactivated fetal bovine serum (FBS)	GIBCO, BRL, UK
	Hexane	Merk, Germany
	Horse Serum	GIBCO, BRL, UK
	Hydrochloric acid	Merk, Germany
	Hydrocortisone	GIBCO, BRL, UK
	Insulin	GIBCO, BRL, UK
	Methanol	Merk, Germany
	n-Butanol	Merk, Germany
	N,N,N',N'-tetramethylethylenediamine (TEMED)	Bio-Rad, USA
	Penicillin/streptomycin stock solution (PenStrap)	GIBCO, BRL, UK
	Phosphate-buffered saline (PBS)	Amresco, Australia
	Precision Plus Protein™ All Blue Prestained Protein Standards	Bio-Rad, USA
	Protease inhibitor cocktail	Bio-Rad, USA
	Potassium chloride	Sigma-Aldrich, USA
	Rabbit anti-human β-actin polyclonal IgG	Abcam, UK
	Rabbit anti-human Bax polyclonal IgG	Abcam, UK
	Rabbit anti-human caspase-8 polyclonal IgG	Abcam, UK
	Rabbit anti-human cytochrome c polyclonal IgG	Abcam, UK
	Rabbit anti-human p53 polyclonal IgG	Abcam, UK
	RIPA Buffer	Bio-Rad, USA
	Roselle's Park Memorial Institute Media (RPMI-1640)	GIBCO, BRL, UK
	Skimmed milk powder	SunLac, Malaysia
	Sodium chloride	Sigma-Aldrich, USA
	Sodium dodecyl sulphate powder	Bio-Rad, USA
	Tamoxifen Citrate Salt	Nacalai tesque, Japan
	Tris base	Vivantis, USA
	Trypan blue	Sigma-Aldrich, USA
	Trypsin (0.25%, w/v)/EDTA	GIBCO, BRL, UK
	Tween-20	Amresco, USA
	Quick Stat Bovine Serum (BSA)	Bio-Rad, USA
	1.0 M Stacking gel buffer	Bio-Rad, USA
	1.5 M Resolving gel buffer	Bio-Rad, USA
	30% (w/v) Acrylamide/Bis solution	Bio-Rad, USA
	Acetonitrile	Merk, Germany
	Aluminium chloride	Acros Organics, USA

Phytochemicals Screening	Folin	Merk, Germany
	Formic acid	Merk, Germany
	Gallic acid	Acros Organics, USA
	Potassium persulfate	Sigma-Aldrich, USA
	Quercetin	Acros Organics, USA
	Sodium carbonate	Sigma-Aldrich, USA
	Trolox	Sigma-Aldrich, USA
	2,2'-Azinobis(3-ethylbenzothiazoline-6-sulphonic Acid) ABTS	Sigma-Aldrich, USA
	2,2-Diphenyl-1-picrylhydrazyl (DPPH)	Sigma-Aldrich, USA

Table 3.2 List of tools and apparatus

Tools and apparatus	Supplier
Analytical balances	Sartorius, Germany
Automated cell counter	Bio-Rad, USA
Agarose gel electrophoresis apparatus	Bio-Rad, USA
Belly dancer	Stovall Life Science, Greensboro
Biomate spectrophotometer	Thermo Fisher Scientific, USA
Biosafety Cabinet Class II	ESCO Global, Singapore
Blender	Panasonic
Centrifuge tube (15 mL and 50 mL)	Thermo Fisher Scientific, USA
Cell scrapper	Cell Biologic, Chicago
Chamber slides	Bio-Rad, USA
Cryogenic vials and Cryoboxes	Nalgene, USA
Culture flasks and dishes	Nunc, Denmark
Filter paper	Bio-Rad, USA
Filter paper (Whatman®)	Schleicher & Schuell, USA
Flow cytometer (FACS Calibur)	Becton Dickinson, USA
Freeze Dryer (GENEVAC LTO, EZ 2.3 ELITE)	SP Scientific
Gas chromatograph (GC)7890A	Agilent, USA
Heraeus Megafuge 16 Centrifuge	Thermo Fisher Scientific, USA
Humidified CO_2 incubator	Thermo Fisher Scientific, USA
Inverted microscope CKX41	OLYMPUS, USA
Ice flakes machine	SASTEC, Malaysia
LC-TOF-MS machine 2795	Waters, USA
Mass spectrometer system (MS) 5973 inert MSD	Agilent, USA
Microcentrifuge (Minispin plus)	Hitachi, Japan
Microplate Reader	BMG LABTECH, Germany
Molecular Imager VersaDoc ™ MP 4000	Bio-Rad, USA
Mechanical pipette	BD Falcon™, USA
Micropipette tips (1 μl, 10 μl, 200 μl and 1000 μl)	Axygen® Scientific, USA

Polyvinylidene fluoride transfer (PVDF) membrane	Axon Scientific, Malaysia
Refrigerator Haier	Haier America
Rotary Evaporator (EYELA, N1100)	Tokyo Rikakikai Co., LTD.
Semi-dry Transfer Cell (Transblot® SD)	Bio-Rad, USA
Serological pipettes (10 mL and 25 mL)	Nunc, Denmark
Shaker (Orbitos)	Infors HT, Switzerland
Syringe	Terumo Medical Corporation
Syringe membrane filter (0.22 μm)	Jet Bio-Filtration, China
Water bath	Jeio Tech Co., Ltd., South Korea
Western blot (PowerPac™ Basic)	Bio-Rad, USA
24-well culture plates	Nunc, Denmark
3 Systems Glass Plates (Mini PROTEAN®)	Bio-Rad, USA
96-well microtiter plates	Nunc, Denmark

3.2 Preparation of 80% MeOH *Physalis minima* leaves extract

P. minima L. was harvested from Kepala Batas, identified and washed with distilled water. The plant was submitted to the USM Herbarium for species identification through morphological taxonomy by Dr Rahmad Zakaria with voucher number 11723 (**APPENDIX 1**). A 200 g weight of leaves were ground finely with 300 mL of distilled water using a mechanical grinder. A 1200 mL of methanol (MeOH) was added, shaken with shaker at 250 rpm and filtered with Whatman #1 filter paper for every 24 hours within three days. Then, the solvent was removed by using the Rotary Evaporator at 140° hPa; 60°C; speed 5 (**APPENDIX 2**) and subjected to freeze dry for a week (**APPENDIX 3**) to obtain dry form of *P. minima* extract. The dried crude 80% MeOH extract (C-MeOH) obtained was weighed using an analytical balances and stored at -20°C until use.