CYTOTOXIC EFFECTS OF *Phyllanthus watsonii* AIRY SHAW EXTRACT IN COMBINATION WITH CISPLATIN ON HUMAN OVARIAN CANCER CELL

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ABSTRACT

Ovarian cancer is the major gynaecological cancer that causes female mortality worldwide and its treatment involved chemotherapy drug of platinum based-drugs. The disadvantages of these treatments are patients experience severe side effects and drug resistance. Drug combination therapy is the gold standard treatment for cancer management with objectives to encounter resistance and decreasing or increasing doses safely without sacrificing efficacy. One such combination is between chemotherapy drug and plant extract. *Phyllanthus watsonii* Airy Shaw, an endemic plant of Peninsular Malaysia exhibited good anticancer effect on various human cancer cell lines. This study is aimed to investigate the cytotoxicity and possible interaction (additive, synergistic or antagonist) of *P. watsonii* and cisplatin (Pt) combination on human ovarian SKOV-3 cancer cell. *P. watsonii* ethyl acetate extract (PW-E) was extracted from the leafy part using a series of solvent extraction and the chemical profile was analysed by LC-MS/MS system. Cytotoxicity of PW-E, Pt and PW-E/Pt combination on SKOV-3 cells were determined using the Neutral Red Uptake assay and the inhibitory concentrations (IC_{50}) of single and combined dosage, Combination Index (CI) and other parameters were analysed using CompuSyn 1.0 software. Cell death mechanism triggered by the single and combination dosage on SKOV-3 cells was determined by morphological imaging under phase-contrast microscope, AO/EB double staining and caspase-3 activation assay. LC-MS/MS analysis of PW-E revealed the presence of gallic acid, strictinin, strictinin isomer, galloyl HHDP hexoside, hyperin, ellagic acid, kaempferol glucoside, kaempferol rhamnoside and quercetin. Cytotoxic effects of PW-E, Pt and PW-E/Pt combination were observed in dose-dependent manner after 72 hours incubation with IC_{50}, 1.78 ± 0.32 µg/ml, 0.69 ± 0.11 µg/ml and 1.34 ± 0.16 µg/ml

(P<0.05) respectively. The combination was considered highly selective towards cancer cell as compared to normal lung fibroblast MRC-5 cells based on the selectivity index (SI>3). CI analysis revealed two important data; (i) synergistic effect (CI<1) was observed at IC_{50}, while at higher doses of the combination exert antagonist effect (CI>1); and (ii) Drug Reduction Index (DRI) revealed a reduction of the extract to about 1.64-fold (1.07 µg/ml) and drug, 2.56-fold (0.27 µg/ml) in combination to achieve similar 50% inhibition as individual treatment, indicating reduction of dosage of both the extract and drug while maintaining efficacy. Morphological examination under phase-contrast of SKOV-3 cells showed typical apoptotic changes i.e, membrane blebbing, cell shrinkage and apoptotic bodies and were enhanced in cells underwent PW-E/Pt combination treatment. Staining with AO/EB revealed condensed and fragmented chromatin more clearly. Caspase-3 activation was increased in Pt, PW-E and PW-E/Pt combination with increment of 38%, 13% and 31% respectively which indicated that caspase-3 may involve in SKOV-3 cell apoptosis. However, the data is not significant (p>0.05). In conclusion, the present study revealed, PW-E/Pt combination exert cytotoxic effect on SKOV-3 cells via doses reduction and with possible synergistic interaction and mediated by apoptotic pathway.

KESAN SITOTOKSIK EKSTRAK *Phyllanthus watsonii* AIRY SHAW DALAM KOMBINASI BERSAMA CISPLATIN TERHADAP SEL KANSER OVARI MANUSIA

ABSTRAK

Kanser ovari adalah kanser ginekologi utama yang menyebabkan kematian wanita di seluruh dunia dan rawatannya melibatkan kombinasi dadah kemoterapi berasaskan platinum. Kelemahan rawatan ini adalah pesakit mengalami kesan sampingan yang teruk dan kerintangan dadah. Terapi kombinasi dadah adalah rawatan dasar pengurusan kanser dengan objektif untuk mengatasi kerintangan serta mengurangkan atau meningkatkan dos selamat tanpa mengorbankan keberkesanannya. Contoh kombinasi tersebut adalah gabungan antara dadah kemoterapi dan ekstrak tumbuhan. *Phyllanthus watsonii* Airy Shaw, tumbuhan endemik di Semenanjung Malaysia yang terbukti mempunyai kesan anti-kanser pada pelbagai sel kanser manusia. Oleh itu, kajian ini adalah untuk menyiasat sitotoksiksiti dan interaksi yang mungkin (aditif, sinergi atau antagonis) oleh gabungan *P. watsonii* dan cisplatin ke atas sel kanser ovari SKOV-3 manusia. Ekstrak *P. watsonii* etil asetat (PW-E) telah diekstrak daripada daunnya menggunakan kaedah pengekstrakan pelarut bersiri dan profil kimia dianalisis menggunakan sistem LC-MS/MS. Seterusnya, kajian sitotoksik PW-E, cisplatin (Pt) dan kombinasi PW-E/Pt pada sel SKOV-3 ditentukan dengan menggunakan kaedah pengambilan "Neutral Red" dan kepekatan perencatan (IC_{50}) dos individu, dos kombinasi, Index Kombinasi (CI) dan parameter lain dikira menggunakan perisian CompuSyn 1.0. Mekanisma kematian sel SKOV-3 yang dicetuskan oleh dos individu dan kombinasi ditentukan dengan pengimejan morfologi di bawah mikroskop fasa kontras, pewarnaan berganda AO/EB dan asei aktiviti caspase-3. Analisa LC-MS/MS terhadap PW-E mendedahkan kehadiran asid gallik, strictinin, strictinin isomer, galloyl HHDP hexoside, hyperin, asid ellagik, kaempferol glucoside, kaempferol rhamnoside

dan quercetin. Sitotoksik terhadap PW-E, Pt dan kombinasi PW-E/Pt adalah didapati bergantung kepada dos selepas inkubasi selama 72 jam dengan IC_{50} masing-masing, 1.78 ± 0.32 μg/ml, 0.69 ± 0.11 μg/ml dan 1.34 ± 0.16 μg/ml ($P<0.05$). Kombinasi ini dianggap sangat selektif terhadap sel kanser berbanding sel fibroblas paru-paru normal MRC-5 berdasarkan indeks pemilihan ($SI>3$). Analisis CI mendedahkan dua data penting; (i) kesan sinergi ($CI<1$) diperhatikan di IC_{50}, manakala pada dos yang lebih tinggi kombinasi menunjukkan kesan antagonis ($CI>1$); dan (ii) Indeks Pengurangan Dadah (DRI) menunjukkan pengurangan ekstrak kepada kira-kira 1.64 kali ganda (1.07 μg/ml) dan dadah, 2.56 kali ganda (0.27 μg/ml) dalam kombinasi untuk mencapai 50% perencatan sama seperti rawatan individu, ini menunjukkan pengurangan dos ekstrak dan dadah disamping mengekalkan keberkesanannya. Pemeriksaan morfologi sel SKOV-3 menunjukkan perubahan tipikal apoptosis i.e membran *blebbing*, pengecutan sel dan jasad apoptotik yang dipertingkatkan dalam sel yang mendapat rawatan kombinasi PW-E/Pt, manakala pewarnaan dengan AO/EB menunjukkan kromatin terpekat dan berpecah. Pengaktifan caspase-3 didapati telah meningkat dalam Pt, PW-E dan kombinasi PW-E/Pt dengan kenaikan sebanyak 33%, 12% dan 28% masing-masing, menunjukkan bahawa caspase-3 berkemungkinan terlibat dalam apoptosis sel SKOV-3. Bagaimanapun data tidak signifikan ($p>0.05$). Kesimpulannya, kajian ini mendedahkan bahawa kombinasi di antara PW-E dan cisplatin menghasilkan kesan sitotoksik pada sel kanser ovari SKOV-3 manusia melalui pengurangan dos dan kemungkinan interaksi sinergi serta perantaraan oleh laluan apoptotik.

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Farhana Raduan
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LIST OF SYMBOLS AND ABBREVIATIONS

List of Symbols;

A-549 : Human lung carcinoma cell line

dH_2O : Distilled water

D_m : Potency of dose-effect relationship

Fa : Effect level

Fa-CI : Combination index – effect level plot

HaCaT : Human keratinocytes cell line

HT-29 : Human colorectal adenocarcinoma cell line

HEPG-2 : Human hepatoma cell line

Huh-7 : Human hepatocarcinoma cell line

MeOH : Methanol

MeWo : Human melanoma cell line

MRC-5 : Human normal lung fibroblast cell line

Na_2SO_4 : Sodium sulphate

PC-3 : Human prostate cancer cell line

Pt : Cisplatin

r : Conformity of rule

Sf : Sensitization factor

SKOV-3 : Human ovarian cancer cell line

List of Abbreviations;

AO	: Acridine orange
CI	: Combination Index
DMEM	: Dulbecco's Modified Eagle Media
DNA	: Deoxyribonucleic acid
DRI	: Drug Reduction Index
EB	: Ethidium bromide
IC	: Inhibitory concentration
LC-MS/MS	: Liquid Chromatography Tandem Mass Spectrometry
NER	: Nucleotide excision repair
NR	: Neutral red
NRU	: Neutral red uptake
PBS	: Phosphate buffer saline
PW	: *Phyllanthus watsonii*
PW-E	: *Phyllanthus watsonii* ethyl acetate extract
PW-H	: *Phyllanthus watsonii* hexane extract
PW-M	: *Phyllanthus watsonii* methanol extract
ROS	: Reactive oxygen species
SI	: Selectivity index
UHPLC	: Ultra high performance liquid chromatography

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CHAPTER 1: INTRODUCTION

1.1 Research Background

Nature has been a source of therapeutic agents for thousands of year and a large number of modern drugs have been derived from natural sources. An example of top selling drugs derived from plant-derived natural product are vincristine from *Vinca rosea* L.*,* morphine from *Papaver somniferum* L., and Taxol® from *Taxus brevifolia* hort. ex Gordon. In the last 20 years, more than 25% of drugs used isolated directly from plant and another 25% are chemically altered natural products (Amin *et al*., 2009).

The concept of combining two or more plant constituents with chemotherapeutic drug(s) to maximize the desire therapeutic effect of both plant constituents and the synthetic drug, has become the new focus in drug discovery research. With fewer side effects as compared to synthetic drugs, plant extracts are enriched with phenolic compounds, flavonoids and other phytochemicals that play an important role as anticancer agents (Amin *et al*., 2009). Combination therapy is currently become the standard treatment for most of advanced cancers. Its main target is to avoid resistance of cancer cell towards chemotherapeutic drugs. The usage of multiple drugs may have multiple targets at molecular level simultaneously because of their wider range of mechanism and possible synergistic interaction. Therefore, drugs-extracts combination with prominent benefits has become the leading choice for cancer treatment for decades (Chou, 2010).

Phyllanthus watsonii Airy Shaw belongs to the family Phyllanthaceae is an endemic species grows on the banks of Endau River, Endau-Rompin National Park, Johor, Malaysia (Kochummen, 1998). Many literatures reported that *Phyllanthus* species had been traditionally used for a long period time in China, India, Brazil and Southeast Asia region for the treatment of digestive disorder, genitourinary, respiratory

problem, skin diseases, hepatitis B, hypertension, jaundice, renal calculus and even malaria (Mao *et al*., 2016). Scientific investigation revealed that extract of *P. watsonii* exhibited high antioxidant activity (Daud, 2006) and possessed remarkable anticancer activity on human gynaecologic and colorectal cancer (Ramasamy *et al*., 2012), breast cancer (Ramasamy *et al*., 2013), prostate cancer (Tang *et al*., 2013) and lung cancer cell lines (Lee *et al*., 2011).

Besides possessed a remarkable cytotoxicity on various human cancer cell lines of different origin, *P. watsonii* shows a high selectivity towards the cancerous cells than the normal cell line (Ramasamy *et al*., 2012; Ramasamy *et al*., 2013). The collective merit of this plant coupled with potential extract/drug combination advantages in cancer management, sparked our interest of the combination of *P. watsonii* extract with chemotherapy drug, cisplatin.

Cisplatin is platinum based drug used as chemotherapy agent against ovarian cancer and other types of cancer including bladder, head, neck and testicular cancer. Cisplatin is a DNA alkylating agent, promotes DNA damages and ultimately triggered the cancer cell death through apoptosis (Siddik, 2003). It is effective against many advanced cancers with dose-limiting side effect such as kidney and neuron damages. Another disadvantage of cisplatin is that, its high resistant rate during chemotherapy among patient who experienced cancer recurrence (Giaccone, 2000).

1.2 Research Objectives

1.2.1 General Objective

Therefore, the present study was carried out to investigate the cytotoxic effects of *Phyllanthus watsonii* Airy Shaw extract in combination with chemotherapeutic drug, cisplatin on human ovarian SKOV-3 cancer cell line and uncover potential mechanisms by which extract of *P. watsonii* regulates and triggered cell death through apoptosis.

1.2.2 Specific Objectives

The specific objectives of the present study were stated as follow:

i. to determine the cytotoxic effect of *P. watsonii* ethyl acetate extract and cisplatin on human ovarian SKOV-3 cancer cell line;
ii. to evaluate potential synergistic, additive or antagonistic effect between combination of *P. watsonii* ethyl acetate extract with cisplatin in their cytotoxicity based on median-effect principle on SKOV-3 cell line; and
iii. to determine the potential mechanisms by which the combination of *P. watsonii* ethyl acetate extract and cisplatin regulates cell death through apoptosis.

CHAPTER 2: LITERATURE REVIEW

2.1 Ovarian Cancer

Ovarian cancer has the highest mortality of all gynaecological malignancy among women. Statistical data from The American Cancer Society stated in 2015, approximately 21,290 women had been diagnosed with ovarian carcinoma in the United States and about 14,180 will die from the disease (www.cancer.org). The lifetime risk of ovarian cancer in the developed world is 1–2% with 75% of cases diagnosed above the age of 55 (Anagnostopoulos *et al.,* 2015).

In Malaysia, ovarian cancer contributed to 6.1% of all women cancer as according to Malaysian National Cancer Registry Report 2007－2011 (National Cancer Institute, 2016). The current treatments for ovarian cancer are debulking surgery and chemotherapy. Early stages ovarian cancer is beneficial to surgery as it provided 90% curative of all cases. While, the standard first line treatment regimen for later stages is the combination of surgery and intravenous administration of platinum based chemotherapy thrice weekly for six cycles (Anagnostopoulos *et al*., 2015).

Ovarian cancer begins at the ovaries and can be categorized into three types according to site of ovarian cells originated from. Epithelial ovarian cancer originated from the cell that covers the surface of the ovary. Its incidence contributes to 85–90% of all ovarian cancer. There are various forms of epithelial ovarian cancers of the ovary which are serous, endometrioid, clear cell, mucinous and undifferentiated or unclassifiable. Other types of rarer ovarian cancer are germ cell tumours and stromal tumours which originated from the cell that produce eggs and structural tissues cell that hold the ovaries in place, respectively. It usually contributed to about 2－7% of all ovarian tumour (Desai *et al*., 2014). Figure 2.1 illustrates various types and origin of ovarian cancer.

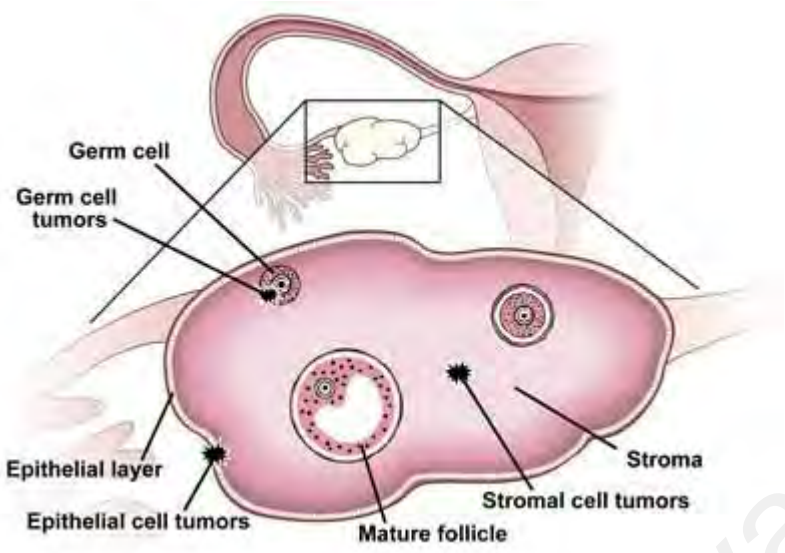

Figure 2.1: Types of ovarian cancer based on its origin (Algeciras-Schimnich, 2013).

2.1.1 Treatment of Ovarian Cancer

Platinum-based chemotherapy agents are class of chemotherapeutics drugs that contains derivatives of platinum. The platinum damages DNA of the cancer cells and stops them from dividing. Cisplatin is the oldest platinum-based drugs that was discovered and approved for cancer treatment followed by carboplatin and oxaliplatin. Apart from ovarian cancer, cisplatin is generally used against advanced, metastatic solid tumours such as colon cancer, breast cancer, endometrial cancer, melanoma, non-Hodgkin lymphoma, small cell lung cancer and testicular cancer (McWhinney *et al*., 2010). The usage of platinum plus taxane regimen (an antimicrotubule anticancer agents originally derived from *Taxus* species) has been the standard for ovarian cancer treatment for over a decade. One meta-analysis reported the inclusion of intraperitoneal (IP) cisplatin in front-line therapy of stage III ovarian cancer had improved patient survival (Hess *et al*., 2007).

Another platinum-based drugs, oxaliplatin (an anticancer nucleoside and an analogue of deoxycytidine), when combined with gemcitabine has high activity and acceptable toxicity in advanced ovarian carcinoma that was considered resistant to most cytotoxic drugs (Ray-Coquard *et al*., 2009). These evidences showed that platinum-based anti-neoplastic agents, either in single or combined dosage are essential in fighting against ovarian cancer.

2.1.2 Human Ovarian SKOV-3 Cancer Cell Line

Human ovarian SKOV-3 cancer cell is an adherent cell line that is used in this current research. SKOV-3 is an epithelial ovarian cancer cell in morphology but histologically is classified under serous type and originated from ascites of an ovarian cancer patient. The cells showed borderline sensitive to resistance towards platinum based treatment while highly sensitive toward taxanes and doxorubicin. It has mutation of the TP53 gene, which is often found in cancer cells (Beaufort *et al*., 2014).

2.2. Drug Combination Strategy in Cancer Treatment

Drug combination strategy is currently the standard treatment for most of advanced cancers and has become the leading choice for cancer treatment for decades (Chou, 2006). It is not only limited to cancer but also diseases such as AIDS, hypertension, malaria and other infectious disease benefited from this multidrug combination treatment (Wagner, 2011). The idea of using combinations in cancer therapy was started way back in the 1960s, prompted by the favourable outcome of combination therapy with antibiotic drugs (Boik, 2001). Its main target is to achieve synergistic drug interaction, avoiding and delaying resistance of cancer cell and to provide safer, more effective treatment.

Malignant cell population consists of mixed population of drug-sensitive cells and drug-resistance cells. As anticancer drugs kill the drug-sensitive cells, a portion of drug-resistance cells was left behind and thrived. When tumour recurrence occurs, the same chemotherapy regimen may not work the second time because the tumour is now resistant to the drug. The usage of multiple drugs will target multiple events at molecular level simultaneously and may effective against resistance cancer cells. Among the possible advantages of synergistic interaction in drug combination is reduced toxicity and lowering doses of drugs to be implemented safely without compromising efficacy (Chou, 2010).

Basically, synergism by definition is the interaction or cooperation of two or more agents or drugs that produced a combined effect more that the total effect of individual agent at separate event. Chou (2010) proposed quantitative definition of synergism (CI<1), defines synergism as more than additive effect and antagonism is less than additive effect. Until today, this definition of synergy is still debatable and has become one's personal preference.

2.3 Natural Product in Cancer Treatment

Today there are many chemotherapy drugs in market which derived from nature. It comes to the question of why the interest to natural compound in cancer treatment surfaced in the first place? Firstly, natural product-derived compounds are widely used in cancer treatment all around the world as part of the complementary and alternative medicine. As an example, in China, cancer treatment in Traditional Chinese Medicine (TCM) was discovered in a 3,500-year-old inscriptions (Liu *et al*., 2015). Secondly, several of *in vitro* and *in vivo* study shows evidence of anticancer potential of natural compounds to fit into the mechanism-based approach of cancer inhibition and lastly natural compound research approach ultimately contribute to a greater understanding of cancer and development of a successful treatment.

Even though a lot of investigations have been performed in development of treatment for cancer, more significant work and improvement is needed. Combined with the main disadvantages of synthetic drugs are the side effect that associated with them, natural compound have been the alternative therapy that 80－85% of global population depend on for health care needs (Ekor, 2014).

According to Boik (2001), there are seven strategies in which natural compounds can be utilized in inhibition of cancer cells. Those strategies are (i) reducing genetic instability, (ii) inhibition of abnormal expression of genes, (iii) inhibition of abnormal signal transduction, (iv) encourage normal cell to cell communication, (v) inhibition of tumour angiogenesis, (vi) inhibition of invasion and metastasis, and (viii) increasing the immune response.

Natural compounds were further classified into three groups according to their mechanism of action; (i) natural compounds that act directly on cancer cells by inhibiting cell proliferation (direct acting compound), (ii) those that inhibit the growth of cancer cells by affecting tissues of compounds outside cancer the cells (indirect

acting compounds) and, (iii) compounds that inhibit cancer cells through immune system stimulation (Boik, 2001).

2.3.1 Natural Product and Anticancer Drug Combination

With so many strategies for cancer inhibition, it is evidence that no single compound is the 'silver bullet' for cancer treatment and combination of multiple compounds is vital. Mother nature has provided us with so many natural bioactive compounds with anticancer potential such as anthocyanidins (red-blue flavonoid pigments found in berries), curcumin (active compound in turmeric), quercetin (flavonoid in many plants) and resveratrol (found in wine and grapes) (Prakash *et al*., 2013). The mild properties of natural compounds allow them to be used at large doses in combination to treat cancer and may able to target all seven cancer inhibition strategies, a role that a single compound could not perform.

Bioactive compounds can contribute to augmenting the efficacy of anticancer therapy through several means. However, according to Ro (1990), before any combination of a natural compound with a chemotherapy agent can be done, researcher must first consider what their aims to be achieved are. There are at least six different aims can be envisioned which are, (i) reducing the side effects of anticancer agents so that higher and more effective doses could be given safely; (ii) to increase drug accumulation or overcoming resistant in cancer cells; (iii) targeting for additive or synergistic anticancer effects; (iv) enhancing local delivery of chemotherapy drugs by modifying the tumour environment; (v) usage of concurrent immune stimulant drugs to enhance the antitumor effects of natural compounds; and (vi) adjuvant natural compound treatment to restore the integrity of immune system for self-cancer cell elimination after chemotherapy (Ro, 1990).

In a natural compound-anticancer agent combination, a bioactive compound that interferes with cell death signal could act additively or synergistically with the chemotherapy drugs to promote DNA damage in the cells. Several *in vivo* studies reported that the combination of natural compounds and chemotherapy drugs produced beneficial result in cancer reduction. For example, Gypenosides (Gyp), a major component from plant *Gynostemma pentaphyllum* (Thunb.) acts synergistically with a chemotherapy drug of 5-flourouracil (5-FU) in inhibition of colon cancer cell proliferation and tumour growth both in *in vitro* and *in vivo* models. Gyp has been shown to elevate intracellular Reactive Oxygen Species (ROS) level, and significantly enhanced 5-FU-triggered DNA damage response. The combination also revealed greater tumour mass and volume inhibition in mouse model (Kong *et al*., 2015). Kilic *et al*. (2015) reported that epigallocatechin-3-gallate (EGCG), a polyphenol extracted from green tea significantly improved cisplatin efficacy in cervical cancer cells inhibition and apoptotic induction. EGCG was shown to inhibit various proteins in signal transduction pathways, and as such enhanced the cells sensitivity to cisplatin. Combination of curcumin with 5-FU augmented the chemotherapy effects against colorectal cancer cells, with possible mechanism of transcription factor nuclear factor-κB (NF-κB) regulated gene expression inhibition (Shakibaei *et al*., 2013).

Numbers of these scientific evidences highlighted the potential of natural compounds to complement conventional chemotherapy and improving the performances and superiority of chemotherapy drugs in cancer treatment. The prospect that natural compounds itself are relatively safe and may synergistically act together with anticancer drugs is a merit in further investigation of potential of natural compound-chemotherapy drug combination in cancer treatment.

2.4 Cisplatin

Cis-diamminedichloroplatinum(II), dichlorodiammineplatinum, platinol, cisplatinum or widely known as cisplatin (Figure 2.2) is a platinum based anti-neoplastic drug that have been used for treatment against solid cancer such as bladder, head and neck, testicular, and ovarian cancer since its approval by Food and Drug Administration (FDA) in 1970s. The first of its class, cisplatin also used to treat various other types of cancer including carcinomas, sarcomas, lymphomas and germ cell tumour (Dasari & Tchounwou, 2014).

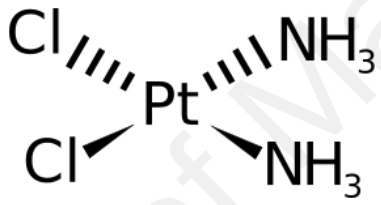

Figure 2.2: Skeletal structure of cisplatin (National Center for Biotechnology Information, 2017).

Cisplatin acts as anticancer agent by first underwent hydrolysis inside cancer cells. The 'aquated' cisplatin will crosslinking to DNA forming DNA adduct, and this lead to the disruption of DNA backbone and finally causes the whole structures of the cancer cells to bend. Eventually, the deformities will activate a series of signalling pathway to repair the damaged DNA, obstructing DNA replication, transcription and subsequently programmed cell death in the event of serious damage (Siddik, 2003).

2.4.1 History and Development of Cisplatin

Cisplatin was first discovered by Italian chemistry Michele Peyrone (1813－1883) in 1844 (Kauffman *et al*., 2010). Initially named after his name 'Peyrone's Chloride', cisplatin does not get significant important until 1965 when Barnett Rosenberg in his experiment accidentally discovered that bacterial (*Escherichia coli*) cell division was inhibited when subjected to an electrical field generated by platinum electrodes (Rosenberg *et al*., 1965). Instead of cellular division, the bacterial cell grew into long filament about 300 times from its normal size (Rosenberg *et al*., 1967).

Extensive research was performed thereafter in order to find out the causative agent for such incident and they identified that $PtII(NH_3)_2Cl_2$ produced by electrolysis of the previously thought inert platinum electrodes was the source (Rosenberg *et al*., 1967). Following the experiment with another group of bacteria it revealed that cisplatin have the ability to regressed large solid sarcoma in animals without obvious damage to the host (Rosenberg & VanCamp, 1970), demonstrating cisplatin potential as anticancer agent.

The first clinical trial of cisplatin on human was initiated by National Cancer Institute in 1970 (Kelland, 2007) and it was shown to be effective against testicular cancer (Rozencweig *et al*., 1977) and advanced ovarian carcinoma (Malpas, 1979; Rossof *et al*., 1979).

2.4.2 Cisplatin-Mechanism of Action

Cisplatin triggered the cell death via multiple signalling pathways beginning with influx into the cells, cytoplasmic activation, adduct formation and apoptosis signalling. The mechanism of action of these event is explained in many reviews elsewhere (Siddik, 2003, Dasari & Tchounwou, 2014;).

Researchers reported that the uptake of cisplatin into cell is regulated by copper membrane transporter 1 (Ctr1) (Lin *et al*., 2002; Holzer *et al*., 2004). Once inside the cytoplasm, the inert cisplatin must be activated in a series of hydrolysis event that displaced the cis-chloride groups in cisplatin with one or two molecules of water (Kelland, 2000). The reaction is facilitated by the low concentration of chloride ion in cytoplasm as compared to extracellular environment (Galluzzi *et al*., 2012). Activated cisplatin then will interact with many different molecules in order to exert its cytotoxicity effect. Figure 2.3 summarized cisplatin main cytotoxicity action.

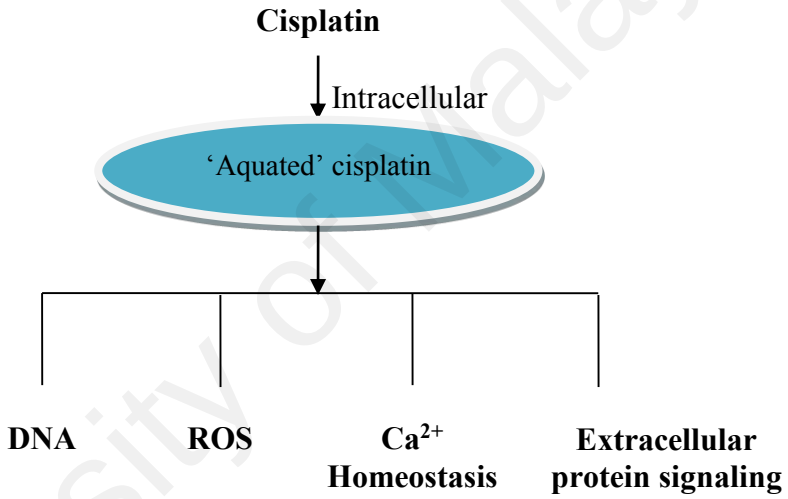

Figure 2.3: Cisplatin main cytotoxic action. DNA-deoxyribonucleic acid, ROS reactive oxygen species, p53-tumour suppressor protein p53.

The activated cisplatin is thousand times more reactive then the inert one. It is a potent electrophile that can react with any nucleophile including sulfhydryl groups on proteins and nitrogen donor atoms in nucleic acid (Dasari & Tchounwou, 2014). Activated cisplatin binds to DNA, at nucleophilic N7-sites of purine bases forming protein-DNA interaction or DNA-DNA inter-strand and intra-strand adduct, and this will activate a series of molecular cascade that determine cisplatin cytotoxicity (Dasari & Tchounwou, 2014). Figure 2.4 shows diagrammatic illustration of the intra-strand crosslink. The binding disrupted DNA conformation (bending the DNA) (Jordan & Carmo-Fonseca, 2000), preventing replication and normal transcription process. Cell

cycle arrest occurs when cisplatin-adduct induced damage is at low extent, allowing repair mechanism especially, nucleotides excision repair (NER) to remove cisplatin-adducts and re-established DNA integrity (Siddik, 2003; Hirakawa *et al.*, 2013). However, if the damages are beyond repairable threshold, apoptotic program pathway will be triggered instead.

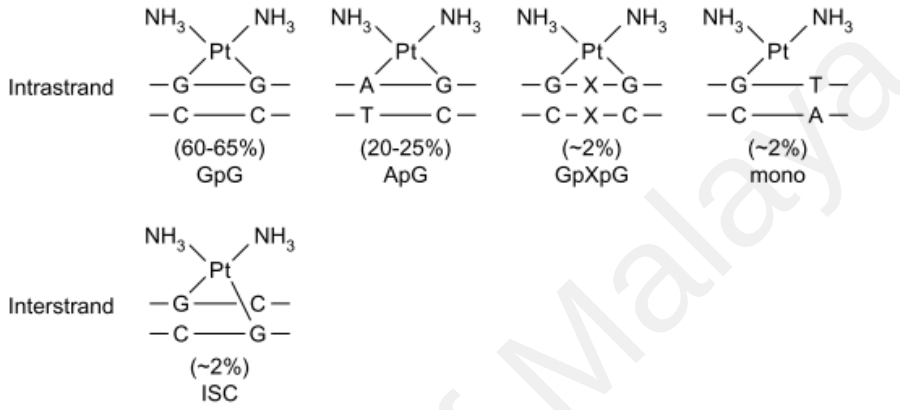

Figure 2.4: Binding of cisplatin adducts to DNA (Kelland, 2000)

Activated cisplatin (bi-aquated and mono-aquated species) disturbed the redox balance towards oxidative stress (which leads to apoptosis) (Siddik, 2003). It disturbed the mitochondrial membrane potential and is correlated with the production of mitochondrial reactive oxygen species (mROS) that influence multiple pathways. Cisplatin inhibited mitochondrial metabolic functions such as glycolysis and tricarboxylic acid cycle (TCA), where both are involved in cellular energy production. Such inhibition lowers ATP production, cell cycle arrest and ultimately programmed cell death (Choi *et al.*, 2015). This was proven that when N-acetylcysteine (NAC), a powerful antioxidant added to normal renal tubular HK-2 cells and exposed to cisplatin, there was significant reduction in ROS production generated by cisplatin toxicity (Choi *et al.*, 2015).

Intracellular calcium homoeostasis plays an important role in regulation and integrity of cellular function. Cisplatin disrupted calcium signalling by increasing the concentration of calcium ion (Ca^{2+}) from intracellular calcium stores such as endoplasmic reticulum into the cytoplasm. Variation in calcium concentration inside cells trigger important cellular process for example; regulation of metabolism, contraction of microfilament during mitosis, hormones and neurotransmitter secretion (Pinton *et al.*, 2008) and failure to sustain Ca^{2+} concentration could result in activation of programmed cell death (Florea & Büsselberg, 2009). Cisplatin has been shown to elevate calcium ion concentration in cytosol and mitochondria and induced apoptosis in recent study on human breast MCF-7 cancer cells (Al-Taweel *et al*., 2014) and human cervical HeLa cancer cells (Shen *et al*., 2016). High load of calcium leads to eventual overload in mitochondria and damages its structure. These leads to activation of signalling cascade such as protein kinase C, activation of calcium-dependent proteases, calpains (cleaves key elements in the apoptotic machinery) and activation of caspase protease family which will culminates to programmed cell death (Rizzuto *et al.*, 2003).

Cellular sensitivity to cisplatin not only regulated by its uptake, efflux or interaction with its primary target DNA but also, cellular responses to cisplatin-induced DNA damage. Although extensive DNA damage by cisplatin adduct binding trigger apoptosis, several signalling pathways, including Akt, protein kinase C (PKC) and mitogen activated protein kinase (MAPKs) can regulate cisplatin-induced apoptosis (Siddik, 2003). MAPKs are a family of structurally related serine/threonine protein kinases that coordinate various extracellular signals to regulate cell growth and survival (Chang & Karin, 2001). It consisted of many protein kinases but the one that mainly involved in cisplatin-induced apoptosis are extracellular signal regulated kinases (ERK), c-Jun N-terminal kinase (JNK) and p38 MAPK (Wang *et al.,* 2000; Johnson *et al.*, 2002; Losa *et al.*, 2003; Mansouri *et al.*, 2003; St. Germain *et al*., 2010; Guegan *et al.*,

2013). Meanwhile tumour protein p53 (TP53) is cisplatin-induced apoptosis via DNA damage signalling. Activated p53 exerts death signalling through cytoplasmic and nuclear mechanisms that culminates to outer mitochondria permeabilization or increased signalling via death receptors followed by cell death (Galluzzi *et al*., 2012).

2.4.3 Cisplatin and Treatment of Ovarian Cancer

Today, cisplatin is used widely in treatment of advanced ovarian cancer stage III and IV, while carboplatin (another platinum-based drug analogue) is used to treat early stages of ovarian carcinoma. Both platinum agents are also used in combination therapy with other anticancer drug such as taxane (Kwa & Muggia, 2014).

One of the earliest clinical trials on cisplatin efficacy to treat ovarian cancer was performed by Young and co-worker in 1979. Young *et al.* (1979) reported that out of 25 patients with advanced adenocarcinoma that resistant to other alkylating agents, 7 (29.0%) patient responded well to cisplatin treatment, with patient who responded survived longer than those who failed. Following that, several other clinical trials were performed to test cisplatin efficacy in treating ovarian cancer (Bruckner *et al.*, 1981; Gershenson *et al.,* 1981; Tsukamoto *et al*., 1982). At initial treatment, most ovarian cancer patient responded well to cisplatin and the respond was up to about 70%.

2.4.4 Cisplatin and Side Effects in Chemotherapy

As effective as cisplatin on many types of tumour, it possessed severe dose-limiting toxicity, mainly kidney and nerve damage, hearing loss, nausea and vomiting (Kelland, 2007). Nephrotoxicity (kidney injury) is one of the first cisplatin-based therapy side effect reported from clinical trials since its introduction over 30 years ago. Its severe toxicity to the kidney is dose-limiting, preventing high-dosage of cisplatin treatment and treatment withdrawal. Most of the nephrotoxicity is acute kidney injury

that happens to about 20－30% of patients (Miller *et al*., 2010). Pathogenesis of cisplatin-induced acute kidney injury could consist of proximal tubular injury, oxidative stress, inflammation and vascular injury to kidney cells (Ozkok & Edelstein, 2014). As kidney is the main organ to remove cisplatin from the body, they accumulated in kidney cells and metabolically activated to more potent toxin that damages the proximal tubule cells.

Another dose-limiting side effect of cisplatin is neurotoxicity (peripheral nerve damage). The cumulative dosage of cisplatin greater than 350 mg/m^2 is at risk of developing neurotoxicity (Park *et al*., 2013). Peripheral neurotoxicity develops in approximately 50% of patients receiving cisplatin treatment with sign and symptoms such as loss of vibration sense, loss of position sense, tingling sensation, and weakness involving the upper and lower extremities. Upon treatment discontinuation, neurological dysfunction may improve gradually or can be permanent (Amptoulach & Tsavaris, 2011).

2.4.5 Cisplatin and Resistance in Chemotherapy

After a long term chemotherapy, patients eventually developed platinum-resistance to cisplatin and also to other platinum-based chemotherapy drugs. This resulting in poor prognosis (Helm & States, 2009). Giaccone (2000) reported an increase in resistant to cisplatin after initial front-line chemotherapy is as high as 95%.

Cisplatin mechanism of resistance is a multifactorial event. No one factor alone could contribute to cisplatin resistance, since its mechanism of cytotoxicity are involving multiple pathways that works together to produce cellular apoptosis. In general, resistance to cisplatin can be divided into three groups, which are (i) factors that decreases the accumulation of cisplatin inside target cells, (ii) factors that limit the formation of DNA adducts, and (iii) increment in DNA repair mechanisms.

Reduction of intracellular accumulation of cisplatin in resistant cancer cells is one of the major mechanisms of cisplatin-resistance described in *in vitro* model (Gately & Howell, 1993). Factors that limiting cisplatin accumulation inside cells can be either inhibition of cisplatin uptake, increase cisplatin efflux, or both. It has been known that the copper transporter-1 (CTR-1) is responsible in regulating cisplatin and other platinum analogue influx into cells (Holzer *et al*., 2006). Thus, it was supported that cisplatin resistance cells showed decreased in cisplatin accumulation and lower CTR1 expression as compared to cisplatin sensitive cells (Kalayda *et al.,* 2012). Decreased cisplatin accumulation in cell also associated with increased efflux of cisplatin from cancer cells. Studies reported that MRP-2 (member of the Adenosine triphosphate-binding cassette, ABC family of plasma membrane transporters) (Borst *et al.*, 2000), participated in the removal of platinum drugs from cancer cells and an increased expression of MRP-2 is associated with cisplatin resistance (Korita *et al.*, 2010; Yamasaki *et al.*, 2011).

The second factor of cisplatin-resistance is caused by the inactivation of reactive cisplatin species by thiol component, thus limiting the formation of DNA adduct. Glutathione (GSH) is an enzyme that involved in GSH synthesis (γ-glutamylcysteine synthetase) is the most abundant with intracellular thiol, detoxifies toxins and drugs such as cisplatin. Therefore, elevated level of GSH involved in conjoining of cisplatin-GSH were observed to be associated with cisplatin resistance (Jansen *et al*., 2002; Chen *et al*., 2008; Chen & Kuo, 2010).

The third factor in cisplatin resistance mechanism is increment of DNA repair in cisplatin resistance cells. The key to cisplatin cytotoxicity is DNA damage promotion due to platinum-DNA adducts binding, cross-linking to DNA and in which lead to the activation of series of apoptotic pathway that eventually leads to cellular death. However, in cisplatin resistance cells, an increased in DNA repair intensity and

tolerance to DNA adducts binding adapted by the cells prevents the effectiveness of cisplatin and its platinum analogue. Platinum-DNA adducts is recognized by nucleotide excision repair (NER) component and the balance between DNA damage and DNA repair shall dictates the fate of the cells (Amable, 2016). In essence, the platinum-DNA adduct is recognized and incised on both side of the lesion before removal from DNA molecule. The process is followed by DNA synthesis and ligation to restore genetic integrity (Galluzzi *et al.*, 2012).

2.5 *Phyllanthus*

The plants classified under the genus *Phyllanthus* belongs to the family of Phyllanthaceae. *Phyllanthus* species are widely distributed throughout most tropical and subtropical countries such as Africa, Asia, Oceania and tropical America. *Phyllanthus* are mostly small and erect herbs that grow up to 30 to 40 cm in height. It can be divided into 11 sub-genera including, *Emblica, Cicca, Phyllanthodendron* and *Phyllanthus* (Calixto *et al*., 1998).

Phyllanthus has been utilized as herbal medicine for a long period in China, India, Brazil and Southeast Asia mainly for the treatment of digestive, genitourinary, respiratory, skin diseases, hepatitis B, hypertension, jaundice, renal calculus and even malaria. The top three species widely used as herbal medicine around the world are *P. emblica, P. reticulatus* and *P. niruri* (Mao *et al.*, 2016). In general, plants belongs to the Phyllanthaceae family are rich with polyphenol with wide biological activities such as rutin, quercetin, gallic acid and geraniin (Qi *et al*., 2014).

2.5.1 Biological Studies of *Phyllanthus* Species

Due to its significant medicinal benefit, it ignited interest in furthering research using *Phyllanthus* species in the form of extract, or purified single compound either in *in vitro* or *in vivo* model Several published works demonstrated its wide biological potential such as an antiviral, antioxidant, antidiabetic, antimalarial and anticancer (Mao *et al*., 2016). Table 2.1 summarized the biological activity of *Phyllanthus* species from various publications.

Extract from the aerial parts of *P. acidus* exhibited good anti-inflammatory activity both on *in vitro* and *in vivo* model (Hossen *et al*., 2015), while essential oil extracted from *P. muellerianus* stem bark was shown to demonstrate antimicrobial activities against multiple bacteria such as *Staphylococcus aureus, Streptococcus pyogenes* and *Escherichia coli* (Brusotti *et al*., 2012). Mediani *et al*. (2016) reported that *P. niruri* or locally known as "pokok dukung anak", effectively decreased serum glucose level and improved lipid profile in obese diabetic rats, exerting the possibilities of using the extract as potential anti-diabetic agent. Additionally, *P. niruri* spray-dried extract demonstrated anti-inflammatory activity in carrageenan-induced paw oedema albino rats by reducing the vascular response (Porto *et al*., 2013) and exerts antioxidant activity if ingested as tea in healthy adults as shown with increased in sera antioxidant markers (Colpo *et al.*, 2014). *P. urinaria* demonstrated potential as antiviral agent against herpes simplex virus (HSV) (Tan *et al*., 2013) and showed anti-hepatitis B virus (HBV) by inhibiting HBV DNA synthesis in drug resistant hepatitis B virus in *in vitro* model (Jung *et al*., 2015).

Previous study also reported that geraniin, compound isolated from *P. urinaria* extracts exhibited anti-hypertensive effects when given orally to hypertensive rats by lowering the systolic and diastolic blood pressure respectively (Lin *et al.,* 2008). Apart from that, extract of *P. amarus* was reported to exhibit antimalarial effects on mice

infected with *Plasmodium yoelii*, justifying its decades utilization in traditional medicine practice for the treatment of malaria (Ajala *et al.*, 2011). The mixture of aqueous and methanolic extracts of *P. amarus, P. urinaria, P. niruri and P. watsonii* have been shown to inhibit Dengue Virus Type 2 (DENV2) with more than 90% of virus reduction and active compounds of gallic acid, geraniin, syringin and corilagen were isolated and identified from all this species (Lee *et al.*, 2013).

Table 2.1: Summarized biological activity of *Phyllanthus* species.

***Phyllanthus* species**	**Biological Activity**	**Details**	**References**
P. acidus	Anti-inflammatory	Suppressing inflammatory marker such as inducible NO synthase and cyclooxygenase-2 in *in vitro* and *in vivo* model.	Hossen *et al.,* 2015
P. muellerianus	Antimicrobial	Tested against multiple bacteria such as *S. aureus, S. pyogenes* and *E. coli.*	Brusotti *et al.,* 2012
P. niruri	Antidiabetic	Effectively decreased serum glucose level and improved lipid profile in obese diabetic rat models.	Mediani *et al.,* 2016
P. niruri	Anti-inflammatory	Reducing the vascular response of carrageenan-induced paw edema albino rats.	Porto *et al.,* 2013
P. niruri	Antioxidant	Increased in sera antioxidant markers when ingested as tea in healthy adults.	Colpo *et al.,* 2014
P. urinaria	Antiviral	Inhibit HSV-1 & HSV-2 in Vero cells.	Tan *et al.,* 2013
P. urinaria	Antiviral	Inhibit HBV DNA synthesis in drug resistant hepatitis B virus in *in vitro* model.	Jung *et al.,* 2015
P. urinaria	Antihypertensive	Geraniin, isolated from its extracts exhibited anti-hypertensive effects when given orally to hypertensive rats.	Lin *et al.,* 2008
P. amarus	Antimalarial	The extract able to provide prophylactic effect and delaying infection against *P. yoelii* on infected mice infected model.	Ajala *et al.,* 2011
Cocktail of *P. amarus, P. urinaria, P. niruri, P. watsonii*	Antiviral	Inhibit DENV2 with more than 90% of virus reduction	Lee *et al.,* 2013

Table 2.1, continued.

P. amarus, *P. urinaria,* *P. niruri,* *P. watsonii*	Anticancer	Anti-metastatic on human lung A549 and breast MCF-7 cancer cell and induced apoptosis on melanoma MeWo and prostate PC-3 cancer cells.	Lee *et al.,* 2011
P. amarus, *P. urinaria,* *P. niruri,* *P. watsonii*	Anticancer	Promotes cell cycle arrest in melanoma MeWo cells and prostate PC-3 cells.	Tang *et al*., 2010
P. emblica	Anticancer	Exert cytotoxicity against human breast MCF-7 cancer cell line.	Luo *et al.,* 2011
P. emblica	Anticancer	Induction apoptosis of cervical SiHa and HeLa cancer cell lines.	Mahata *et al.,* 2013
P. niruri	Anticancer	Induced apoptosis and selectively cytotoxic towards human hepatocellular HEPG2 and Huh7 cancer cells.	de Araujo Jr. *et al.,* 2012

*information was compiled based on its order mentioned in text.

Another interesting biological potential of genus *Phyllanthus* is, they are invaluable natural resources with remarkable anticancer activities against cancer cell lines of different origin such as lung, liver, melanoma, leukaemia, prostate and breast (Tang *et al.,* 2014). Studies by Lee *et al.* (2011) shows a wide range of anti-metastatic effect of extract of *P. niruri, P. urinaria, P. watsonii* and *P. amarus* on human lung A549 and breast MCF-7 cancer cell lines and the extracts were able to induced the cell death through apoptosis in melanoma MeWo and prostate PC-3 cancer cells. The extracts also promotes cell cycle arrest at S phase for MeWo cells and at G0/G1 phase for PC-3 cells (Tang *et al.,* 2010). Phenolic compounds extracted from the fruits of *P. emblica* was shown to exert cytotoxicity against human breast MCF-7 cancer cell line (Luo *et al.*, 2011), while Mahata *et al.* (2013) reported that *P. emblica* induced apoptosis in cervical SiHa and HeLa cancer cell lines through inhibition of activator protein-1 (AP-1) activity and suppression of human papillomavirus (HPV) transcription

factor. Spray-dried extract of *P. niruri,* induced apoptosis and selectively cytotoxic towards human hepatocellular HEPG2 and Huh7 cancer cells, while exerting cytoprotective effect on non-cancerous keratinocytes HaCaT cells (de Araújo Jr *et al.,* 2012).

With more than 700 species of *Phyllanthus* (Mao *et al.*, 2016), more yet to be discover and the potential to explore and develop new and novel anti-tumour drugs from *Phyllanthus* species are limitless.

2.6 *Phyllanthus watsonii* Airy Shaw

Phyllanthus watsonii Airy Shaw (Figure 2.5) is a small shrub or herbs grow to about 100 cm in height that can be found near river bank. The plant is an endemic species of Peninsular Malaysia and commonly found on the banks of Endau River in Endau-Rompin National Park, Johor, Malaysia. The leaves of this plant have thread-like stalks, at least 5 mm long, while the flowers and fruits are in clusters (Figure 2.6), situated on main branches behind the leafy twigs (Daud, 2006).

Figure 2.5: *Phyllanthus watsonii* Airy Shaw

Figure 2.6: The thread-like stalk of the leafy parts of *P. watsonii*

2.6.1 *Phyllanthus watsonii* and Its Biological Activities

Limited scientific studies reported on the potency of *P. watsonii* in exerting various therapeutic benefits such as anti-viral, anti-diabetic and more importantly anticancer properties whether in *in vitro* or *in vivo* model when compared with other *Phyllanthus* species such as *Phyllanthus niruri* (de Araujo Jr *et al.,* 2012; Colpo *et al.,* 2014; Mediani *et al.*, 2016) and *Phyllanthus urinaria* (Lin *et al.,* 2008; Jung *et al.,* 2015). *P. watsonii* aqueous extracts had shown high cytotoxic effect on Vero cells infected with herpes simplex virus, HSV-1 and HSV-2, and this is the first reported work on the antiviral activity of *P. watsonii* (Tan *et al*., 2013). While in a more recent article, ethanolic and petroleum ether extracts of *P. watsonii* showed significant blood glucose lowering effects in type 2 diabetic rats (Rao *et al.,* 2016).

P. watsonii was shown to exert significant cytotoxic effect on skin melanoma (MeWo) and prostate cancer (PC-3) cells as compared to normal human cells (Tang *et al.*, 2010) and anti-metastatic effect on lung and breast cancer cells (Lee *et al.*, 2011).

Both studies were performed simultaneously with three other *Phyllanthus* species which are *P. amarus, P. niruri* and *P. urinaria*. Published work by Tang and colleagues was the earliest clinical investigation of *P. watsonii* cytoxicity on cancer cell lines using aqueous and methanolic extracts. Apart from that, more recent publication revealed, *P. watsonii* extracts was shown to demonstrated cytotoxicity on human ovarian SKOV-3 cells with IC_{50} of 8.51±0.50 µg/ml (extract prepared in methanol), 5.79±0.29 µg/ml (extract prepared in hexane) and 5.52±0.50 µg/ml (extract prepared in ethyl acetate) (Ramasamy *et al*., 2013).

2.7. Cell Death Mechanism

Cells that were triggered to die can undergo different morphological changes and fates including (i) apoptosis, (ii) autophagy (iii) necrosis and (iv) necroptosis. Apoptosis is one of programmed cell death; also termed type I cell death defined by the changes in nuclear morphology that include chromatin condensation and nuclear fragmentation. Apoptotic cells also showed changes in cell morphology including cell shrinkage, plasma membrane blebbing, loss of adhesion to neighbouring cells and the formation of apoptotic bodies (Ouyang *et al*., 2012).

Autophagy, also termed as type II cell death, is defined by the accumulation of autophagosomes, double-membrane vacuoles that encircling cytoplasmic materials. Autophagy often associated with survival mechanism cells adapted during starvation in order to prolonged the functionality of the whole organism (Tait *et al*., 2014).

Necrosis, also known as type III cell death, is characterized when the cell death lacks the characteristic of type I and type II process, distinguished by rupture of the plasma membrane accompany by subsequent immunological response (Schulze-Osthoff, 2008). Necrosis often occurs due to external stimuli that cause cells to die without proper regulated responses.

Another new classification of cell death is necroptosis. Necroptosis is programmed cell death version of necrosis (Tait *et al.*, 2014). Having morphological feature of necrosis with apoptosis mechanistically. However, unlike apoptosis, necroptosis is a caspase-independent and its process dependent on signalling by the receptor-interacting protein-kinase-3 (RIPK-3) complex (Linkermann, 2014). Cell death via necroptosis is often triggered by ligation of TNF1 and viral proteins (RNA & DNA viruses) (Hanson, 2016). Overall morphological difference of these different types of cell deaths is illustrated in Figure 2.7.

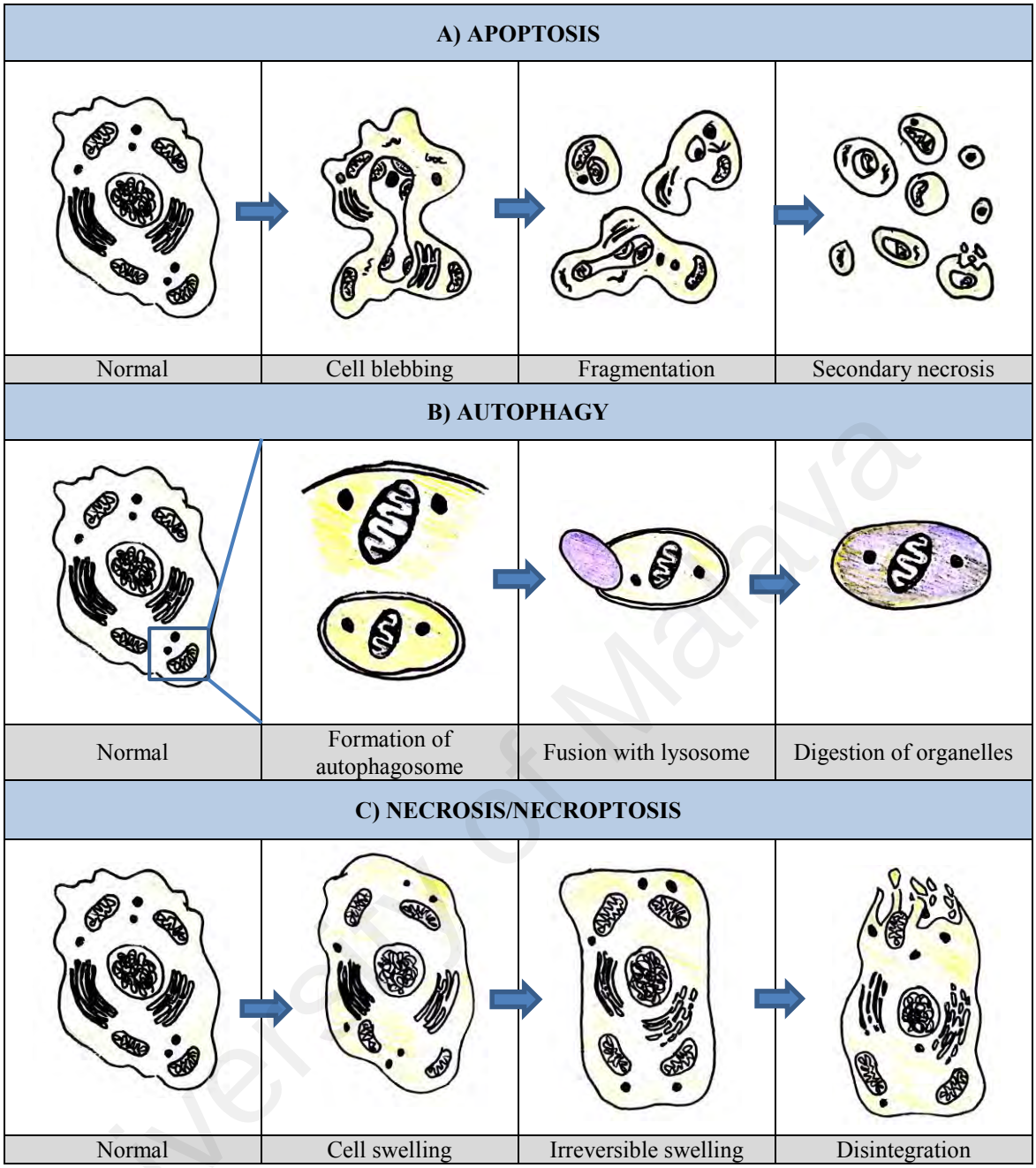

Figure 2.7: Cell death mechanism. A) Apoptotic cells revealed characteristic morphology of cell shrinkage and membrane blebbing, nuclear condensation and the formation of apoptotic bodies that each containing fragments of nuclei, genetic materials and organelles. B) Autophagy is characterized by the formation of autophagosomes, double-membrane vacuoles than enclosing cytoplasmic materials. It will fuse with a lysosome, initiating the degradation of its content and eventually cell death. C) Necrosis/Necroptosis is defined by the characteristic of cell swelling with undisturbed chromatin pattern, dilation of organelles and cell rupture. Affected cells release its content into the surrounding and triggered inflammatory responses (Illustration adapted from Schulze-Osthoff (2008)).

2.7.1 Features of Apoptosis

In normal regulated condition, apoptosis is the main cell death mechanism for removing unwanted and harmful cells in a clean and silent manner especially during embryonic development, immune regulation and overall tissue homoeostasis mechanism to control cell population (Schulze-Osthoff, 2008). It also occurs due to various DNA damaging factor such as chemotherapeutic drugs, hypoxia and ultraviolet radiations (Baig *et al.,* 2016).

Apoptosis process is started with chromatin condensation and nuclear fragmentation, followed by rounding up cell, decrease in cytoplasmic volume and pseudopods retraction. Chromatin condensation starts at the nuclear membrane periphery, forming a ring/crescent-like form. It further condenses until finally breaks up inside a cell with an intact plasma membrane. Late apoptosis shows more drastic morphological changes such as plasma membrane blebbing, loss of membrane integrity and organelles modification (Wong, 2011).

Apart from morphological changes, major biochemical changes also occur during apoptosis. In early apoptosis, the inner layer phosphatidylserine is translocated to the outer layers, making an early apoptotic signal 'eat-me' to the circulating phagocytes (Kerr *et al.,* 1972). Apoptotic cells *in vitro* will undergo a secondary apoptosis instead. Late apoptosis will mark the breakdown of DNA into large fragments and further smaller base pairs by endonucleases (Wong, 2011).

Another specific feature of apoptosis is the activation of a group of enzymes belonging to the family of protease enzymes known as caspases. Caspases are the demolition machinery that involved in both initiation and execution of apoptotic mechanism. They are so called caspases because the cysteine in their active site cleaves a target protein only after an aspartic acid residue. Caspases carries various other role that are not limited to cell death including proliferation, differentiation, inflammatory

reaction, neural development, tumour suppression and aging (Sollberger *et al.,* 2014). Caspases that play a role in apoptosis are called apoptotic caspases. Depending on the sequence they were recruited into the apoptotic pathway, apoptotic caspases are further classified into initiator and executioner caspases. The initiator caspases consisted of caspase-2, caspase-8, caspase-9, and caspase-10, while executioner caspases are caspase-3, caspase-6, and caspase-7 (Shalini *et al.,* 2015). In response to stress or apoptotic stimulus, the initiator caspases are the first to be activated. Once activated, the machinery produces a chain reaction by attacking and activating executor caspases and degrading over 400 cellular components in order to produce morphological changes for cell death (Fuentes-Prior *et al.,* 2004). These carefully orchestrated processes of apoptosis are usually occurring without the spillage of cytoplasmic contents therefore preventing the triggering of the inflammatory response. At such cases, DNA repair mechanism and cell cycle checkpoints will ensure that the integrity of the cell is preserved and cell underwent apoptosis if the damage is severe, however, should the mechanism is disturbed, those damages will pass onto the daughter cells and may initiate carcinogenesis (Baig *et al.*, 2016).

Even though, failure of the apoptosis process being one of the causes of most cancer formation, apoptosis plays an important role in cancer therapy as a strategy to exterminate tumour cells without damaging normal cells. Apoptosis studies become a part in development of cancer drug that targeted specific apoptosis pathway that existed in rapidly dividing tumour cells (Kasibhatla & Tseng, 2003).

2.7.2 Apoptosis Pathways

The two commonly described apoptosis pathway are (i) intrinsic mitochondrial pathway, and (ii) extrinsic death receptor pathway, both finality will lead to the execution phase of apoptosis (Figure 2.8). Intrinsic mitochondrial pathway, as its name implies start inside the cells in response to cellular upset such as hypoxia, high cytosolic calcium, oxidative stress and irreparable DNA damage, while death receptor pathway plays an important role in normal tissue homeostasis maintenance mainly immune system, begins when death ligands bind to death receptor (Danial & Korsmeyer, 2004). Regardless of any of the stimuli, they cause an increased in permeability of mitochondrial membrane and thus increased the release of pro-apoptotic factor cytochrome c into the cytoplasm (Wong, 2011). In normal condition, cytochrome c is the one that responsible in generating ATP in electron transport chain. However, in cell undergoing apoptosis, it acts as the cofactor for the adaptor protein Apaf-1 (Schulze-Osthoff, 2008). The binding of cytochrome c to the protein changes its structure and promoting the employment of caspase-9 to induce the formation of apoptosome. Apoptosome complex that consisted of cytochrome c, Apaf-1 and caspase-9 activates caspase-3 and subsequently triggers the apoptosis cascade (Wong, 2011).

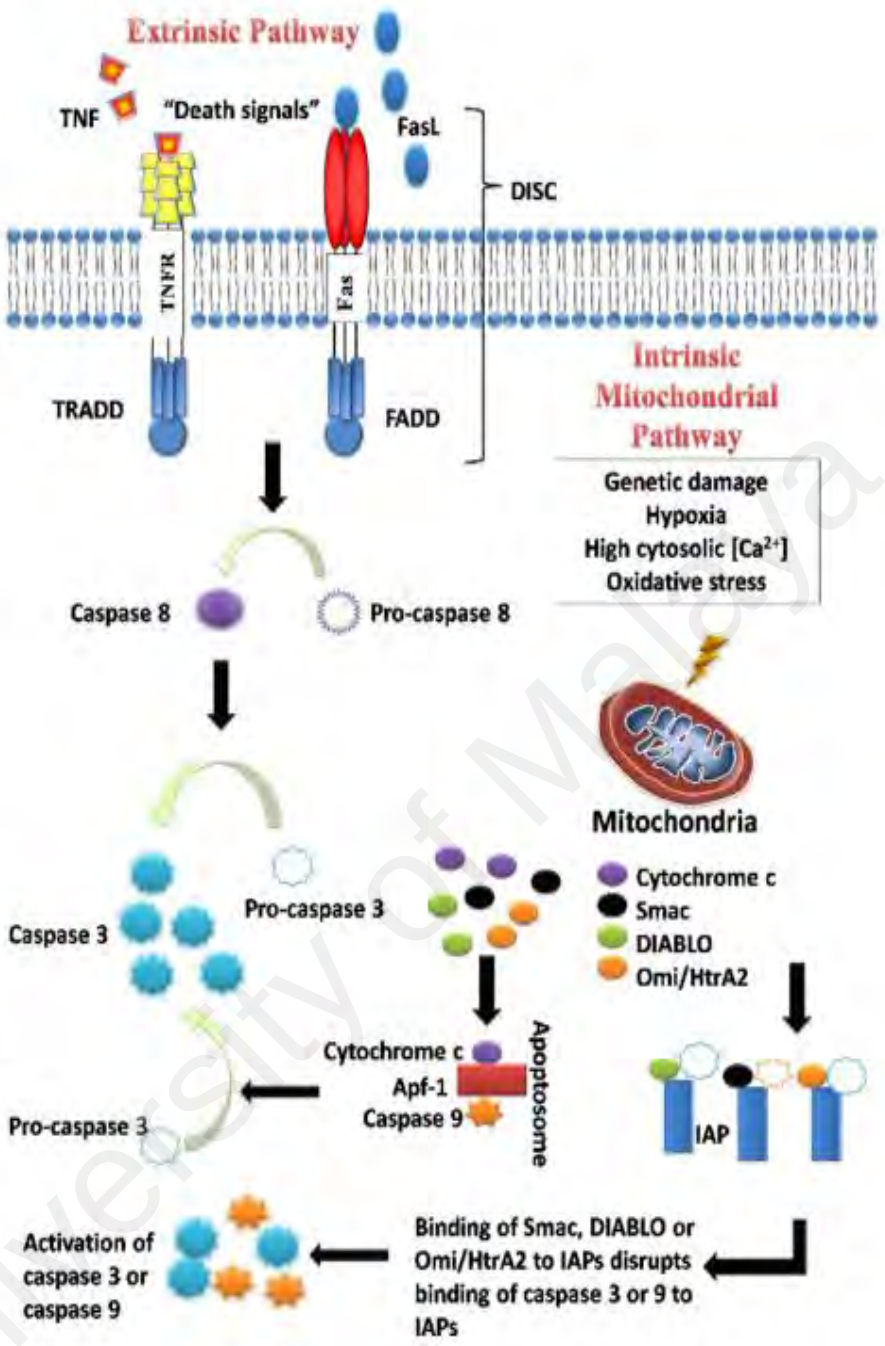

Figure 2.8: Extrinsic and intrinsic pathway of apoptosis (Wong, 2011)

In extrinsic death receptor pathway the most known death receptors are type 1 Tumour Necrosis Factor receptor (TNFR1) and a protein called FAS (CD95) with each of their ligands, TNF and Fas ligand (FasL) respectively (Elmore, 2007). Death receptors transmitted death signal from cell surface to intercellular signalling pathway and each of the receptors have their own intracellular death domain. Upon binding of

ligands onto the death receptors, the death domains form its binding site for adaptor protein recruitment and the entire complex (ligand-receptor-adaptor) is called death-inducing signalling complex (DISC) (Schneider & Tschopp, 2000). Several adaptor proteins are TNR-receptor associated death domain (TRADD), Fas-associated death domain (FADD) and cysteine proteases such as caspase-8 (Schneider & Tschopp, 2000). The death complex begins the activation of procaspase-8 to caspase-8, an initiator caspase which then cleaves executioner caspases, initiating apoptosis (Wong, 2011).

The final event of apoptosis is the execution phase begins with the activation of execution caspases-3, caspase-6 and caspase-7. Caspase-3 is considered the most important executioner caspase and it is activated by initiator caspase-8, caspase-9 or caspase-10 in both intrinsic and extrinsic pathways. Caspase-3 activates substrates for example endonuclease caspase-activated DNase (CAD) by cleaving inhibitor of caspase-activated DNase (ICAD) and in turn, the substrate degrade DNA, causes chromatin condensation, cytoskeletal reorganization (Fulda & Debatin, 2006) and cell disintegration as well as possible involvement in the relocation of phosphatidylserine from inner membrane to cell surface (Mandal *et al.*, 2005) to facilitate recognition and disposal by the phagocytes.

Both caspase activation pathway appears to induce apoptosis in different manner, however, it is not absolute and sometimes a divergent can occur. For example, in some cell types, activation of caspase-8 at DISC is insufficient to stimulate executor caspases activation and amplification with cytochrome c-dependent apoptosome formation is required. This evidence that, in some cases intrinsic and extrinsic pathway are linked together to produce apoptosis (Roy & Nicholson, 2000; Winter *et al*., 2014).

In addition, a number of other pathways have also been reported and reviewed elsewhere, including the intrinsic endoplasmic reticulum (ER) pathway; which was

believed to be caspase-12 dependent and mitochondria-dependent (Winter *et al.,* 2014). An adaptor protein TNF receptor associated 2 (TRAF2) will be cleaved from procaspase-12, activates it for the apoptotic cascade (Wong, 2011). However, unlike the well-established role of the death receptor and mitochondrial pathway, the physiological relevance on other apoptosis pathway remains controversial.

CHAPTER 3: MATERIALS & METHODS

3.1 Materials

3.1.1 Plant Materials

Phyllanthus watsonii leaves were collected from Endau Rompin National Park, Johor (Peninsular Malaysia). Authentication of the plant species was performed by Dr Sugumaran Manickam from Rimba Ilmu Botanical Garden, Institute of Biological Sciences, University of Malaya and voucher specimen (Reference no. KLU46068) was deposited at the same herbarium.

3.1.2 Cis-diamminedichloroplatinum(II) (Cisplatin)

Cisplatin (Sigma, USA) in powdered form diluted in 0.9% NaCl (Systerm, Malaysia) to yield a 2 mg/ml stock solution and kept at -20 ºC (Hall *et al.,* 2014). Stock solution was then diluted in culture media supplemented with 10% Fetal Bovine Serum (FBS) to desired working concentration to use in the assays.

3.1.3 Neutral Red Stock and Medium

Neutral Red (NR) (R&M Chemicals, UK) stock (4 mg/ml) was prepared by dissolving 0.04 g NR powder in 10 ml sterile distilled water. Stock solution was vortex and stored at 4 ºC. At the end of the incubation period of NR Uptake Assay, NR media (50 µg/ml) was prepared by diluting NR stock with culture media supplemented with 10% FBS.

3.1.4 Neutral Red Washing Solution

10% of calcium chloride (Sigma, USA) was dissolved in 89 ml of distilled water and 1 ml formaldehyde (Sigma, USA) and kept at 4 ºC.

3.1.5 Neutral Red Resorb Solution

Neutral Red Resorb Solution was prepared by mixing ethanol, distilled water and acetic acid at ratio 50:49:1 and kept at 4 ºC.

3.2 Extract Preparation

3.2.1 Preparation of Ethyl Acetate Extract of *P. watsonii*

Ethyl acetate extract was extracted from the leaves of *P. watsonii* according to the method described by Ramasamy *et al.* (2012). Selection of ethyl acetate extract of *P. watsonii* (PW-E) in this study was based on the reported work of the effectiveness cytotoxicity of PW-E against human ovarian SKOV-3 cancer cell line (Ramasamy *et al.,* 2012).

The leaves of *P. watsonii* were washed, sliced, dried at 40 °C, ground, and extracted three times with methanol at room temperature for 72 hours. The solvent-containing extract was filtered and the excess solvent was removed under reduced pressure using a rotary evaporator (Buchi, Switzerland) to produce dark-greenish methanol extract. The dried extract portion were added with hexane and shaken vigorously until the hexane appeared almost colourless. Chemical compounds that soluble in hexane were filtered and pooled, followed by concentration under reduced pressure by a rotary evaporator to yield hexane extract.

Hexane insoluble compound was then subjected to solvent–solvent extraction with a mixture of ethyl acetate and distilled water in a ratio of 1:1 followed by vigorous mixing. The mixture was fractionated in succession using a separating funnel to produce distinct layers. The residual water at bottom layer (water layer), was discarded while the ethyl acetate phase (top layer) released into a clean collection beaker followed by concentration under reduced pressure using a rotary evaporator to yield the PW-E. Figure 3.1 summarized the extraction step for PW-E. For bioassay test, PW-E was dissolved in dimethylsulfoxide (DMSO) (R&M Chemicals, UK) to yield a stock solution of 20 mg/ml and further diluted in 10% DMSO to obtain the desired working solution.

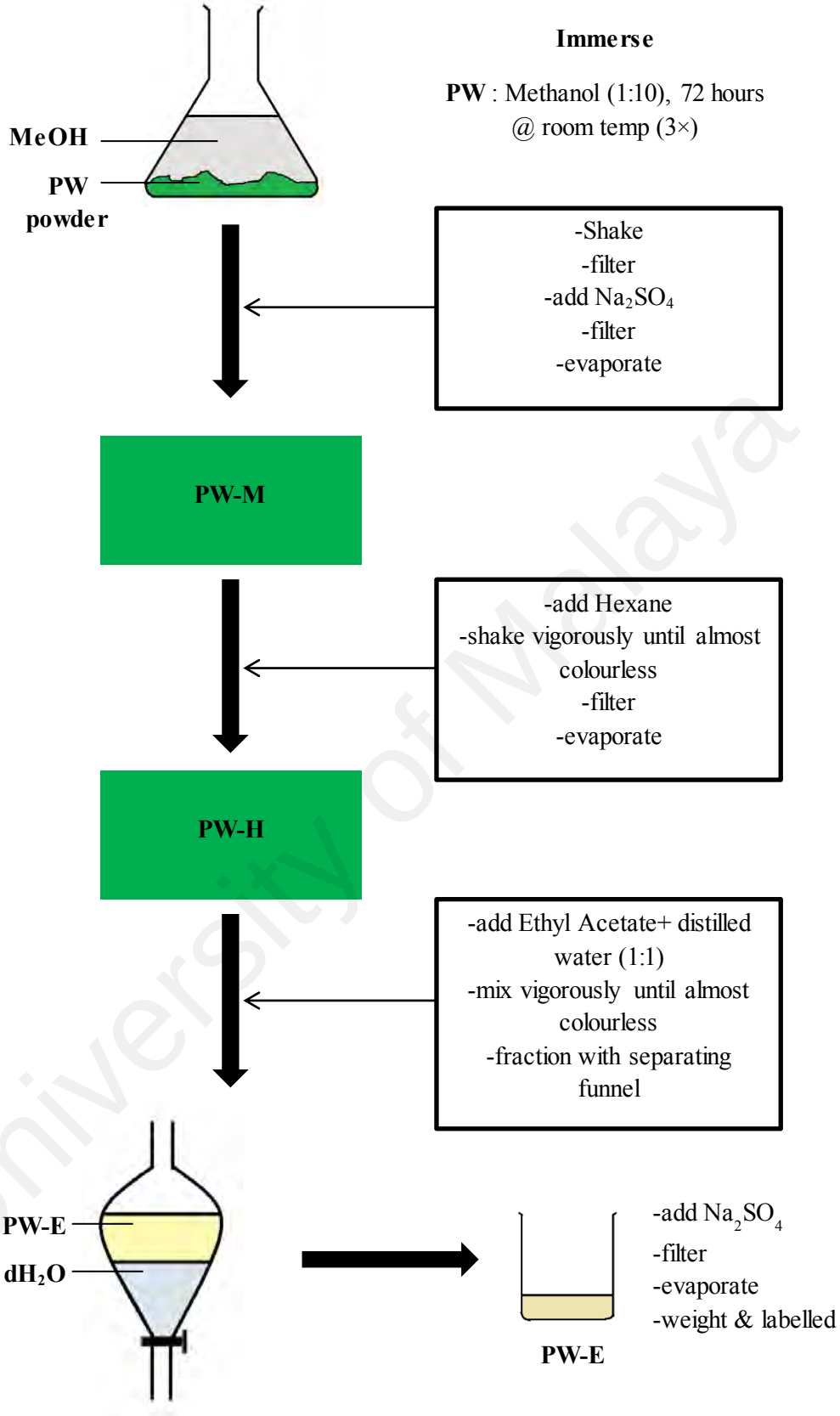

Figure 3.1: Illustration of the PW-E extraction steps. PW - *P. watsonii* powder; PW-M – *P. watsonii* methanolic extract; PW-H – *P.watsonii* hexane extract; PW-E – *P. watsonii* ethyl acetate extract.

3.2.2 Liquid Chromatography Mass Spectrometry (LC-MS/MS) Analysis

In order to determine the phytochemical compound(s) present in PW-E, the extract was analysed using LC-MS/MS system, 3200QTrap mass spectrophotometer (AB Sciex, USA) with Perkin Elmer FX15 UHPLC system installed. The chromatographic separation performed on a 150 mm × 4.6 mm × 5 µm Zrbax C18 column (Agilent, USA), eluted with a mobile phase consisting of water (A) and acetonitrile (B), both containing 0.1% formic acid and 5 mM ammonium formate. A gradient elution of different concentration (starting from 10% of A to 90% of B, from 0.01 minutes to 8.0 minutes, hold for 3 minutes and back to 10% of A in 0.1 minutes and re-equilibrated for 5 minutes) of A & B was used to separate the compounds of interest before to mass spectral analysis. The mass spectrometer analysis performed in a negative ion mode for detection of secondary compounds. Identities of the compounds were obtained by matching their molecular ions (m/z) with reference standards and also by correlation with previous published data on chemical component of *Phyllanthus* sp.

3.3 Cell Culture

The human ovarian SKOV-3 and normal lung fibroblast MRC-5 cell lines were purchased from American Type Culture Collection (ATCC, USA). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) and Minimum Essential Medium (MEM) (Sigma, USA) respectively. The culture medium was supplemented with 10% (v/v) fetal bovine serum (FBS) (TIC Europe). Cells were maintained in 25 cm^3 flasks (Nunc, Denmark) with 10 ml of DMEM media and incubated at 37 ºC in an incubator with 5% CO_2 in a humidified atmosphere. The cells were sub-cultured every 2 or 3 days and routinely checked under Leica DMI 3000 B phase contrast inverted microscope (Leica Microsystems, Germany) for any contamination.

Prior the assay, cells were detached from flask with 1.0 ml solution of accutase (iCT, CA) in phosphate buffer solution (PBS at pH 7.4). This was followed by centrifugation (Kubota, Japan) at 1,000 rpm for 5 minutes to obtained cell pellet. The numbers of the viable cells were counted by tryphan blue exclusion method using haemocytometer and adjusted to a desired concentration per well.

3.4 Neutral Red Uptake (NRU) Cytotoxicity Assay

Neutral Red Uptake (NRU) assay was performed to measure cytotoxicity effect of PW-E and the method was adapted from Ramasamy *et al.,* 2012. The NRU assay was based on the uptake of supravital dye neutral red, followed by its lysosomal accumulation in the viable and uninjured cells. The quantification of the dye extracted from the cells has shown to be directly proportional with the cell numbers (Repetto *et al.,* 2008). SKOV-3 cells were plated in 96-well micro titre plate (Orange Scientific, Belgium), at seeding density of 30,000 cells/ml followed-by incubation in a CO_2 incubator at 37 ºC for 4 hours to allow the cells to adhere before addition of the test agents. The seeding density for normal lung fibroblast MRC-5 cell was 100,000 cells/ml.

The experiments consisted of four groups: Group I: negative control-cells only without addition of any test agents; Group II: positive control - cancer cells treated with cisplatin (concentration of 0.3123, 0.625, 1.25, 2.5, 5 and 10 µg/ml); Group III: cancer cells treated with PW-E (concentration of 0.3123, 0.625, 1.25, 2.5, 5 and 10 µg/ml); Group IV: Combination of PW-E and cisplatin. Combination concentration ratio were determined to be 4:1 (PW-E: Pt) based on IC_{50} of individual treatments (Table 4.3), i.e PW-E+Pt (0.25+0.0625, 0.5+0.125, 1.0+0.25, 2.0+0.5, 4.0+1.0, 8.0+2.0 µg/ml).

At the end of the incubation period, the medium was replaced with NR medium and incubated for another 3 hours to allow for uptake of the vital dye into the lysosomes

of viable and uninjured cells. Then, the medium was removed and rapidly washed with the calcium chloride-formaldehyde mixture. NR resorb solution was added to each well to elute out the dye within viable cells. This followed by rapid agitation and optical density (OD) measurement at 540 nm using Multiskan Go micro-plate reader (Thermo Fisher Scientific, USA). The experiment was performed in three replicates. The red colour intensity and the absorbance readings are expected to be proportional to the number of viable cells within the wells. Percentage of SKOV-3 cells inhibition was calculated according to the following formula:

$$\% \text{ of inhibition} = \frac{\text{Absorbance of negative control - absorbance of treated cells}}{\text{Absorbance of negative control}} \times 100$$

3.4.1 Cytotoxicity (IC_{50}) Analysis

The percentage inhibitions were converted to effect level, Fa value (0－1.0) before being submitted into CompuSyn 1.0 (Chou & Martin, 2007) for further analysis. Potency, Dm (IC_{50}, μg/ml) values (concentration required to inhibit cells viability by 50% as compared to the negative control) for PW-E was obtained from antilog of x-intercept of the median-effect plot generated by CompuSyn 1.0 computerized stimulation. Based on the US National Cancer Institute guidelines, a crude extract is generally considered to have *in vitro* cytotoxic activity if the IC_{50} value in carcinoma cells, following incubation between 48 and 72 hours, is ≤ 20 μg/ml, while for a pure compound the IC_{50} value is ≤ 4 μg/ml (Boik, 2001).

3.4.2 Selectivity Index (SI) Analysis

The selectivity index (SI) was determined by the ratio between IC_{50} value of the extract on normal lung fibroblast MRC-5 cell and IC_{50} value of the extract on human ovarian SKOV-3 cell. Extract with SI value greater than 3 were considered to have a high selectivity towards cancerous cells (Bézivin *et al.*, 2003).

3.5 Combination Index (CI) Analysis

Cytotoxicity (IC_{50}) of the extract-drug combination was further analysed for CI analysis in order to assess its possible synergistic effect. The IC_{50} of each PW-E and cisplatin individually i.e 2.0 µg/ml and 0.5 µg/ml respectively were the benchmark for 4:1 constant combination ratio. CI analysis is determined by the Chou and Talalay equation, using CompuSyn 1.0 software (Chou, 2006; Chou & Martin, 2007).

In equation stated below, D1 and D2 are the doses of individual drugs in combination, i.e., the concentration of cisplatin and PW-E that gives 50% inhibition, Fa is the effect level and m is the slope. Result were interpreted as CI in which, CI>1, CI=1, and CI<1 indicate antagonistic, additive, and synergistic effects, respectively; Isobologram and Dose Reduction Index (DRI), DRI>1. All experiment was performed in three independent replicates.

$$CI = [(D)1/(Dx)1] + [(D)2/(Dx)2] \text{ where } Dx = Dm[fa/(1-fa)]1/m$$

CI is the quantitative measure the degree of drugs interaction at given endpoint of effect measurement, while DRI is the measure of how many fold dose of drugs in synergistic combination is reduced as compared to the individual dosage (Chou & Martin, 2007).

3.6 Degree of Sensitization

The degree of sensitization (S_f) to cisplatin by PW-E was calculated as the ratio of inhibitory concentration of drug (cisplatin) to inhibitory concentration of extract/drug combination at IC_{50}, IC_{75} and IC_{90} (Motiwala & Rangari, 2015).

3.7 Cell Apoptosis Determination

Cell apoptosis determination was performed and standardized for all assays with four groups of treatments based on the IC_{50} values of individual agents (close to the IC_{50} to ease calculations) i.e Group I: negative control, cells without treatment; Group II: positive control, cells treated with cisplatin at 0.5 μg/ml; Group III: PW-E at 2.0 μg/ml and Group IV: PW-E/cisplatin combination (2.0 + 0.5 μg/ml).

3.7.1 Morphological Analysis under Phase-Contrast Microscope.

The individual or/and combined effects of cisplatin and PW-E on morphological changes of SKOV-3 cells were observed under Leica CTR 6000 phase contrast inverted microscope (Leica Microsystems, Germany) at 200× magnification. SKOV-3 cells were cultured in 6-well plates (Orange Scientific, California) at 30,000 cells/ml density and treated with the IC_{50} of individual agents and extract/drug combination for 48 hours. Cytotoxicity of the different treatments on the cells was evaluated according to the variation in cell morphological changes and the amount of adherent cells.

3.7.2 Analysis of Cell Morphology by Acridine Orange/Ethidium Bromide (AO/EB) Fluorescence Staining.

Cell morphology changes in response to apoptosis agent were also evaluated using acridine orange/ethidium bromide (AO/EB) double fluorescence staining according to the method described by Navanesan *et al.* (2015) with slight modifications. About 3×10^4 cells/ml was plated into 6-well tissue culture plate followed-by incubation for 24 hours prior to treatment with PW-E, cisplatin and PW-E/cisplatin combination. After 48 hours' incubation, SKOV-3 cells were detached from plate and centrifuged to pellet. The cells were resuspended with 50 µl cold PBS and 4 µl AO/EB solution mix (1 part of 100 µg/ml of AO in PBS; 1 part of 100 µg/ml of EB in PBS) was added prior to microscopic examination at 2:25 ratio (AO/EB mix: Media). Cell suspension with dye were added to 2-well teflon coated microscopic slide and observed with Leica CTR 6000 phase contrast inverted microscope (Leica Microsystems, Germany) under fluorescence illumination. Images of the cell morphology were recorded and analysed.

3.7.3 Caspase-3 Activation Detection

Caspase-3 activity in cells-treated with extract/drug were determined using the Caspase-3 DEVD-R110 Fluorometric and Colorimetric assay kit (Biotium, CA) according to manufacturer protocol. The assay based on a colorimetric detection of the chromophore, rhodamine 110 (R110), after its cleavage from the labelled substrate (Ac-DEVD) 2-R110. The assay was performed in three replicates. SKOV-3 cells at seeding density of 1×10^5 cells/ml in 24-wells plate (Nunc, Denmark) were treated with extracts and drug alone, and extract/drug combination for 48 hours before being lysed with 100 µl of chilled cell lyses buffer and incubated on ice for 10 minutes.

After incubation, the cell suspension was transferred to microcentrifuge tube and centrifuged for 5 minutes at 12,000 g in 4 °C (Thermo Fisher Scientific, USA) to pellet insoluble cell debris. The supernatant (cytosolic extract) was aspirated to a fresh tube and 50 µl of Assay Buffer was added followed by incubation on ice for 30 minutes. Then, 5 µl of Enzyme Substrate was added to each sample and mixed well before final incubation at 37 °C for 60 minutes. The formations of R110 in samples were measured with micro-plate reader (Thermo Fisher Scientific, USA) at absorbance 495 nm. Activity of caspase-3 in treated cells were determined by comparing the results with controls (untreated cells) and presented as percentage.

3.8 Statistical Analysis

Data from cytotoxicity analysis and caspase-3 detection were presented as mean ± standard deviation from three independent experiments. The differences between SKOV-3 cells treated with PWE, cisplatin and PW-E/cisplatin combination from the mentioned experiment were evaluated using One-way Anova. *$P<0.05$ denoted a statistically significant.

CHAPTER 4: RESULTS

4.1 Cytotoxicity of PW-E, Pt and PW-E/Pt against SKOV-3 cells

Cytotoxicity potential of *Phyllanthus watsonii* ethyl acetate extract (PW-E), cisplatin (Pt) and PW-E combined with cisplatin (PW-E/Pt) against human ovarian SKOV-3 cancer cell line was assessed using the Neutral Red Uptake (NRU) assay after 72 hours of incubation period. Selection of PW-E as a test agent in this current study was undertaken based on reported work by Ramasamy *et al*. (2012), which reported PW-E was the best performer in cytotoxicity against ovarian cancer cells in comparison with PW extracts in different solvent. Percentages of inhibition of the SKOV-3 cells triggered by the test agents in various concentrations in three independent experiment were entered into CompuSyn1.0 software and the D_m value expressed as IC_{50}, µg/ml (cytotoxicity effect) was generated from the antilog of x-intercept of median-effect plot (Chou & Martin, 2007).

Table 4.1 shows the individual cytotoxicities of PW-E and Pt were observed at 1.78 ± 0.32 µg/ml and 0.69 ± 0.11 µg/ml respectively. Based on the IC_{50} values obtained for individual PW-E and Pt, combination of PW-E/Pt was done in a constant ratio of 4:1 as shown in Table 4.2 and applied in drug-extract combination cytotoxic effect study. Cytotoxicity (IC_{50}) of PW-E/Pt combination against SKOV-3 cells was 1.34 ± 0.16 µg/ml. PW-E and PW-E/Pt demonstrated 50% inhibitory concentration in lower concentration ($IC_{50} \leq 20$ µg/ml) when compared with anticancer drug, Pt on SKOV-3 cells.

Selectivity index (SI) was calculated as the ratio of the IC_{50} values of extract/drug on normal lung fibroblast MRC-5 cells to SKOV-3 cells. According to Mahavorasirikul *et al*. (2010), extracts or drugs that exhibited SI of greater than 3 was considered highly selective in their inhibitory effect towards cancer cell tested. In the

present study, the selectivity index (SI) of PW-E/Pt combination was 4.33. PW-E/Pt combination showed an interesting selectivity index value of more than three, which indicated that the combination provided greater selectivity toward ovarian cancer cells than normal noncancerous cells.

Figures 4.1 and 4.2 showed the sensitivity of SKOV-3 cells towards PW-E, Pt and PW-E/Pt combination and presented as percentage inhibition of the cells after 72 hours of exposure towards the test agents in a dose-dependent manner, with the later shown detailed breakdown of each combination concentration. Statistically significant result ($P<0.05$) was observed when comparing combination group with PW-E and Pt individual cytotoxic data using Student's T-test. Overall, PW-E and Pt groups showed significant inhibition of SKOV-3 cell after 72 hours exposure in different concentrations of 0.3125, 0.625, 1.25, 2.5, 5 and 10 µg/ml.

Additional dose-effect curve graph (Figure 4.3) generated by Compusyn 1.0 software showed that for PW-E, Pt and PW-E/Pt combination its conformity to rule, *r* (i.e reliability of the data) is almost to a straight line r=1 (Table 4.3) and the graph shape was confirmed almost identical to excel generated graph in Figure 4.1.

Table 4.1: Cytotoxicity (IC_{50}, µg/ml) of *P. watsonii* ethyl acetate extract, cisplatin and their combination

	IC_{50} (µg/ml)[a]			
Cell lines[b]	**PW-E[c]**	**Pt[d]**	**PW-E/Pt[e]**	**SI[f]**
SKOV-3	1.78 ± 0.32	0.69 ± 0.11	1.34 ± 0.16	4.33
MRC-5	3.62 ± 0.15	11 ± 0.73	5.80 ± 1.83	

[a]Data are represented as mean±SD from three independent experiments, triplicate each; [b]SKOV-3 (ovarian cancer); and MRC-5 (normal lung fibroblast); [c]*P. watsonii* ethyl acetate extract; [d]Cis-diamminedichloroplatinum (II), cisplatin; [e]combination at ratio 4:1; [f]SI, selectivity index is the ratio of the IC_{50} values of extract/drug on MRC-5 cells to SKOV-3. Samples with SI greater than 3 were considered to have high selectivity (Mahavorasirikul *et al.*, 2010).

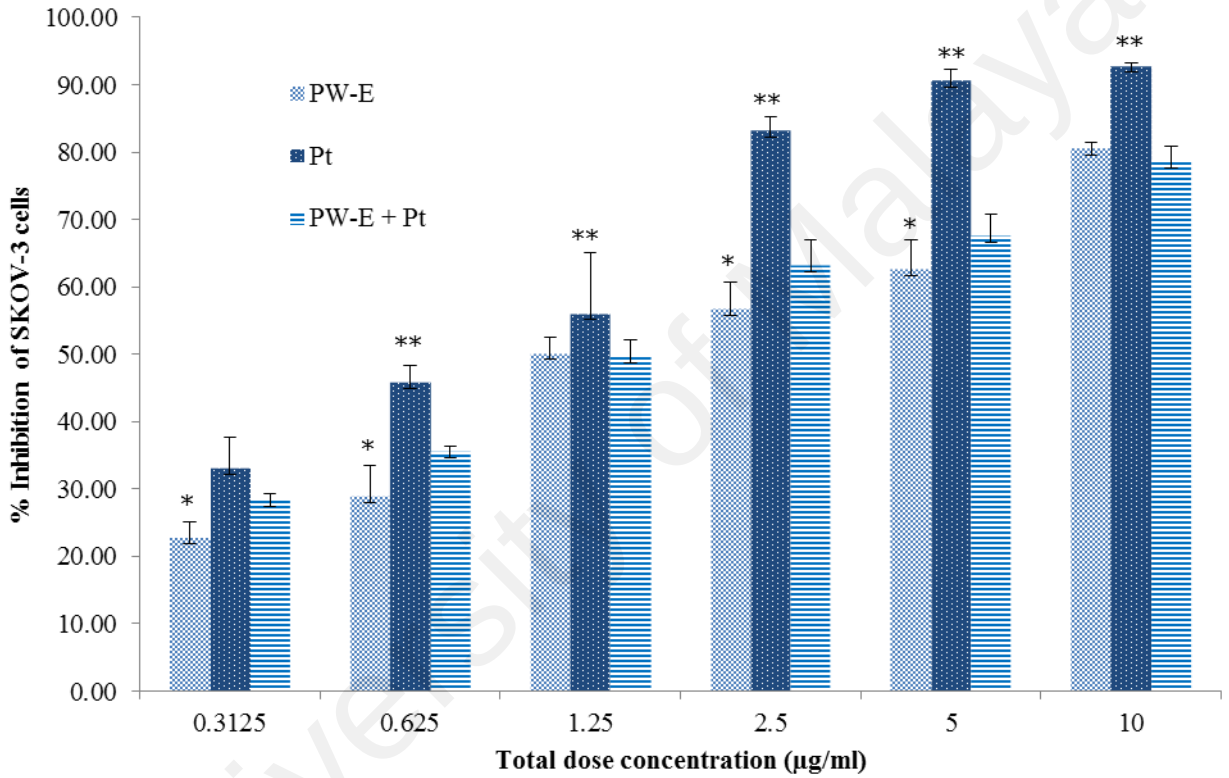

Figure 4.1: Sensitivity of SKOV-3 cells towards PW-E, Pt and PW-E/Pt combination presented as percentage of cells inhibition after 72 hours of exposure assayed by NRU assay. All values are expressed as mean ± SD of three independent experiments. PW-E: *P. watsonii* ethyl acetate extract; Pt: cisplatin; PW-E/Pt: combination at ratio 4:1; $^{**}p<0.05$, Pt group vs PW-E/Pt group; $^{*}p<0.05$, PW-E group vs PW-E/Pt group.

Table 4.2: Concentration of individual PW-E and Pt in ratio of 4:1 for PW-E/Pt combination cytotoxicity analysis on human ovarian SKOV-3 cancer cell

Test Agents	Concentrations (µg/ml)					
PW-E[a]	0.25	0.5	1.0	2.0	4.0	8.0
Pt[b]	0.0625	0.125	0.25	0.5	1.0	2.0

[a]*P. watsonii* ethyl acetate extract, [b]Cis-diamminedichloroplatinum(II), cisplatin

Table 4.3: Dose-effect relationship of PW-E/Pt combination on human ovarian SKOV-3 cancer cell

Test Agents	Dm[c] (µg/ml)	m[d]	r[e]
PW-E[a]	1.78 ± 0.32	0.734	0.977
Pt[b]	0.69 ± 0.11	1.037	0.974
PW-E/Pt (4:1)	1.34 ± 0.16	0.646	0.994

[a]*P. watsonii* ethyl acetate extract; [b]Cis-diamminedichloroplatinum(II), cisplatin; [c]median-effect dose that produces 50% cell inhibition; [d]shapes of dose-effect curve; [e]conformity parameter for goodness of fit, for *in vitro* experiment r>0.95 are considered good;

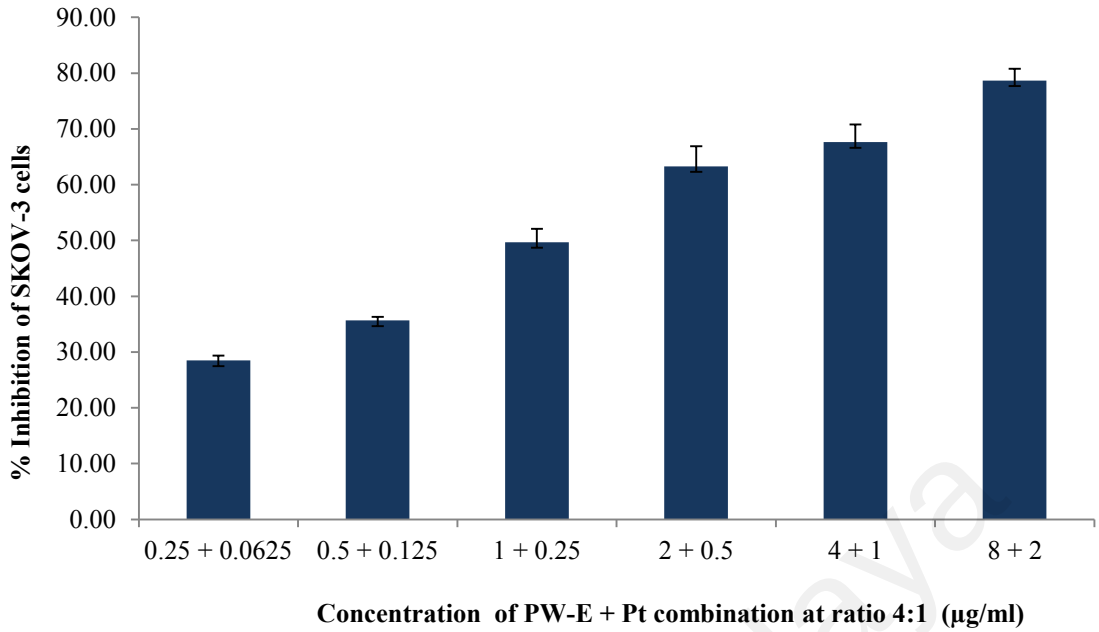

Figure 4.2: Graph of percentages inhibition of SKOV-3 cell after 72 hours' incubation with PW-E/Pt combination at 4:1 ratio in different concentrations. The inhibition potentials were in dose-dependent manner and data presented as mean ± SD from three independent experiments.

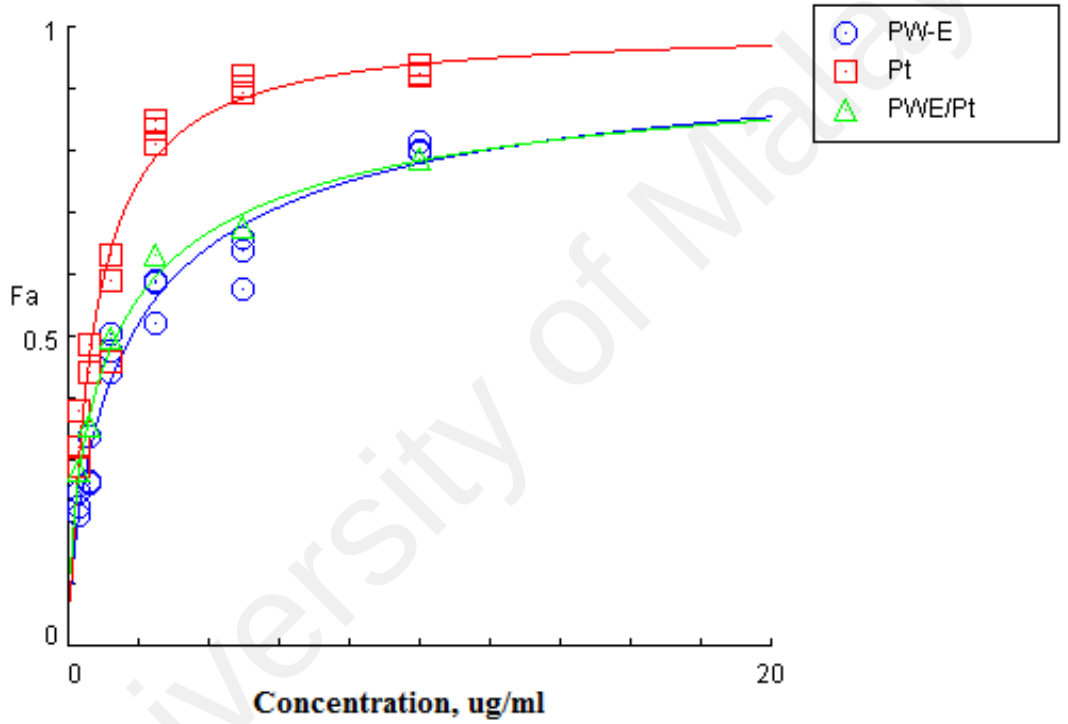

Figure 4.3: Dose-effect curve of cytotoxicity effect of PW-E, Pt and PW-E/Pt combination against human ovarian SKOV-3 cancer cells generated from Compusyn 1.0 software. PW-E: *P. watsonii* ethyl acetate extract; Pt: cisplatin; PW-E/Pt: *P. watsonii* ethyl acetate extract/cisplatin combination; and Fa: effect level (SKOV-3 cells growth inhibition percentages/100).

4.2 Combination Index (CI) Analysis

To further explore the combination effect of PW-E with cisplatin (Pt) on SKOV-3 cells, Combination Index (CI) analysis was performed and CI values, isobologram and Drug Reduction Index were generated by CompuSyn 1.0 software. Combination between two drugs or agents can interact to either reduce or increase its inhibitory efficacy towards the tested cells thus producing three possible effects; (i) synergism, in which the combine effect is more than additive effect; (ii) additive, the combined effect predicted by the mass-action law principle in the absence of synergistic and antagonistic effect; and (iii) antagonism, the combine effect is lesser than additive effect (Chou & Martin, 2007).

CI of PW-E/Pt combination at $IC_{50,}$ IC_{75} and IC_{90} are 0.998, 1.487, 2.321 respectively (Table 4.4). According to the guidelines given by Chou and Martin (2007), any combination with CI>1, indicate antagonism interaction, CI=1, additive interaction and CI<1 indicate synergistic interaction between the two agents. In the present study, it shows that PW-E/Pt combination exerts synergistic interaction at 50% cell inhibition and antagonism interaction between the extract and drug at 75% and 90% cell inhibition.

Data in Table 4.4 also summarized the sensitization factor (S_f) of Pt towards SKOV-3 by PW-E and was calculated as the ratio of $IC_{50/75/90}$ of individual Pt/$IC_{50/75/90}$ of PW-E/Pt combination. It can be clearly seen that there was a decrease in S_f values at higher inhibitory concentration. It indicates that the present of PW-E at high concentration (> 2µg/ml) in PW-E/Pt combination has no sensitizing effect in increasing cisplatin inhibitory activity towards SKOV-3 cells.

The diagonal lines in the isobologram presented in Figure 4.4 indicated additive interaction between the extract and drug at three different inhibitory effects, 50%, 75% and 90%. Any points or the inhibitory effect values that fall above each diagonal lines indicating antagonism interaction, while points or inhibitory effect values that fall below each diagonal lines indicating synergism interaction. In this present study at 50% inhibitory, PW-E/Pt combination exert synergistic with nearly additive effect with CI value of 0.998, antagonism effect at 75% and 90% cells inhibition with CI value of 1.487 and 2.321 respectively.

The nature of interaction between PW-E and Pt at each point of combination with their Fa value (effect level) were graphically presented in Figure 4.5 and summarized in Table 4.5. Synergistic effects were only observed at lower concentration combination dosages at Fa<0.6, while at higher concentration that with Fa>0.6, PW-E/Pt combination exerted an antagonist interaction. Figure 4.5 revealed that among six effect levels, four combination points showed synergism while two combination points with antagonism effect. Among the ones that shown synergism, three of the combination points showed a nearly additive effect.

Table 4.4: Combination index (CI) and sensitization factor of cisplatin in PW-E/Pt combination on human ovarian SKOV-3 cancer cell line

Inhibitory Concentration at 72 hours	PW-E[a] (µg/ml)	Pt[b] (µg/ml)	PW-E/Pt(µg/ml)	CI[c]	S_f[d]
IC_{50}	1.78 ± 0.32	0.69 ± 0.11	1.34 ± 0.16	0.998	0.52
IC_{75}	7.89± 1.25	1.99 ± 0.19	7.49± 1.54	1.487	0.27
IC_{90}	35.31± 5.08	5.73± 0.47	41.72± 12.53	2.321	0.14

[a]*P. watsonii* ethyl acetate extract; [b]Cis-diamminedichloroplatinum(II), cisplatin; [c]combination index which measures the degree of PW-E/Pt interaction, CI was measured at the affected fractions of 50% (IC_{50}), 70% (IC_{70}) and 90% (IC_{90}), CI＜1 indicates synergism interaction, CI>1 indicates antagonism interaction and CI=1 indicates additive interaction; [d]sensitization factor of Pt towards cancer cell (SKOV-3) by PW-E was calculated as the ratio of $IC_{50/75/90}$of individual Pt /$IC_{50/75/90}$of PW-E/Pt combination (Motiwala & Rangari, 2015).

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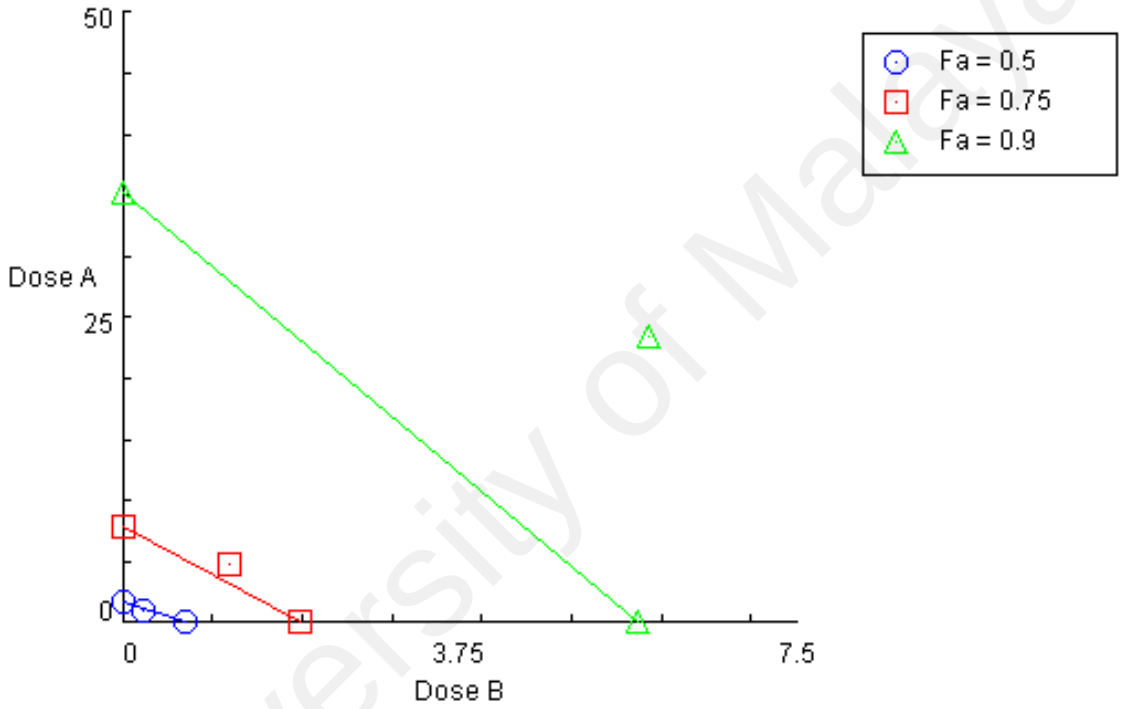


Figure 4.4: Isobologram of cytotoxic effect level at 50% inhibition (Fa=0.5), at 75% (Fa=0.75) and at 90% (Fa=0.9). The lines indicated the degree of interaction between PW-E (Dose A) and Pt (Dose B). Effect level that fall on the diagonal lines of its colour indicated additive, below the lines synergism and above the lines, antagonism interaction. In present study, at 50%, 75% and 90% inhibition showed nearly additive, antagonistic and moderate antagonistic respectively. PW-E: *P. watsonii* ethyl acetate extract; Pt: Cis-diamminedichloroplatinum(II), cisplatin.

Table 4.5: Combination Index (CI) at different points of PW-E/Pt combination

Combination Ratio (4:1) (µg/ml)		Fa[c]	CI[d]	Interpretation
PW-E[a]	Pt[b]			
0.25	0.0625	0.2849	0.7166	Synergism
0.5	0.125	0.3566	0.9534	Synergism
1.0	0.25	0.4968	0.9436	Synergism
2.0	0.5	0.6331	0.9672	Synergism
4.0	1.0	0.6762	1.5437	Antagonism
8.0	2.0	0.7868	1.5882	Antagonism

[a]*P. watsonii* ethyl acetate extract; [b]Cis-diamminedichloroplatinum(II), cisplatin; [c]effect level; [d]Combination index CI was measured at the affected fractions of 50% (IC_{50}), 70% (IC_{70}) and 90% (IC_{90}), CI＜1 indicates synergism interaction, CI>1 indicates antagonism interaction and CI=1 indicates additive interaction.

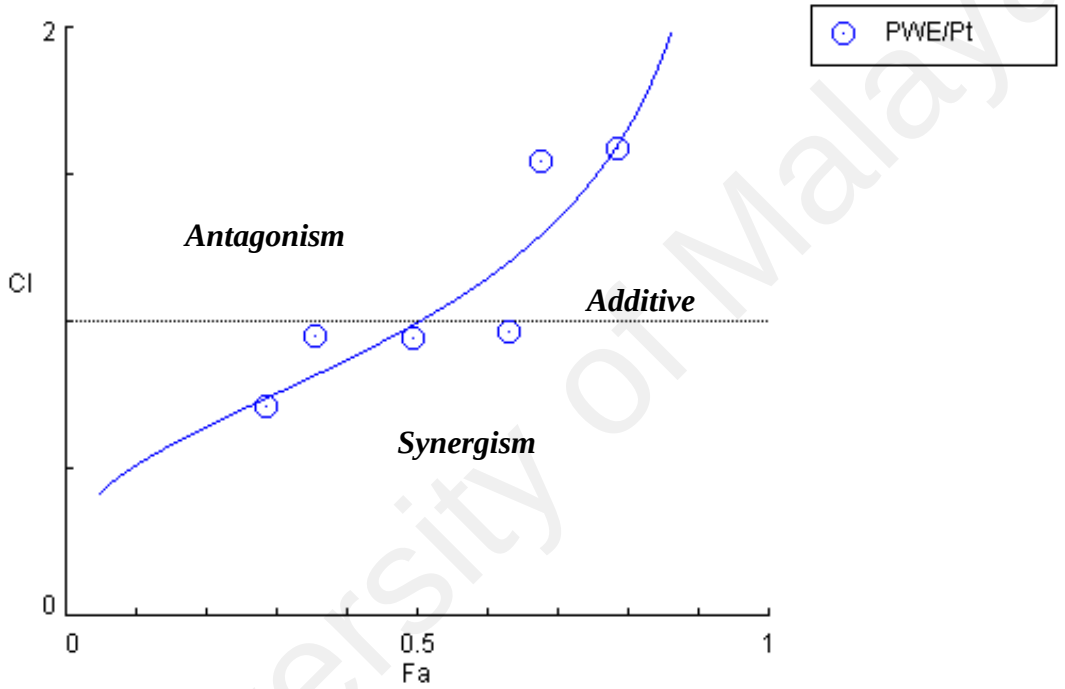


Figure 4.5: Combination index (Fa-CI) plot, effect level, Fa is at x-axis, while CI is at y-axis. The horizontal line signifies additive effect, CI=1. Combination points of PW-E/Pt situated at below and upper level of the line are synergistic and antagonistic respectively. PW-E: *P. watsonii* ethyl acetate extract; Pt: Cis-diamminedichloroplatinum(II), cisplatin. Fa: effect level (SKOV-3 cells growth inhibition percentages/100).

Table 4.6 summarizes the Drug Reduction Index (DRI) for PW-E, Pt and PW-E/Pt combination. DRI of drug-agent combination is a measure on how much (-fold) the dose of a drug or agent (e.g. PW-E or Pt) in synergistic combination may be reduced at a given effect level compared with the dose of each individual drug. The DRI is another mathematical interpretation of the CI, and CI= $1/(DRI)_1 + 1(DRI)_2$. The value of DRI>1 indicates that the combination is favourable in terms of clinical therapy (Chou, 2010).

The data showed that to exert 50% inhibition of the cells, it requires 1.76648 µg/ml of PW-E, and 0.68868 µg/ml of Pt of each of the individual extract and drug. However, when combined, there are reduction of doses at 1.6440-fold of PW-E plus 2.5637-fold Pt to achieve the same 50% inhibition (i.e. 1.0745 µg/ml of PWE added to 0.2686 µg/ml of Pt at combination ratio of 4:1 will achieve 50% of cell inhibition). This indicates that, the PW-E/Pt combination at lower dosage of drugs at ratio of 4:1 still providing the same therapeutic effect in comparison with the Pt or PW-E individually. Figure 4.6 depicts the DRI values for PW-E, Pt and PW-E/Pt combination and it clearly can be seen that the DRI>1, i.e. indicating favourable dose reduction and also suggesting that PW-E/Pt combination is favourable in cancer therapy.

Table 4.6: Drug Reduction Index (DRI) values for PW-E, Pt and PW-E/Pt combination

Fa[a]	Combination Ratio (4:1) (µg/ml)		DRI[e]	
	PW-E[c]	Pt[d]	PW-E[c]	Pt[d]
0.2849	0.25	0.0625	2.0154	4.5362
0.3566	0.5	0.125	1.5804	3.1184
0.4968	1.0	0.25	1.7359	2.7209
0.6331	2.0	0.5	1.8580	2.3310
0.6762	4.0	1.0	1.2050	1.4010
0.7868	8.0	2.0	1.3092	1.2130
0.5000[b]	**1.76648**	**0.68868**	**1.6440**	**2.5637**

[a]Fa: Fractional inhibition; [b]Fa=0.500 is Compusyn computerized stimulation of the DRI at 50% inhibition based on the IC_{50} of individual test agents ***(Bold values)***; [c]*P. watsonii* ethyl acetate extract; [d]Cis-diamminedichloroplatinum(II), cisplatin; [e]a measure of how many folds the dose of Pt in a synergistic combination with PW-E may be reduced at a given level when compared with the doses of each alone, DRI>1 indicates favourable dose reduction, DRI<1 indicates not-favourable reduction and DRI=1 symbolizes no dose reduction.

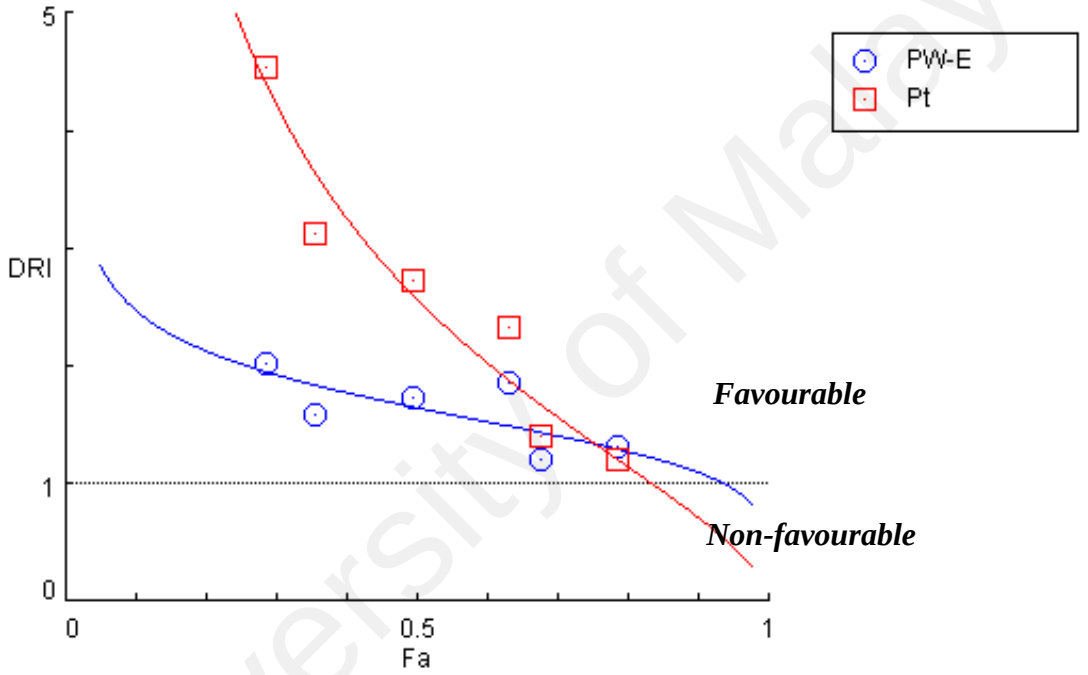


Figure 4.6: Drug Reduction Index (DRI) plot of PW-E, Pt and PW-E/Pt combination (4:1). DRI>1 indicates favourable dose reduction and the combination is favourable in terms of clinical therapy (Chou, 2010). PW-E: *P. watsonii* ethyl acetate extract; Pt: Cis-diamminedichloroplatinum(II), cisplatin. Fa: effect level (SKOV-3 cells growth inhibition percentages/100).

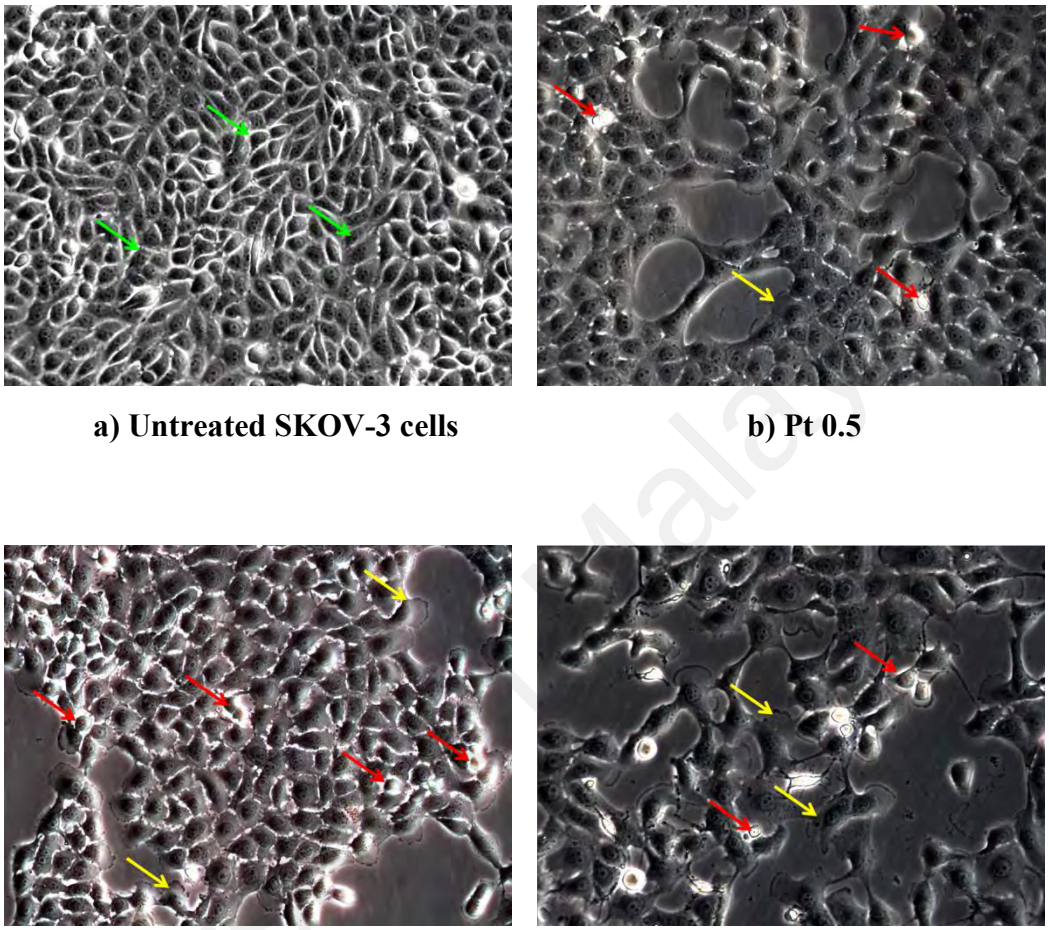
4.3 Cell Morphological Analysis under Phase-Contrast Microscope

The morphological changes of SKOV-3 cells treated with PW-E, cisplatin (Pt), and PW-E/Pt combination at 48 hours were observed under inverted microscope fitted with phase contrast objective. The cell morphological changes were compared with non-treated cells (without addition of any test agent) that were showing uniform, epithelial-like cells and solid adherence onto the surface of the well-plate.

Figure 4.7 shows that at magnification 100×, SKOV-3 cells exhibited loss of cell populations when treated with Pt and PW-E individually; and was shown increased in population loss at combined treatment. The treated-SKOV-3 cells showed morphological changes atypical to cells undergoing apoptosis such as membrane blebbing (protrusion of the plasma membrane due to disruption of membrane integrity) and loss of contact between neighbouring cells.

Figure 4.8 shows that at magnifications 200×, untreated SKOV-3 cells showed smooth and intact cell membrane while cells treated with PW-E showed changes in terms of cellular shape and cells treated with Pt individually showed more appearance of apoptotic cells which tend to float inside the culture media. Overall, cells in PW-E and Pt group revealed morphological changes related to apoptosis such as cell shrinkage, blebs of membrane and loss of adherence towards neighbouring cell as seen under magnification 100×.

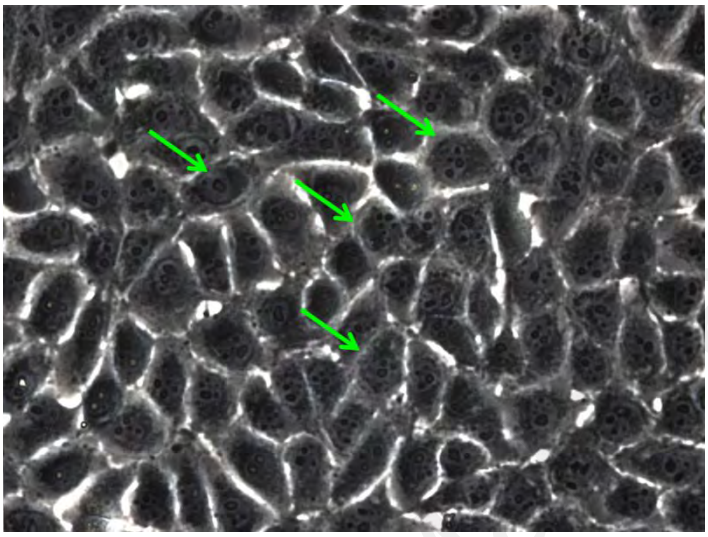
SKOV-3 cells treated with combination of PW-E/Pt revealed extensive shape alteration, blebbings and late apoptotic cells as compared to individual treatments with formation of dark ring-like formation (Wong, 2011) in the nucleus which may suggest chromatin condensation. These results suggested that the cytotoxicity of *P. watsonii* ethyl acetate extract and cisplatin combination were able to induce apoptosis in human ovarian SKOV-3 cells and PW-E/Pt combination increases apoptotic cells as compared to individual treatment alone.



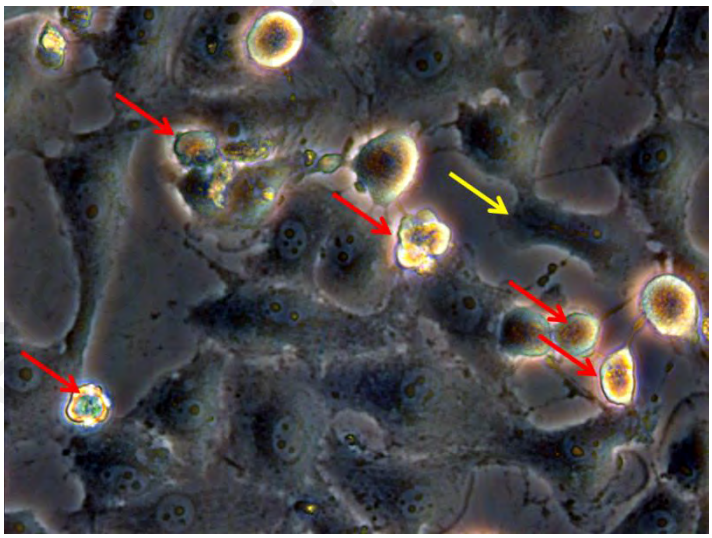
a) Untreated SKOV-3 cells **b) Pt 0.5**

c) PW-E 2.0 **d) Pt 0.5 + PW-E 2.0**

Figure 4.7: Morphological changes on SKOV-3 cells after treatment with IC_{50} dosages of individual agents and drug/extract combination for 48 hours view under inverted microscope fitted with phase-contrast objective (100×magnifications). **a)** Untreated cells (without addition of any test agents), **b)** SKOV-3 cells with cisplatin 0.5 µg/ml, **c)** SKOV-3 cells with PW-E 2.0 µg/ml, **d)** SKOV-3 cells with PW-E + cisplatin (2.0+0.5 µg/ml). There are marked changes in cell population and morphology seen when compared PW-E/Pt combination with individual treatments. Green arrow indicates normal, red arrow showing apoptotic cells with shrinkage characteristic; and yellow arrow showing cells with membrane blebbing. Images are the representatives from three independent experiments.

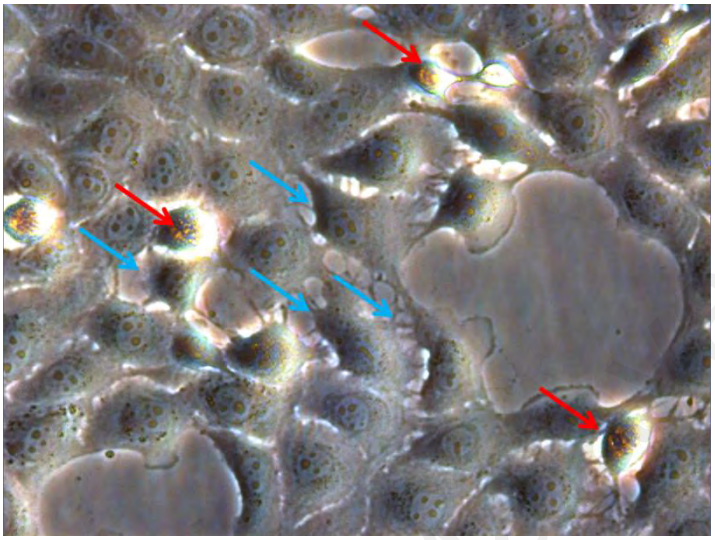


a) Untreated SKOV-3 cells

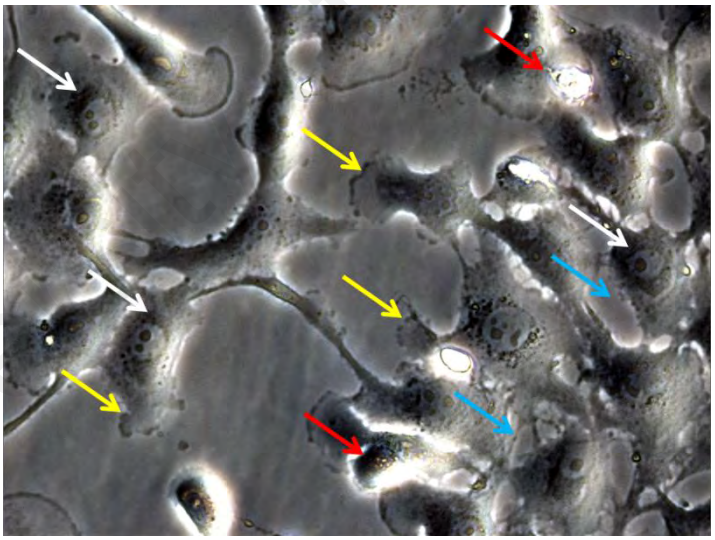


b) Pt (0.5 µg/ml)

Figure 4.8: Morphological changes on SKOV-3 cells after treatment with IC_{50} doses of individual agents and drug/extract combination for 48 hours view under inverted microscope fitted with phase-contrast objective (200×magnifications). **a)** Untreated SKOV-3 cell shows intact smooth membrane (green arrow), while **b), c)** and **d)** treated SKOV-3 cells show atypical pattern of cell undergoing apoptosis i.e. loss of adherence to neighbouring cells (blue arrow), membrane blebbing (yellow arrow), apoptotic cells with shrinkage characteristic (red arrow) and nuclei condensation (white arrow), shown here in a ring-like formation, could be seen. Images are representatives from three independent experiments.



c) PW-E (2.0 µg/ml)



d) Pt + PW-E (0.5+2.0 µg/ml)

Figure 4.8, continued.

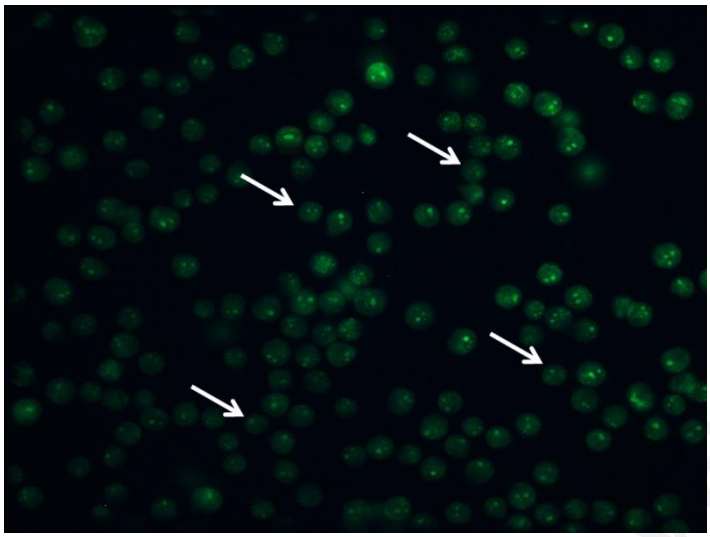
4.4 Cell Morphological Analysis using Acridine Orange/Ethidium Bromide (AO/EB) Double Staining

Acridine orange is a vital dye that will stain the membrane and nucleus of both viable and early apoptotic cells green, while ethidium bromide is a DNA stain that stained dead cells pale orange to bright red colour (Citalingam *et al.*, 2015; Navanesan *et al.*, 2015). Live or viable cells will be stained in pale green, while early apoptotic cells will be stained in green to bright green that usually centred inside the nucleus indicating chromatin condensation or fragmentation. Late apoptotic cells in which the cell loss its membrane integrity, will be stained green with a little touch of orange to red nuclei and necrotic cells usually will appear in uniformly orange to bright red nucleus. Figure 4.9 shows the images of SKOV-3 cells stained with AO/EB and viewed under inverted fluorescence microscope fitted with phase contrast objective. Double staining with AO/EB coupled with fluorescence microscopy examination was performed to further confirmed that the individual (PW-E and Pt) and combination (PW-E/Pt) treatments promote SKOV-3 cell death via apoptosis mechanism and not necrosis.

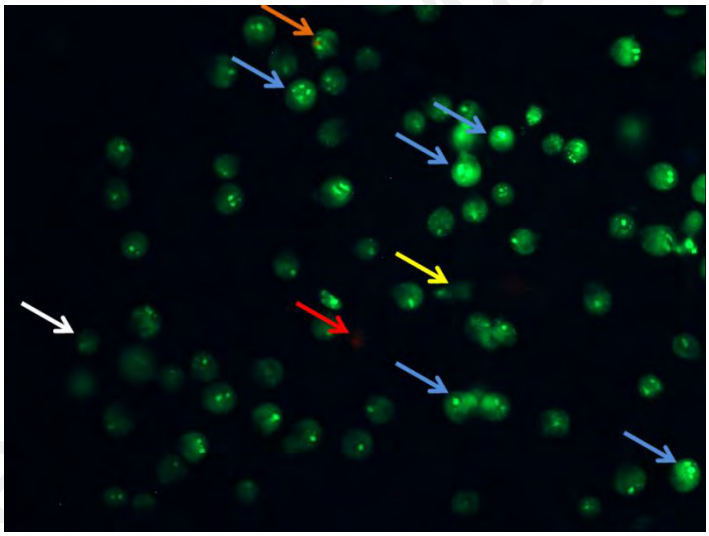
Human ovarian SKOV-3 cancer cells were treated with PW-E at concentration of 2.0 µg/ml, Pt at 0.5 µg/ml and PW-E/Pt combination at 2.0+0.5 µg/ml and after 48 hours incubation, cells were stained with mixture of AO/EB solution. The resulting images clearly differentiated between untreated cells (without addition of any test agent) and treated cells. In Figure 4.9a, untreated cells displayed intact round to oval shape and smooth membrane stained uniformly with pale green colour. In contrast, SKOV-3 cells that underwent individual treatment with extract and cisplatin (Figure 4.9b and 4.9c) respectively, showed bright green nuclei that exhibited condensed and fragmented appearances as compared to untreated group, with a few of the cells exhibited blebbings of the membrane. SKOV-3 cells that were treated with PW-E/Pt combination (Figure

4.9d) showed an increased in the number of cells underwent apoptosis and cells with membrane blebbings, and with more necrotic cells observed in fluoresce red.

The images revealed SKOV-3 cells showed atypical apoptotic changes when treated with PW-E at 2.0 µg/ml, Pt at 0.5 µg/ml and PW-E/Pt combination. There was reduction in the number of viable (live) cells in each treatment groups with the combination treatment has even lesser to none of viable cells. In comparison with individual treatment groups, more numbers of cells population in combination treatment group showed early apoptotic appearances characterized by membrane blebbings and condensed or fragmented chromatin (stained bright green colour) with smaller number of cells at late apoptosis stage (stained orange inside). Necrotic cells were stained in red. Our observation suggested that, the combination of extract/drug treatment elevates SKOV-3 cells promotion towards apoptosis.

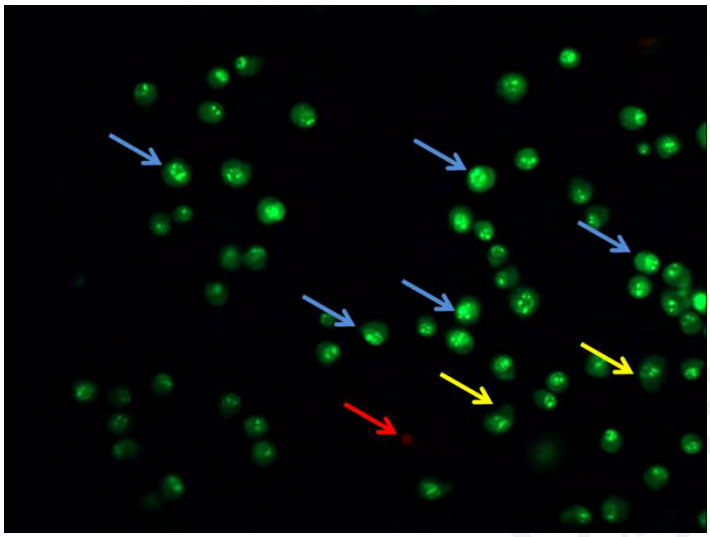


a) Untreated SKOV-3 cells

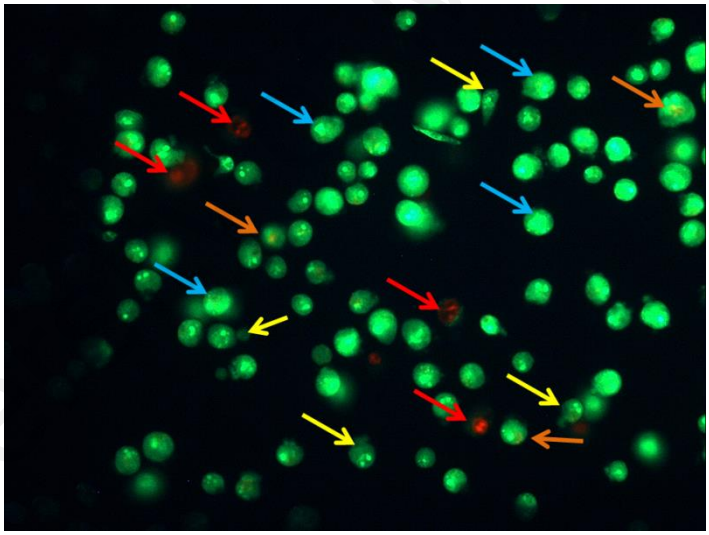


b) Pt (0.5 µg/ml)

Figure 4.9: Morphological observation of SKOV-3 cells after 48 hours treatments with PW-E, Pt and PW-E/Pt combination stained with AO/EB view under inverted microscope using fluorescence fitted with phase-contrast objective at 200× magnification. Viable cells stained pale green colour (white arrow), condensed and fragmented chromatin (blue arrow), membrane blebbing (yellow arrow), late apoptotic cells (orange arrow) and necrotic cells (red arrow). Images are representatives from three independent experiments.



c) PW-E (2.0 µg/ml)



d) PW-E + Pt (2.0 + 0.5 µg/ml)

Figure 4.9, continued.

4.5 Caspase-3 Activity Determination

The determination whether caspase-3 activation was involved in the cellular pathway of apoptosis in SKOV-3 cells treated with PW-E, cisplatin and their combination, caspase-3 activity was measured after 48 hours of treatment with each agent at its individual IC_{50} i.e Pt at concentration of 0.5 µg/ml, PW-E 2 at µg/ml and PW-E+Pt at 2.0 + 0.5 µg/ml. The absorbance readings were expressed as percentage of enzyme activity compared with untreated SKOV-3 cells (without addition of any test agents) with the assumption that concentration of caspase-3 in untreated SKOV-3 cells is 100% (Ramasamy *et al.,* 2012) for relative companion to treated group.

Data in Figure 4.10 revealed multiple increased in the activation of caspase-3 in SKOV-3 cells treated with test agents when compared with untreated cells. There was an increase of 13% and 31% of caspase-3 in SKOV-3 cells treated with PW-E and PW-E/Pt combination respectively, while the positive control, cisplatin showed a caspase-3 increment of 38%. The combination between PW-E and Pt produced 2-folds increment in caspase-3 activity as compared to individual treatment of PW-E. However, an analysis of variance showed the data as not significant due to limited number of sample, $F(3,8)=1.806$, $p=0.224$ ($p>0.05$). The present study confirmed, that caspase-3 activation may involve in SKOV-3 cell death process.

These data suggest that, the combination of PW-E and cisplatin exerted cytotoxic effect on SKOV-3 cells with possible activation of caspase-3 activity, a common executor caspase in apoptotic pathway.

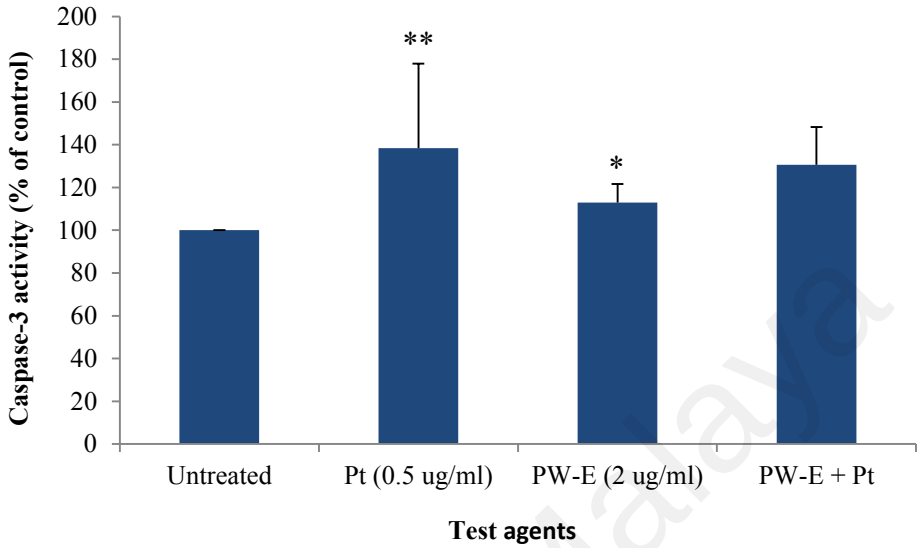


Figure 4.10: Caspase-3 activity in SKOV-3 cells with and without addition of different test agents and analysed using Caspase-3 DEVD-R110 Fluorometric and Colorimetric assay kit. Pt (Cisplatin) = 138 ± 40%, PW-E (*P. watsonii* ethyl acetate extract) = 113 ± 9% and PW-E+Pt = 131 ± 18%. The values are expressed as percentage mean ± S.D compared with control from three independent experiments. **p>0.05, Pt vs PW-E+ Pt; *p>0.05, PW-E vs PW-E+Pt. Data is not significant.

4.6 LC-MS/MS Analysis

P. watsonii ethyl acetate extract (PW-E) obtained from maceration was analysed by LC-MS/MS system in order to allow the detection of the major component(s) present in the extract. The raw data obtained were compared with previous published data of chemical constituents of *P. watsonii*. The LC-MS/MS-TIC profiles of major compounds in PW-E was shown in Figure 4.11 and major component were identified based on the mass spectrometric analysis and summarized in Table 4.7.

Data from the mass spectrometry analysis revealed there were at least ten identified compounds present in PW-E. The data showed the extract is rich with phenolic and flavonoids compounds. Compound with the highest retention time at 9.80 minutes was identified as quercetin (MW m/z = 301.0) and with the lowest retention time at 2.43 minutes was identified as gallic acid (MW m/z = 169.0) (Appendix D1). Other compounds identified in PW-E were (from highest to lowest retention time), kaempferol rhamnoside (8.46 minutes, MW m/z = 431.1) (Appendix D9), kaempferol glucoside (8.00 minutes, MW m/z = 447.1) (Appendix D8), ellagic acid (7.50 minutes, MW m/z = 301.0) (Appendix D7), hyperin (7.45 minutes, MW m/z = 463.1) (Appendix D6), strictinin isomer (5.81 minutes, MW m/z = 633.1) (Appendix D5), galloyl HHDP hexoside (5.14 minutes, MW m/z = 633.1) (Appendix D4), strictinin isomer (4.85 minutes, MW m/z = 634.1) (Appendix D3) and strictinin (4.46 minutes, MW m/z = 633.1) (Appendix D2). The full chromatographic profile also showed that the major component in PW-E is kaempferol rhamnoside and minor compound is strictinin.

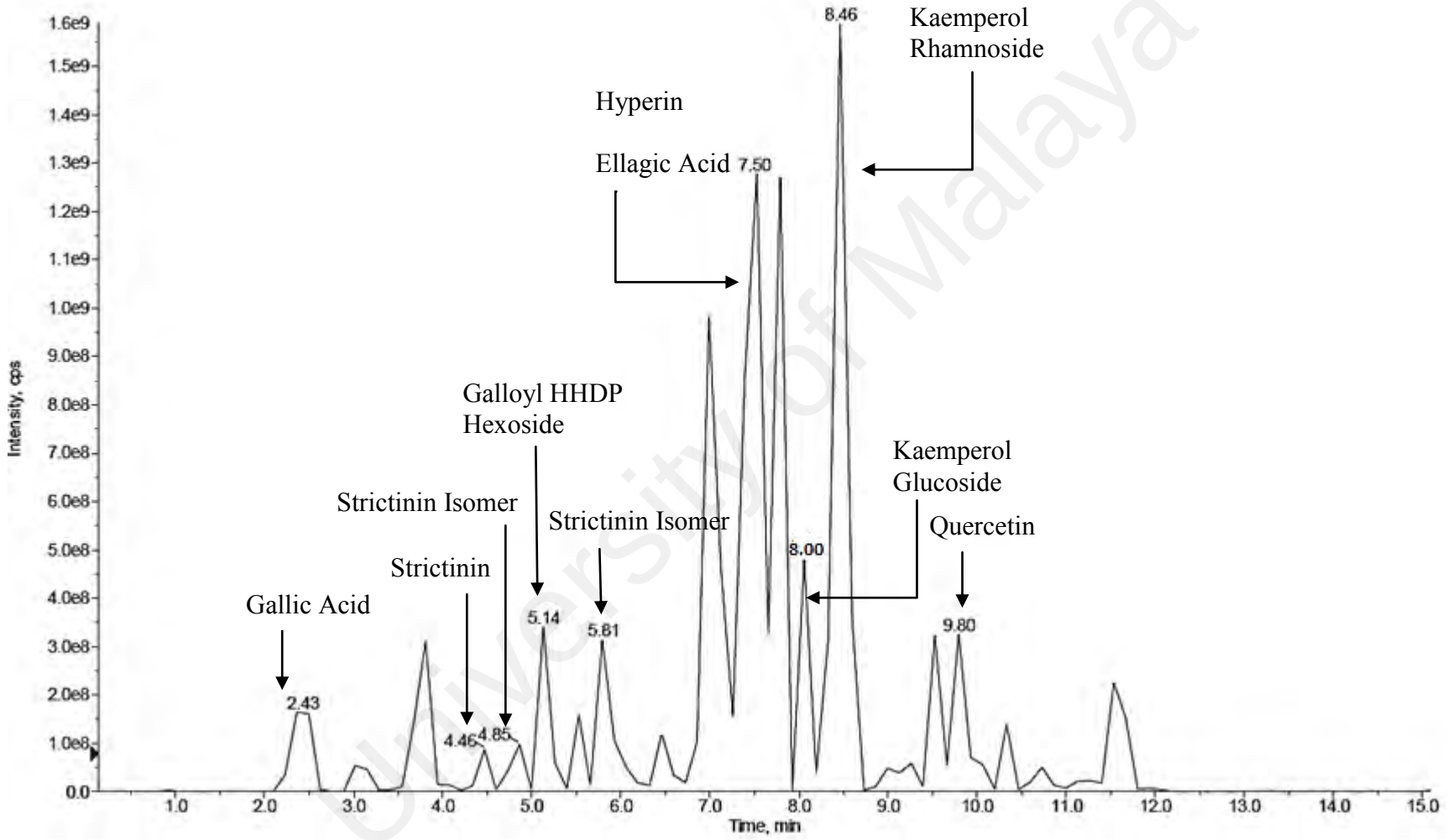


Figure 4.11:LC-MS/MS profile of chemical compounds in *P. watsonii* ethyl acetate extract

Table 4.7: Chemical compounds of PW-E identified using LC-MS/MS analysis

Retention Time	MW (m/z)	Tentative ID of Compounds
2.43	169.0	Gallic acid
4.46	633.1	Strictinin
4.85	634.1	Strictinin isomer
5.14	633.1	Galloyl HHDP hexoside
5.81	633.1	Strictinin isomer
7.45	463.1	Hyperin
7.50	301.0	Ellagic acid
8.00	447.1	Kaempferol glucoside
8.46	431.1	Kaempferol rhamnoside
9.80	301.0	Quercetin

*identification of the compound was facilitated by comparing with reference standards by correlation with previous literature reports. PW-E: *P. watsonii* ethyl acetate extract. MW: molecular weight.

CHAPTER 5: DISCUSSION

5.1 Extraction of Phytochemicals from *Phyllanthus watsonii*

In the present study, *Phyllanthus watsonii* were collected, dried, ground and extracted using cold extraction method in three organic solvents which are methanol, hexane and ethyl acetate of increasing polarity. The drying process of *P. watsonii* leaves involved quick drying in oven at relatively low temperature (40 - 50 $^{\circ}$C) for two days to avoid fungus or microbial growth that may result in loss or degradation of certain valuable compounds. Higher temperature may also result in decomposition of compounds as some of the plant's constituent may susceptible to extreme temperature treatment.

The utilization of organic solvent has been found to be effective in extraction of phytochemical due to their ability to exploit the various solubility of plant constituents (Sarker *et al.,* 2006). This method known as maceration, involved soaking the powdered plants in a suitable solvent at room temperature. Different class of phytochemical can be extracted using solvents with different polarity. Methanol extraction (high polarity solvent) able to yield high polarity compounds such as flavonoids glycosides, tannins, some alkaloids, while hexane (non-polar solvent) are used to solubilize most lipophilic compounds such as alkanes, fatty acids, pigments, waxes, sterols, alkaloids, some terpenoids and coumarins. Ethyl acetate (medium polarity solvent) is used to extract intermediate polarity compounds for example flavonoids and some alkaloids (Sarker *et al.*, 2006).The main drawback of maceration technique is time consuming, which ranges from a few days to weeks to complete.

5.2 Cytotoxicity Assay of PW-E, Cisplatin and PW-E/Cisplatin Combination

In order to discover the potential of natural product as anticancer agents, *in vitro* cytotoxicity screening of the extract towards cancer cell lines are important stepping stone. There are several well-known assays for *in vitro* cytotoxicity assay such as methyl tetrazolium (MTT), Neutral Red Uptake, lactate dehydrogenase leakage (LDH) and protein assay (Fotakis & Timbrell, 2006). The choice of which assay dependent on researchers specific goal of either cytotoxicity or viability (Niles *et al.*, 2008).

Neutral Red Uptake assay was chosen in this study because its reproducible result especially at large scale assay apart from highly sensitive to detect cytotoxicity, cheaper, and fairly quick. The entire assay can be completed in just less than four hours (Fotakis & Timbrell, 2006). Neutral Red Uptake assay is a colorimetric assay to quantify membrane permeability and lysosomal activity of cells in response to chemical, pharmaceutical and environmental compounds. It is based on the ability of viable cells to incorporate and bind neutral red dye within its lysosomes matrix and generally performed on adherent cells. Therefore, it is possible to distinguish between alive and death cells as they can no longer retain neutral red inside their lysosomes. The quantity of the dye absorbed by the lysosomes of the viable cells is measured by spectrometry at 540 nm and the absorbance value is directly proportional to the number of cells with intact membrane (Repetto *et al.,* 2008).

Natural sources have become 'the medicine' for treatment of many ailments thousands of years. The genus *Phyllanthus*, where *P. watsonii* was classified into were utilized as herbal medicine in many part of Asia for the treatment of diseases such as digestive, respiratory, hypertension, liver, hepatitis and even malaria. Limited publication reported on the potential of *P. watsonii* as anticancer agents. Study by Tang *et al.* (2010) reported that aqueous and methanolic extract of *P. watsonii* was able to induce apoptosis on melanoma MeWo cells and prostate PC-3 cancer cells by

promoting cell cycle arrest at various phases. Methanolic and aqueous extract of *P. watsonii* exhibited anti-metastatic activities by reducing invasion, migration and adhesion of both human breast MCF-7 and human lung A-549 cancer cells with IC_{50} ranging from 40 - 70 µg/ml and 100 - 200 µg/ml respectively (Lee *et al*., 2011). More recent studies reported the *P. watsonii* extract (prepared in methanol, hexane, ethyl acetate) and fractions from hexane extracts showed cytotoxicity <20 µg/ml on human ovarian SKOV-3, cervical Ca Ski and colon HT-29 cancer cells (Ramasamy *et al*., 2012). These cited works, proved that *P. watsonii* have tremendous potential to be developed as anticancer drug in the future.

P. watsonii ethyl acetate extract (PW-E) was selected in this present study based on previous work reported on the cytotoxicity potential of PW-E (Ramasamy *et al*., 2012). Among three *P. watsonii* extract prepared in methanol, hexane, ethyl acetate, extract prepared in ethyl acetate was found to have strong cytotoxicity against human ovarian SKOV-3 cells (Ramasamy *et al*., 2012). Our main objective is to investigate the cytotoxic effect of PW-E and anticancer drug, cisplatin combination on human ovarian SKOV-3 cancer cells. IC_{50} of individual treatment (PW-E and cisplatin) became the basis for the combination ratio. In the present study, PW-E, cisplatin and PW-E/cisplatin combination showed cytotoxic effect in a dose-dependent manner. The result showed combination of extract/cisplatin has higher cytotoxicity as compared to individual treatments alone. Ramasamy *et al*. (2012) reported the IC_{50} of PW-E against SKOV-3 was 5.52 ± 0.50 µg/ml, a higher value compared to this present work which is 1.78 ± 0.32 µg/ml. Different in the cytotoxicity values of the same plant species can be affected due to several factors such as differences in plant's batches, age of the plant when harvested and perhaps where it were collected (near or further to the riverside) or the environment (temperature, sunlight and humidity) (Chandradevan & Bala, 2014).

Comparison of cytotoxicity of PW-E/cisplatin combination against SKOV-3 cells and human lung fibroblast MRC-5 cells showed an interesting insight. PW-E/cisplatin combination revealed a selectivity index (SI) of more than 3 towards SKOV-3 cancer cells when compared with the non-cancerous cells. SI value is to measure on the safety of extract/drug on non-cancerous cells. At present study, the result indicated that the combination treatment proven to be more selective towards cancer cells as compared to normal non-cancerous cells. The significance to this observation is that cisplatin was known to cause toxicity on normal cell (Jordan & Carmo-Fonseca, 2000) and this combination of PW-E/cisplatin may provide more tolerable treatment to patient undergone cisplatin chemotherapy. It was earlier reported that *P. watsonii* extracts were more selective toward cancer cells as compare to normal cells (Ramasamy *et al*., 2012; Ramasamy *et al*., 2013), thus motivating further work to determine the combination effect of the extract and anticancer drug.

The first of its class of platinum-based drugs, cisplatin is the oldest drug that is used in the treatment of advanced cancer such as ovarian cancer and prostate cancer. Combination therapy of cisplatin with other anticancer drugs is current standard in cancer therapy in order to overcome or delay cancer resistance and reducing cytotoxicity of one drug. The first clinical trial on cisplatin combination is with drug adriamycin (doxorubicin). In early study showed that the combination of cisplatin and adriamycin improved survival rate of advanced gynaecological tumour (Peters *et al*., 1989). Following years showed various clinical trial and publication on various anticancer drug combination to provide advantages that are able to encounter tumour resistance, safer treatment while providing greater efficacy of cisplatin. Among them are drug such as paclitaxel (Taxol), which is used to treat breast, ovarian, lung and other cancers (Bocci *et al*., 2013).

Both adriamycin and paclitaxel were initially discovered from natural resources, with adriamycin produced by bacteria (Arcamone *et al*., 1969) and paclitaxel isolated from plant (Wall & Wani, 1995). Researches combined cisplatin with other natural product either raw extract or single compound, and the combination reported produces synergistic inhibition (He *et al*., 2016) or even enhanced cisplatin-induced cytotoxicity towards cancer cell lines (Wang *et al*., 2016). One study reported compound isolated from *Lithospermum erythrorhizon*, shikonin, combined with cisplatin exhibited synergistic anticancer efficacy and achieved greater selectivity towards colon cancer cells in comparison with normal cells (He *et al*., 2016), while in another published work, compound isolated from Chinese herbal medicine *Triptergyium wilfordii* combined with cisplatin produces synergistic effect and enhances cisplatin-induced cytotoxicity in gastric cancer cell line (Li *et al.,* 2012). Combination of *Aloe vera* crude extract (Hussain *et al*., 2015), gallic acid (Wang *et al*., 2016) and epigallocatechin-3-gallate from green tea (Kilic *et al*., 2015) alone with cisplatin, demonstrated synergistic effects in inhibiting the growth of breast cancer, small cell lung cancer and cervical cancer cells. These are evidences that supported the hypothesis that combination of cisplatin with natural products able to produce cytotoxic effect on various cancer cell lines of different origins and at the same time safer and tolerable in cancer therapy.

5.3 Combination Index Analysis

In the present study, cytotoxicity data of individual treatment of SKOV-3 cells with PW-E and cisplatin and the combination of PW-E/cisplatin at ratio at 4:1 were entered into CompuSyn 1.0 software for combination index (CI) analysis. The ratio was derived from each IC_{50} of individual treatments, based on recommendation by Chou and Martin for the Combination Index (CI) analysis (Chou & Martin, 2007). According to the guidelines, any combination values with CI>1, CI=1 and CI<1, indicate antagonism

interaction, additive interaction and synergistic interaction respectively between the two or more agents. Analyzed data showed that different interaction was observed at particular point of the PW-E/cisplatin combination, at 50% inhibition or lower concentration revealed synergistic effect, while antagonistic interaction was observed at higher concentration or at 75% and 90% inhibition of the cells.

Drug Reduction Index (DRI) was generated from the Compusyn 1.0 software. A DRI of drug/agent combination is a measure of how much (-fold) the dose of a drug or agent (e.g. PW-E or cisplatin) in synergistic combination may be reduced at a given effect level compared with the dose of each individual drug (Chou & Martin, 2007). DRI at each combination doses in the present study are more than one which indicate favourable dose reduction in cancer therapy. A favourable dose reduction suggests that PW-E/cisplatin combination has the ability to maintain therapy efficacy at lower doses as compared to individual agents, thus reducing the treatment toxicity.

Combination treatment between two or more drugs for diseases is intended to ward off resistance and reducing side effect of the drug. Treatment with a single drug is discouraged for the fear of the cancer cells or microbes becoming resistant towards the drugs. Multiple drug treatment will not only allow multiple targets in fighting the disease and promoting greater efficacy, they are also impossible to become resistance to multiple drugs with multiple targets simultaneously.

To date, there are no published works on the combination interaction between *P. watsonii* extract/compound with anticancer drug reported. However, limited research reported on the cytotoxic effect of extracts from different *Phyllanthus* species combined with anticancer drug. Study by de Araujo Jr *et al.* (2012) discovered that combination of *P. niruri* extract with cisplatin induces a synergistic increased in cellular apoptosis in both colon HT-29 and hepatocellular HepG2 cancer cells. Combination of *P. emblica* extract with anticancer drugs, doxorubicin or cisplatin was reported to produce

synergistic growth inhibitory effects against human hepatocellular HepG2 and lung A-549 cancer cells (Pinmai *et al.,* 2008). While another *Phyllanthus* species; *P. niruri* and *P. amarus* enhanced cell cycle arrest at S phase in human liver HepG2 cancer cells when combined with anticancer drug 5-fluorouracil (Guo *et al*., 2016).

In the present study, PW-E/cisplatin combination produces synergistic interaction at lower concentration, and antagonistic interaction at higher concentration. According to Yin *et al.* (2014) in their article on developing network topology of drug combination effects and drug targets prediction, treatments with multiple drugs depends on the interaction of their targets in network manner. This could contribute to the interaction to behave in unexpected ways and result in multitude of outcomes. Extract used in this study, PW-E are literally a cocktail of many compounds. Each one of them has their own bioactivity and its own molecular targets. Therefore, considering the claimed from Yin and colleague, combination of two or more compounds with drug could resulted in boosting or suppressing the effects of one or more agents due to the differences in molecular targets and unexpected network topology. In addition, compounds with similar chemogenomic profile or targeting neighbouring biological network such as the MAPK and P13K/Akt/mTOR pathways will more likely to produce synergistic interaction (Yin *et al*., 2014).

Cisplatin most well-known mechanism of action is it causing the DNA damages by forming platinum-DNA adduct and bind to the DNA molecules, in turn initiating ROS formation and multiple signalling pathways that will ultimately lead to apoptosis activation; while *P. watsonii* mode of action is still ambiguous and limited research was found. Several published articles from different authors explored *P. watsonii* possible mechanism of action in inhibiting the growth of the cancer cells. A study by Tang *et al*. (2010) reported that aqueous and methanolic extract of *P. watsonii* able to induced apoptosis on melanoma MeWo cells and prostate PC-3 cancer cells by promoting cell

cycle arrest at S phase for MeWo cells and G0/G1 phase for PC-3 cells. Through continuation of the study, the authors found out that the extracts mode of action disturbs multiple tumorigenesis signalling cascades in prostate PC-3 cancer cells that eventually leads to apoptosis of the cells (Tang *et al.*, 2013). Extract of *P. watsonii* also reported exerting anti-metastasis in A-549 cells (Lee *et al*., 2013) and MCF-7 cells (Lee *et al*., 2016) through extracellular signal-related kinase (ERK) and hypoxia pathways inhibition. The differences between extract and drug molecular targets may perhaps the deciding factor to its final outcome. However, true mechanism in which the combination of PW-E and cisplatin took to exert growth inhibitory effect on human ovarian SKOV-3 cells at this point of data is currently unknown and further study to ascertain the mechanism is needed in the future.

Antagonism, not necessarily suppressed the action of other drug, it may involve positive feedback loop or downstream link of various protein or targets. One interesting work reported that the pre-treatment of dexamethasone (a corticosteroid drug) in non-small cell lung cancer antagonizes paclitaxel-induced cytotoxicity through the suppression of ERK and dephosphorylation of pRB and raised the possibility to prevent paclitaxel adverse reaction during treatment (Morita *et al.*, 2007). While in study reported by Yeh *et al.* (2009), antagonistic interactions are promising to counteract against antibiotic resistant. According to Yeh and colleague, there are two types of antagonism interaction; buffering antagonism (when one drug completely hinder the effect of another) and suppression antagonism (when the drugs combination yields a higher inhibition rate than at least one of the single drugs by itself) (Yeh *et al.*, 2006). CI values that fall between 1.0 – 2.0 are considered as buffering antagonism and >2.0 are considered as suppression antagonism (Yin *et al.,* 2014). In the case of the present study, at IC_{75} and IC_{90} the CI were >1.45 and >2.0, which may indicate a buffering antagonism and suppression antagonism respectively.

Based on the sensitization factor value, it can be concluded that the present of PW-E in high concentration in PW-E/cisplatin combination has no sensitizing effect in increasing cisplatin inhibitory activity towards SKOV-3 cells. The sensitization factor affirmed that at higher doses, *P. watsonii* ethyl acetate extract unable to increase cisplatin cytotoxicity against human ovarian SKOV-3 cells. However, the combination also showed synergistic interaction at its IC_{50} and favourable dose reduction at all six-point combination concentrations, which suggest by combining the two test agents in treatments, raised the possibility to reduce cisplatin toxicity against normal cells and maintain the drugs' efficacy in chemotherapy.

5.4 Morphological Observation using Phase-Contrast Inverted Microscope

Morphological assessment of cells using inverted microscope fitted with phase-contrast objective are the simplest and cheapest method for apoptosis cells recognition. At present study, SKOV-3 treated with PW-E at 2 µg/ml, cisplatin at 0.5 µg/ml and PW-E/cisplatin combination at 2 + 0.5 µg/ml and after 48 hours' incubation cells were rinsed and observed under phase-contrast inverted microscope. Key morphological features of the cells observed in this experiment were membrane changes, reduction of cytoplasmic volume, adherence among sister cells, nuclei and chromatin changes. SKOV-3 without addition of any test agents displayed smooth and intact plasma membrane, while cells treated with extract, drug and extract/drug combination treatment were all exhibited apoptosis related morphological changes such as membrane blebbing, loss of contact with surrounding, shrinkage of cells and apoptotic bodies sighting. Ramasamy *et al*. (2012), reported human ovarian SKOV-3 cells that were treated with PW-E showed apoptotic features such as formation of apoptotic bodies, membrane budding and loss of adherence with neighbouring cells. Some of the cells also dislodged from the surface growth, a feature dubbed as anoikis, unique in adherent type cell. *In*

vivo, the feature may ensure the survival of the cells at other site (i.e metastasis), but *in vitro*, it is a signal of the downfall of the cells (Liotta & Kohn, 2004).

Morphological observation under phase-contrast microscope is convenient, yet effective screening method before other experimentation in order to examine possible apoptosis induction in cells underwent treatments with natural product or drugs, however, its main limitation entails that it is only qualitative and as such other method for apoptosis detection must be done to solidify the result (Gao *et al*., 2011).

5.5 Acridine Orange/Ethidium Bromide (AO/EB) Double Staining

In the present study, morphological changes of SKOV-3 cells treated with PW-E, cisplatin and PW-E/cisplatin combination (i.e 2 µg/ml, 0.5 µg/ml and 2 + 0.5 µg/ml respectively) after 48 hours incubation and followed by staining the cells with the mixture of double AO/EB and mounted on Teflon-coated glass slide, were immediately observed under inverted fluorescence microscope fitted with phase-contrast objective. Key features of the cells observed in this experiment were chromatin condensation and DNA fragmentation (Atale *et al*., 2014), which previously cannot be determined completely with simple morphological observation.

Acridine orange (AO) is vital stain where both viable and early apoptotic cells will fluoresce pale green to bright green under fluorescent microscope. Ethidium bromide (EB) is sensitive, easy stain for DNA. It is a DNA intercalator, inserting itself into the spaces between base pairs in the DNA double helix and fluoresce the cell orange to bright red. Among the two, only AO can diffuses into and differentiate between viable and early apoptotic cells, while EB only diffuses into the cells when the integrity of the membrane is loss (Kasibhatla *et al*., 2006). As such, normal, early apoptotic, late apoptotic and necrotic cells can be examined accurately.

In this study, live cells exhibited pale green colour with intact membrane, early apoptotic cells showed bright green centre with dense or crescent shape nuclei signify condensed or fragmented chromatin, late apoptotic cells display almost the same as early apoptotic cell except with orange chromatin and death cells showed bright orange or red nuclei (Atale *et al*., 2014). Few other published work using AO/EB staining method reported the similar observation (Citalingam *et al*., 2015; Liu *et al*., 2015; Navanesan *et al*., 2015). In conclusion, AO/EB double staining is inexpensive and easy to perform. It does not require large volumes of cultures nor wide range of instrument and can provide data immediately (Ogle, 2010). Its main drawbacks are extra caution must be excised, as they are suspected to be a potent mutagen, easily absorbs into the skin upon exposure and irritate the skin and eyes on direct contact.

5.6 Caspase-3 Activity in Individual and PW-E/Cisplatin Combination Treatments on Human Ovarian SKOV-3 Cancer Cells

Apoptosis is highly sophisticated and tightly regulated process as such; there are multiple mechanisms and various pathways that cells follow to execute apoptosis. The process is necessary in order to remove unhealthy or unwanted cell in an organism and allow the cells to maintain its normal functionality (Hanahan & Weinberg, 2011). The knowledge of how plant extract induce apoptosis in cancer cells is important in chemotherapy drug development. In general, a natural compound could induce apoptosis through various means among them were cell cycle alteration, disturbances of signal transduction pathways (i.e MAPK, NF-KB, COX-2), microtubules interference, and increment in caspases signalling (Safarzadeh *et al.,* 2014).

The present study would like to ascertain at which pathways, PW-E/cisplatin combination took in order to exert cell death mechanism. Initial data from morphological analysis and AO/EB double staining suggest SKOV-3 cells underwent

possible apoptosis process upon treatment with PW-E/cisplatin combination. During the cells undergoing apoptosis, morphological changes such as chromatin condensation, DNA fragmentation, membrane budding and apoptotic bodies formation are observed (Porter & Janicke, 1999).

Caspase-3 activity detection was selected due to the main factors that caspase-3 is the main marker that indicates apoptosis machinery, the central executioner (Wong, 2011) and its activation is by far the most frequent in mammalian cell apoptosis (Porter & Janicke, 1999). Cells treated with the test agents were lysed and its content was assayed using fluorometric kit to detect caspase-3 activity. Caspase-3 activity was determined using Caspase-3 DEVD-R110 Fluorometric and Colorimetric Assay Kit which exploits activated caspase-3 ability to hydrolyse flourogenic substrate (Ac-DEVD)-R110 to release fluorescent dye R110 and subsequent fluorescence detection is correlated with increased of caspase-3 activity (Hug *et al*., 1999).

The present study suggest caspase-3 was involved in triggering human ovarian SKOV-3 cell death mechanism when treated with PW-E/cisplatin combination at 72 hours. Caspase-3 activation increased with PW-E/cisplatin combination treatment and is as high as cell treated with cisplatin (positive control) and also when compared with untreated cells. This study is in agreement with work published by Ramasamy *et al*. (2012), though not a combination study, indicated that SKOV-3 cells treated with *P. watsonii* ethyl acetate extract at 10 µg/ml for 48 hours showed an increment of 2.9-fold higher than untreated SKOV-3 cells.

There is a limited amount of caspase-3 detection in combined treatment involving *P. watsonii* and cancer drugs, however, a few studies otherwise explored caspase-3 activities in cisplatin combination in other natural products. For example, the combined treatment of triptolide (compound isolated from *Tripterygium wilfordii*) and cisplatin showed an elevation of caspase-3 activities at 4.9 folds as compared to control

untreated cells, while cisplatin treatment alone at 2.0 folds after 24 hours treatment (C. Li et al., 2012).

Caspase-3 is activated dependent or independent with the release of cytochrome c from mitochondrion and activation of caspase-9. Its activation plays the central role in the execution phase of cell apoptosis (Porter & Janicke, 1999). At normal condition, caspase-3 existed as an inactive caspase-3 zymogen, a feature that will hinder it's activation from killing indiscriminately. Its existence is important for normal brain development and cell death of remarkable tissue. Caspase-3 is essential in process associated to cells dismantling, apoptotic bodies formation, chromatin condensation and DNA fragmentation (Porter & Janicke, 1999). Caspases, from initiator to executioner, are always activated during apoptosis induction and at its centre are the dominant caspase-3. Therefore, measuring caspase-3 activity in cell undergoing treatments is a convenient way of ascertaining apoptosis event occurrences.

5.7 LC-MS/MS Analysis of PW-E Chemical Components

P. watsonii was found to contain rich amount of polyphenols and flavonoids. Earlier phytochemical study showed that, chloroform extract of *P. watsonii* constitutes of multiple bioactive compound, such as friedelin, glochidone, glochidonol, lupenyl palmitate and lupeol which are triterpenes class (Matsunaga *et al.*, 1992). Tang and colleagues reported the detection of polyphenol compounds from both aqueous and methanol extract of *P. watsonii* using high-performance liquid chromatography (HPLC). The compound identified were gallic acid, corilagin, geraniin, rutin, quercetin glucoside, quercetin rhamnoside and caffeolquinic acid (Tang *et al.,* 2010). In a more recent study reported the presence of ellagic acid, betulin, phyllanthin and sterol glucoside component by LC-MS/MS analysis of *P. watsonii* hexane extract and fractions (Ramasamy *et al.,* 2012).

LC-MS/MS (Liquid Chromatography-Tandem Mass Spectrometry) at present offers far superior analytical sensitivity as compared to conventional HPLC and higher-throughput than Gas Chromatography Mass Spectrometry (GCMS). The system is so sensitive that it can analyse compounds with the same m/z and differentiated them. The system combined the liquid chromatography (LC) and two mass analysers in one mass spectrometry instrument (MS/MS) and quickly becomes the most preferred instrument for separating and analysing chemical component in laboratory (Grebe & Singh, 2011). In this study, LC-MS/MS analysis was performed on *P. watsonii* ethyl acetate extract and the analysis revealing the extract phenolic and flavonoids content. The detected compounds were gallic acid, strictinin, strictinin isomer, galloyl HHDP hexoside, strictinin isomer 2, hyperin, ellagic acid, kaempferol glucoside, kaempferol rhamnoside and quercetin and presence of some of this compounds may be attributed for *P. watsonii* cytotoxicity on cancer cells.

Gallic acid and ellagic acid from hydrolysed tannin class are naturally occurring phenolic content in many flowering plants (Maas *et al.,* 1991). Gallic acid itself is found in oak gall of various oak species where it gets its name (Mammela *et al*., 2000) and is usually bonded to form dimers such as ellagic acid. High content of ellagic acid naturally found in fruits from Rosaceae family (strawberry, raspberry, rosehip, blueberry) and pomegranates (Landete, 2011). Both are a potent antioxidant (Priyadarsini *et al*., 2002) and anti-inflammatory agents (BenSaad *et al*., 2017). Strictinin and its isomers (strictinin isomer, strictinin isomer 2, galloyl HHDP hexoside) are other components identified in *P. watsonii* which also belongs to the class of hydrolysable tannins. Strictinin was first discovered by Gen-Ichiro *et al.* (1983) in green tea leaf. It exhibits antioxidative effects against lipid peroxidation on human red blood cells (Zhou *et al.,* 2004). Strictinin also exhibited antiviral activities on influenza B virus and human parainfluenza virus type-1 *in vitro* (Saha *et al*., 2010).

Kaempferol glucoside, kaempferol rhamnoside and quercetin are flavonoids constituent reported in *P. watsonii* in this present study. Kaempferol and quercetin are most commonly found flavonoids in various plants, fruits and vegetables (Erlund, 2004) and reported to have wide range of biological activities such as anti-inflammatory, anti-allergy, antimicrobial, antidiabetic, neuroprotective, antiestrogenic, and antioxidant effects (Calderon-Montano *et al*., 2011). Kaempferol rhamnoside originated from crude ethyl acetate extract of *B. pinnatu* was shown to have antimicrobial activity on various bacteria i.e *Salmonella aureus, Pseudomonas aeruginosa, Salmonella. Typhi* (Tatsimo *et al*., 2012). Kaempferol rhamnoside and quercetin found in *Rosa damascena* Mill. petals was shown to have strong free radical scavenging ability (Yassa *et al*., 2009).

Among the detected flavonoids and tannins in the present study, most of them were reported to have anticancer effect. Kaempferol was reported to promote apoptosis in cancer cell while providing protective effect on normal cells (Chen & Chen, 2013). Kaempferol induced apoptosis in human cervical HeLa cancer cells through P13K/AKT and telomerase pathway with IC_{50} value 10.48 μM as compared to anticancer drug 5-fluorouracil at 1.40 μM (Kashafi *et al*., 2017).

While another flavonoids, quercetin was shown to induce apoptosis in human lung A-549 cancer cell via increasing the expression of apoptotic related gene bax (Zheng *et al*., 2012). In one extensive work that showcases its anticancer effects; Srivastava *et al*. (2016) reported that quercetin induced cytotoxicity on leukemic and breast cancer cell. It induces cell cycle arrest, binds to the DNA and significantly decreased tumour mass in mice model (Srivastava *et al.,* 2016). A potent antioxidant, ellagic acid from *Punica granatum* L. juice promotes apoptosis in HT-29 and HCT-116 colon cells at 100 μg/ml (Seeram *et al*., 2005). Gallic acid also known as an antioxidant agent, induced DNA damage and inhibited DNA repair gene expression in prostate cancer cell PC-3 (Liu *et al*., 2011) and inhibit lung cancer cell proliferation through increased ROS elevation and glutathione reduction (You *et al*., 2011).

In view to caspase-3 activation potential of the chemical constituents, several studies reported Kaempferol rhamnoside isolated from the leaves of *Schima wallichii* Korth. inhibited breast cancer cell growth (Diantini *et al.,* 2012) and prostate cancer cell line (Halimah *et al.*, 2015) through activation and upregulation of the caspase cascade pathway that includes caspase-3, caspase-8 and caspase-9. In another study, quercetin induced apoptosis by activating caspase-3 in human acute leukemia HL-60 cell line (Niu *et al.,* 2011) and also in human osteosarcoma MG-63 cells via mitochondrial-dependent pathway which includes activation of caspse-9 and caspase-3 (Liang *et al.,*

2011). Another component, ellagic acid promoted caspase-3 activation in apoptosis of human bladder cancer T24 cells (Li *et al.*, 2005).

LC-MS/MS analysis of *P. watsonii* has proven that a crude extract contains multiple chemical constituents and each one of them has its own biological effect that can acts as a potent anticancer agent. These components are potentially the reason for *P. watsonii* high cytotoxicity on human ovarian SKOV-3 cell.

CHAPTER 6: CONCLUSION

Natural product including plant-derived extract was used as medicine since the ancient time and still was today. For example, Eastern medicine was still popular alternatives in many parts of the world for treatments such as cancer and infection. Many of the current modern medicines were developed from natural product either through accidental discovery, nature scouting or extensive research. Either way, the nature is invaluable source for potential chemical constituent to be discovered and developed as super medicine.

The standard treatment for cancer always involves the combination of two or more anticancer drugs with the specific goal for achieving synergistic effect, resistance avoidance and providing a safer treatment to patient. However, the main drawbacks in modern chemotherapy drugs are they possess high toxicity and cancer cell resistant despite some of the regimens effectiveness in initial response of cancer treatment.

In this present study, cytotoxic effects of *Phyllanthus watsonii* ethyl acetate extract in combination with cisplatin on human ovarian SKOV-3 cells were investigated. Combination strategy of drug and plant extract is the current potential approach in cancer treatment. Combination concentrations in this study was based upon IC_{50} of the individual treatments, as recommended by the US NCI drug development guidelines that the concentration ideal for anticancer screening is 50% inhibitory concentration (Boyd, 1995). Plants enriched in polyphenols and flavonoids content and the usage of plant extract for many years had proven that they were relatively safe as long as responsibly utilized. Synergistic combination of the plant-derived extract/compound with anticancer drugs is expected to provide protective effect to normal cells due to its potent antioxidant activity, allowing the usage of anticancer drug at high doses with minimal side effects; reducing the doses of anticancer drug while still

maintain the same efficacy as individual treatments; and enhancing or boosting the sensitivity of anticancer drugs on cancer cells.

The evidence in our study revealed that the individual treatment of *P. watsonii* and cisplatin inhibited the growth of SKOV-3 cells was observed at dose-dependent manner and their combination produces greater cytotoxicity against the ovarian cancer cell. Moreover, combination index analysis through median-effect method showed that *P. watsonii*/cisplatin combination is able to reduce the dosage of individual test agents, in turn reducing toxicity while still give out the same inhibitory efficacy on SKOV-3 cells. The combination also showed greater selectivity towards ovarian cancer cells as compared to normal cells and revealed that the cell death is by initiation of apoptosis program through caspase-3 activation.

In order to explore more on the combination potential of *P. watsonii* in terms of synergism and antagonism, experimenting on cisplatin-resistance ovarian cancer cells line as resistance model is needed. In addition, the usage of sub-effective concentration ($<IC_{50}$) such as IC_{20}, IC_{25} are recommended for both extract and cisplatin in reducing cytotoxicity towards normal cell with more prominent synergistic interaction may also be observed. Detail work needs to be carried out in order to understand the mechanism of action undertake by *P. watsonii* to synergize with cisplatin on growth inhibition effect towards SKOV-3 cells especially on the study of the expression of protein or enzyme related to cisplatin resistance. Lastly, as *in vitro* and *in vivo* conditions are different, further investigation in animal model should be explored as well to find out if the dose reduction can be sustained in animal study or even true synergism can be achieved.

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