CYTOTOXIC EFFECTS OF *Phyllanthus watsonii* AIRY SHAW EXTRACT IN COMBINATION WITH 5-FLUOROURACIL ON HUMAN COLON CANCER CELLS

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CYTOTOXIC EFFECTS OF *Phyllanthus watsonii* AIRY SHAW EXTRACT IN COMBINATION WITH 5-FLUOROURACIL ON HUMAN COLON CANCER CELLS

ABSTRACT

Colorectal cancer (CRC) is one of the most common cancers that affects both men and women in Malaysia and is considered as one of the leading causes of death in the world. The chemotherapeutic agent that is used to treat CRC is usually 5-Fluorouracil (5-FU). However, the resistance of CRC cells to this drug and the side effects caused by this drug have prompted new strategies to overcome the shortcomings. The aims of this study are (i) to investigate the cytotoxic effects of *Phyllanthus watsonii* ethyl acetate extract (PW-E) in combination with 5-FU on human colon cancer cell lines HT-29 and HCT-116, (ii) to evaluate the possible interaction (synergistic, antagonistic or additive) occurs during combination between PW-E and 5-FU in their cytotoxicity based on the median-effect principle, and (iii) to determine the potential cell death mechanisms via apoptosis that is triggered by the combination of PW-E and 5-FU. The cytotoxic effects of PW-E, 5-FU and PW-E/5-FU combination on HCT-116 and HT-29 after 72 hours of were determined by Neutral Red Uptake (NRU) assay. The IC_{50} of PW-E, 5-FU and PW-E/5-FU combination (at a ratio of 3:1) on HCT-116 cell were 1.522 ± 0.16 µg/ml, 1.588 ± 0.46 µg/ml and 1.482 ± 0.25 µg/ml respectively while the IC_{50} on HT-29 were 0.030 ± 0.003 µg/ml, 19.70 ± 1.72 µg/ml and a combination of 0.03 µg/ml of PW-E and 10.0 µg/ml of 5-FU respectively. By combining PW-E with 5-FU, lower dose of drug was needed, indicating that the cytotoxicity effect of 5-FU towards the cancer cells was enhanced in the presence of PW-E. Our result also shows that the Combination Index (CI) of PW-E/5-FU combination analysed by Compusyn 1.0 software was < 1 on both HCT-116 and HT-29 cells. This indicates that PW-E/5-FU combination exerts a synergistic

effect on both CRC cells. In addition, PW-E/5-FU combination is selectively cytotoxic towards the CRC cells in comparison with the normal lung fibroblast MRC-5 cells. Drug Reduction Index (DRI) for PW-E/5-FU combination was measured on how much (-fold) the dose of a drug or agent in synergistic combination may be reduced at a given effect level compared with the dose of each drug alone. The DRI value for PW-E/5-FU combination on both HCT-116 and HT-29 is > 1 and shows that the combination is favourable in terms of clinical therapy. Morphological assessment by Acridine Orange / Ethidium Bromide (AO/EB) double staining showed that cell death mainly occurs by apoptosis instead of necrosis. Increase in caspase-3 activity was observed in CRC cells treated with PW-E/5-FU combination. PW-E was subjected to Liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) analysis and six main compounds were identified. The compounds detected were quercetin, kaempferol rhamnoside, kaempferol glucoside, ellagic acid, hyperin and strictinin isomer. In conclusion, herb-drug combination enhances the cytotoxic activity of 5-FU towards colorectal cancer cells and the cell death is mediated by apoptosis. Hence, further studies should be done to understand further the interactions between the herb and drug.

KESAN SITOTOKSIK EKSTRAK *Phyllanthus watsonii* AIRY SHAW DALAM KOMBINASI BERSAMA 5-FLUOROURACIL TERHADAP SEL KANSER KOLOREKTAL MANUSIA

ABSTRAK

Kanser kolorektal (CRC) merupakan salah satu jenis kanser yang paling kerap dihidapi oleh lelaki dan juga wanita di Malaysia dan juga merupakan salah satu penyebab kematian yang paling utama di seluruh dunia. Agen kimoterapi yang sering digunakan untuk merawat CRC adalah 5-Fluorouracil (5-FU). Tetapi, disebabkan oleh daya ketahanan sel CRC terhadap agen tersebut dan kesan sampingan yang dialami akibat penggunaan agen ini, stategi baru harus diformulasikan bagi mengatasi batas keupayaan agen kimoterapi ini. Tujuan kajian ini adalah untuk (i) mengkaji kesan sitotoksik ekstrak etil asetat daripada daun *Phyllanthus watsonii* yang digabungkan dengan 5-FU terhadap sel-sel CRC iaitu HT-29 dan HCT-116, (ii) untuk mengenal pasti interaksi (sinergi, antagonistic atau tambahan) yang terhasil apabila PW-E digabungkan dengan 5-FU terhadap kesan sitotoksisiti berdasarkan kepada prinsip kesan-median, dan (iii) untuk mengenalpasti mekanisma kematian sel yang dicetuskan oleh gabungan dos PW-E dan 5-FU. Kesan sitotoksik oleh dos tunggal PW-E, 5-FU dan dos gabungan PW-E dan 5-FU terhadap sel CRC HCT-116 dan HT-29 setelah 72 jam rawatan telah dikenalpasti melalui eset sitotoksik “Neutral Red” (NR). IC_{50} bagi PW-E, 5-FU dan kombinasi PW-E/5-FU pada nisbah (3:1) terhadap sel HCT-116 adalah masing-masing 1.522 ± 0.16 µg/ml, 1.588 ± 0.46 µg/mL and 1.482 ± 0.25 µg/ml, manakala IC_{50} terhadap sel HT-29 pula adalah masing-masing 0.030 ± 0.003 µg/ml, 19.70 ± 1.72 µg/ml dan campuran 0.03 µg/ml PW-E serta 10.00 µg/ml 5-FU. Dengan menggabungkan ekstrak PW-E dan 5-FU, dos ekstrak dan ubat telah dapat dikurangkan menandakan bahawa kesan sitotoksik agen kimoterapi tersebut telah dipertingkatkan. Nilai Gabungan Indeks yang dianalisis oleh

perisian Compusyn 1.0 menunjukkan nilai < 1 bagi kombinasi PW-E/5-FU yang diuji terhadap kedua-dua sel HCT-116 dan HT-29. Ini menandakan bahawa PW-E menyebabkan kesan sinergi apabila digabungkan dengan 5-FU terhadap sel CRC. Selain itu, kombinasi ubat dan ekstrak tersebut juga didapati lebih memilih ke arah sel CRC berbanding sel paru-paru MRC-5. Indeks Pengurangan Dos Ubat (DRI) telah diukur berdasarkan berapa kuantiti dos bagi kombinasi ubat dan ekstrak yang memberi kesan sinergi yang boleh dikurangkan tetapi masih memberi kesan yang sama seperti apabila ubat atau ekstrak digunakan secara berasingan. Nilai DRI bagi kombinasi PW-E/5-FU terhadap kedua-dua sel HCT-116 dan HT-29 adalah < 1, memberi indikasi bahawa kombinasi tersebut adalah bersesuaian dalam aplikasi terhadap terapi klinikal. Penilaian morfologi melalui perwarnaan dengan akridin oren dan etidium bromide AO/EB mendapati bahawa kematian sel adalah disebabkan apoptosis, bukannya nekrosis. Aktiviti caspase-3 juga menigkat apabila PW-E dan 5-FU digabungkan berbanding apabila ubat dan ekstrak digunakan secara individu. Kesimpulannya, gabungan ubat-herba meningkatkan kesan sitotoksisiti 5-FU terhadap sel kanser kolorektal. Interaksi sinergistik juga dapat dilihat apabila ekstrak digabungkan bersama agen kimoterapi dapam proses mematikan sel kanser. Kombinasi tersebut juga lebih kearah membunuh sel kanser dan meingkatkan aktiviti caspase-3 menandakan bahawa proses kematian sel meningkat. PW-E disubjekkan ke atas analisa LC-MS/MS dan enam sebatian utama dapat dikenalpasti. Sebatian-sebatian yang dikesan adalah kuercetin, kaempferol rhamnosida, kaempferol glucosida, asid ellagik, hiperin dan isomer striktinin. Kesimpulannya, kombinasi herba-ubat meningkatkan lagi aktiviti sitotoksik 5-FU terhadap sel kanser kolorektal dan kematian sel adalah disebabkan oleh apoptosis. Justeru, lebih banyak kajian harus dilakukan bagi memahami lagi interaksi antara herba dan ubat.

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Amira Nadirah

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LIST OF SYMBOLS AND ABBREVIATIONS

List of Symbols

ºC	:	Degree Celcius
≤	:	Less than or equals to
≥	:	More than or equals to
dH_2O	:	Distilled water
g	:	Gram
h	:	Hour
L	:	Litre
µg	:	Microgram
µg/ml	:	Microgram per mililitre
µl	:	Microlitere
mM	:	Micromolar
µM	:	Micromole
mg	:	Miligram
ml	:	Mililitre
min	:	Minute
MW	:	Molecular weight
m/z	:	Average molecular weight
%	:	Percentage
pH	:	Potential of hydrogen
v/v	:	Volume per volume

List of Abbreviations

5-FU	:	5-Fluorouracil
AIF	:	Apoptosis inducing factor
AO/EB	:	Acridine Orange/Ethidium Bromide
APC	:	Adenomatous polyposis coli
ATCC	:	American Culture Collection
Ca^{2+}	:	Calcium ion
CaCl	:	Calcium chloride
CI	:	Combination Index
CRC	:	Colorectal cancer
DHFU	:	Dihydrofluorouracil
DISC	:	Death-inducing-signalling-complex
DMSO	:	Dimethlysulfoxide
DNA	:	Deoxyribonucleic acid
DPD	:	Dihydropyrimidine dehydrogenase
DRI	:	Drug Reduction Index
DTT	:	Dithioteritol
ELISA	:	Enzyme-linked immunosorbent assay
EtOAc	:	Ethyl acetate
FBS	:	Foetal bovine serum
HCT-116	:	Human colorectal adenocarcinoma
HEPES	:	N-2-Hydroxylethyl-Piperazine-N-2-Ethane-Sulfonic acid
HHDP	:	Hexahydroxydiphenic acid
HT-29	:	Human colorectal carcinoma
IC	:	Inhibition concentration
LC-MS/MS	:	Liquid chromatography-mass spectrometry/mass spectrometry

LOH	:	Loss of heterozygocity
MeOH	:	Methanol
$NaHCO_3$	:	Sodium bicarbonate
NR	:	Neutral Red
NRU	:	Neutral Red Uptake
OD	:	Optical density
PBS	:	Phosphate buffer saline
PW	:	*Phyllanthus watsonii*
PW-E	:	*Phyllanthus watsonii* ethyl acetate extract
RNA	:	Ribonucleic acid
rpm	:	Rotation per minute
Rt	:	Retention time
RT-PCR	:	Real-time polymerase chain reaction
SBE	:	*Scutellaria barbata* D. Don extract
SFRE	:	Supercritical fluid rosemary extract
SI	:	Selectivity Index
sp.	:	Species
TS	:	Thymidylate synthase
UHPLC	:	Ultra-high performance liquid chromatography
UK	:	United Kingdom
USA	:	United States of America
WHO	:	World Health Organization

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

According to the World Health Organization (WHO) in 2017, cancer is one of the leading causes of death worldwide and was to be blamed for the death of 8.8 million of the world population in 2015. American Cancer Society defines cancer as a group of diseases characterized by out-of-control growth of the cells. The uncontrolled cell division harms the body by forming lumps or masses of tissues called tumours which can either be benign (accumulate in only one part of the body) or malignant (spreading to other parts of the body and destroying healthy tissues) (Lodish, 2000).

One of the most common cancers that affects both men and women is colorectal cancer (CRC). According to the National Cancer Registry Report, colorectal cancer is the most common cancer among the men and the third most common among women reported in Peninsular Malaysia (Lim *et al*., 2008). The cancer starts either in the colon or the rectum and is found on the epithelial lining of the large intestine or rectum. Rectal bleeding, pain of the abdomen, anemia, occult bleeding (gastrointestinal bleeding that is not visible) and change in the bowel habit are some of the symptoms of CRC (Majumdar *et al*., 1999). Early detection of colorectal cancer symptoms through intensive case-finding, raising awareness of the public and an open-access facility of sigmoidoscopy can help in decreasing the mortality rate of CRC (Mulcahy & O'Donohue, 1997).

There are various types of cancer treatments available nowadays. Treatments depends on the type and stage of the cancer. American National Institute of Health (2015) stated that most patients undergo combination treatments such as surgery with radiotherapy or chemotherapy and some might only need a single treatment such as either surgery or chemotherapy.

In the treatment of colorectal cancer, 5-Fluorouracil (5-FU) has been the focal drug used during chemotherapy (Carethers *et al*., 2004). Studies on several human colon cancer cell lines shows that 5-FU cause the cell death by promoting apoptosis (Violette *et al*., 2002). However, several disadvantages arise in the use of 5-FU in colorectal cancer chemotherapy, namely drug resistance and severe side effects such as irritation, reddening and excessive lacrimation (tear production) of the eyes (Christophidis *et al*., 1979), diarrhea, nausea and also low blood count in patients. Cardiac toxicity has also been associated with the use of 5-FU in cancer treatment (Freeman & Costanza, 1988). Therefore, researchers are finding better solution in improving colorectal cancer chemotherapy and overcome the disadvantages of using this drug.

One of the most widely used treatments for cancer is chemotherapy. Chemotherapy is the use of drugs to destroy cells, or commonly referred to as cytotoxic medication (Nordqvist, 2015). The agents used in existing clinical practice have played a substantial role in reducing mortality/morbidity and in increasing patient's quality of life (Suggitt & Bibby, 2005). During chemotherapy, patient will either be given only one specific type of drug (monotherapy) or a combination of more than one type of drugs (combination therapy). Chemotherapy drugs works by disrupting the cancer cell's capability to divide and reproduce (Nordqvist, 2015). Despite the recent advances of early cancer diagnosis and the improvement in clinical protocols for cancer treatment, the development of antineoplastic agents that combine the effectiveness, safety and convenience for the patient remains a great challenge (Ismael *et al*., 2008). However, one of the major problems in cancer chemotherapy is the toxicity effects of the drug (Prakash *et al*., 2013). In addition, the inability of the chemotherapy drugs to differentiate between healthy and cancer cells also cause adverse side effects such as stomach upset and hair loss (Fischer *et al*. 2003; Skeel *et al*., 2003). Most anticancer drugs also able to develop

multidrug resistance (MDR), have narrow therapeutic index, present unspecific biodistribution upon intravenous administration triggering undesirable side effects to healthy tissues. These drawbacks of conventional chemotherapeutic strategies commonly result in suboptimal dosing of chemotherapeutic agents, patients delaying and discontinuing treatments and even refusing to comply to the planned therapy (Ismael *et al*., 2008).

Plant-derived compounds have long been an important source of numerous clinically beneficial anti-cancer agents as they have been proven to be effective and safe in the treatment and management of cancers (Prakash *et al*., 2013). Over 60 % of the anti-cancer agents that are currently being used are derived from natural resources such as plants, marine organisms and microorganisms (Cragg & Newman, 2005). Convincing outcome in *in vitro* and *in vivo* preclinical studies have been reported to support the use of plant-derived compunds to treat several forms of cancers (Juàrez, 2014). There are four main classes of plant-derived anti-cancer agents that are currently being used clinically which are vinca alkaloids, epidophyllothxins, taxanes and camptotecins. These drugs have been used in the treatment of leukemias, lymphomas, breast, lung and Kaposi's sarcoma and testicular cancer for more than 40 years (Juàrez, 2014). The search for anti-cancer agents derived from plants began in the 1950s which lead to the discovery and development of vinca alkaloids isolated from the Madagascar periwinkle (*Catharanthus roseus* synonym *Vinca rosea*) and the isolation of cytotoxic podophyllotoxins from *Podophyllum* species (Cragg & Newman, 2005). These alkaloids and their semi-synthetic derivatives triggers the cancer cell death by blocking the metaphase stage of the cancer cells and also causes cell apoptosis (Juàrez, 2014).

Today, researchers have designed a new method in cancer chemotherapy treatment and management, which is by combining chemotherapeutic agent with plant extract/compound. Positive result has been shown where combination of the chemotherapeutic agent with plant extract/compound helps in reducing side effects, overcoming drug resistance, and enhancing the effect of the anti-cancer drug by synergistic interaction.

Hence, in this study, 5-FU was combined with ethyl acetate extract of *Phyllanthus watsonii* Airy Shaw (family: Phyllanthaceae), an endemic plant species grows in Peninsular Malaysia and the cytotoxic and interaction effects of these drug-extract combination towards selected colon cancer cell lines were investigated. Previous studies showed that the *P. watsonii* ethyl acetate extract selectively inhibited the growth of human colon HT-29 cancer cells via apoptosis and cell cycle modulation. *P. watsonii* ethyl acetate extract also had been reported to induce apoptosis in breast MCF-7, ovarian SKOV-3 and cervical Ca Ski cancer cell lines and the cytotoxicity is selective towards the cancer cell compared to normal cells (Ramasamy *et al*., 2012; Ramasamy *et al*., 2013).

Therefore, the specific objectives of this study were:

i) to investigate the cytotoxic effects of individual *Phyllanthus watsonii* ethyl acetate extract (PW-E) and 5-Fluorouracil (5-FU) on human colon cancer cell line, HT-29 and HCT-116;

ii) to evaluate the possible interaction (synergistic, antagonistic or additive) occurs during combination between PW-E and 5-FU in their cytotoxicity based on the median-effect principle; and

iii) to determine the potential cell death mechanisms via apoptosis that was triggered by the combination of PW-E and 5-FU.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction to Cancer

In 2012, it was estimated that about 14.1 million new cases of cancer occurred globally (United Kingdom National Health Service, 2016). Cancers can be defined as a group of diseases caused by an uncontrolled growth of cells, which will then producing tumour and invade other parts of the body (WHO, 2013). Cancer can affect almost every parts of the body. Cancer can either be benign in which the cancerous cells accumulate in only one part of the body or malignant, the cancerous cells spreading to other parts of the body and destroying healthy tissues (Lodish, 2000).

Cancers have their own traits, also known as the hallmarks of cancer, which are common in every types of cancer. The traits are biological capabilities attained during the multistep development of human tumours. The six hallmarks of cancer are (i) self-sufficient in growth signals, (ii) evading apoptosis or programmed cell death, (iii) insensitive to anti-growth signals, (iv) sustained angiogenesis by stimulating the growth of blood vessels to supply nutrients to tumours, (v) having a limitless capability to multiply, and (vi) able to invade local tissue and metastasize to other tissues (Hanahan & Weinberg, 2011) For a normal cell to progress into a neoplastic state, they acquire a succession of these traits. Radiotherapy, surgery, chemotherapy, hormone therapy, biological therapy and targeted therapy are some of the treatments used to cure cancer patients and the chances to cure is higher if the cancer is detected early (American Cancer Society, 2012; WHO, 2013).

2.1.1 Colorectal Cancer

Colorectal cancer (CRC) is one of the most common forms of gastrointestinal cancer (Goh *et al*., 2005) and is the third leading cause of cancer mortality throughout the world (Yu & Li, 2006). According to the National Cancer Registry Report 2003–2005, in Peninsular Malaysia, CRC is the most common cancer among the men and the third most common cancer among the women (Lim, 2014). Cancer starts to develop either in the colon or the rectum and is found on the epithelial lining of the large intestine or rectum (Hong *et al*., 2015).

Human digestive system including colon and rectum turns the food we eat into energy and packages the residue for excretion. Human colon is lined with rapidly dividing epithelial cells that are separated into individual compartment called crypts. A normal cell develops into a tumour when lesions occur on the individual crypts. The lesions on the crypts then progress into small adenomatous polyps and increase in size over time. These adenomas (benign tumour) can transform into carcinomas (malignancies that begin from the epithelial tissues), which will advance into metastatic colorectal cancer if left untreated (Kruzelock & Short, 2007). For a colorectal cancer to develop from an adenomatous polyp into an invasive cancer, it took approximately 8–10 years (Sung, 2007; Ramasamy, 2012).

The abnormal structure and numbers of chromosomes in CRC is largely due to chromosomal instability. The loss of heterozygocity (loss of one of the two parental alleles in the chromosome, LOH) is one of the consequences of chromosomal instability. LOH usually happens in the tumour suppressor gene p53, adenomatous polyposis coli (APC) and SMAD family member 4 (SMAD4). Similar to other types of cancer,

neoplastic cells of CRC are also observed having mutation of oncogenes and p53 genes and genetic instability (Teoh, 2016).

Treatment of colorectal cancer is usually by surgery, followed by radiotherapy and then chemotherapy using the chemotherapeutic agents (Li *et al*., 2009). Most of the chemotherapeutic agents that had been widely used in cancer treatment are usually single isolate constituents or combinations of drugs. 5-Fluorouracil, raltitrexed, irinotecan and oxaliplatin are some of the common chemotherapeutic agents used in the treatment of CRC (Gill *et al*., 2003). Among all the anti-cancer drugs listed, 5-Fluorouracil has been mainstay in the treatment of CRC and is the first significantly effective monotherapy agent for stage 3 of CRC (Kruzelock & Short, 2007).

2.1.2 5-Fluorouracil

5-FU is an analogue of uracil with a fluorine atom at the C-5 position in place of hydrogen and having a structure similar to the pyrimidine molecules of DNA and RNA (Zhang *et al*., 2008). Because of its structure, 5-FU are able to be converted into fluorouridine triphosphate (FUTP), fluorodeoxyuridine monophosphate (FdUMP) and fluorodeoxyuridine triphosphate (FdUTP).

FUTP, an active metabolite, disrupts RNA processing and function by being incorporated into the RNA, and lead to cell death. Meanwhile, FdUMP and FdUTP induces cell death by disrupting DNA synthesis (Longley *et al*., 2003). FdUMP forms a stable complex with thymidylate synthase (TS), thus inhibiting deoxythymidine monophosphate (dTMP) production. dTMP is essential for DNA replication and repair and its depletion therefore causes cytotoxicity (Parker & Cheng, 1990; Longley *et al*., 2003). Many studies show that 5-FU has been shown to promote cell death in several

colon cancer cell lines through apoptosis (Petak *et al*. 2000; Longley *et al*., 2004; Borralho *et al*., 2007).

2.1.3 Limitations and Side Effects of 5-FU

Despite its advantages, clinical applications of 5-FU have been greatly limited due to drug resistance (Zhang *et al*., 2008). Resistance to 5-FU is a multifactorial event (Zhang *et al*., 2008). There are reports stating that 5-FU depends on the p53 tumour suppressor protein to trigger apoptosis (Pritchard *et al*., 1998; Bunz *et al*., 1999; Petak *et al*. 2000; Borralho *et al*., 2007). p53 is a tumour suppressor protein that aids in regulating feedback towards 5-FU. If p53 protein is disrupted, it will cause the colon cancer cells to be resistant to 5-FU and cancer cells will fail to undergo apoptosis and cell cycle arrest (Mader *et al*., 1998; Bunz *et al*., 1999; Borralho *et al*., 2007; Ng *et al*., 2014).

In addition, the resistance of colorectal cancer cells towards 5-FU is also attributed to the increase dosage of 5-FU during treatment (Mader *et al*., 1998; Ng *et al*., 2014). Besides the resistance of cancer cells towards 5-FU, other major drawbacks of 5-FU are (i) adverse side effects; (ii) the dose-limiting toxicity of using this drug (Li *et al*., 2009); and (iii) its short half-life (Mader *et al*., 1998; Jin *et al*., 2002; Ng *et al*., 2014). 5-FU has a short half-life due to a rate-limiting step of 5-FU catabolism in normal and tumour cells in which dihydropyrimidine dehydrogenase (DPD)-mediated conversion of 5-FU to dihydrofluorouracil (DHFU). Up to 80% of administered 5-FU is broken down by DPD to DHFU in the liver causing it to have a short half-life (He *et al*., 2008; Zhang *et al*., 2008). These side effects worsen the life quality of the patients and contributes to the patient's refusal to continue treatment (Delval & Klastersky, 2002; Viale & Yamamoto, 2008; Li *et al*., 2009).

2.2 Cell Death

2.2.1 Cell Cycle

Cell cycle is a series of developmental stages that both normal and cancer cells go through, which plays an important role in forming new cells to replace injured or dead cells. During cell cycle, two identical cells will be reproduced. These two new identical cells produced can enter the cycle again and reproduce new cells again when needed.

The five stages of cell cycle are G_1 phase, S phase, G_2 phase, M phase and G_0 phase (American Cancer Society, 2015). The cycle is shown as a circle as the cell reproduction happens recurrently starting from the G_0 phase and ending at the M phase then circles back to the G_0 phase (Figure 2.1).

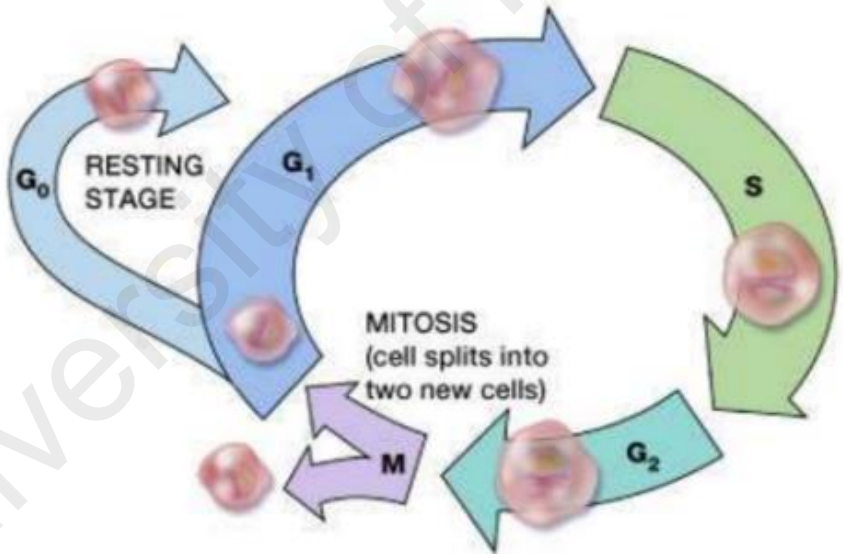


Figure 2.1: Progression through the five phases of the cell cycle, G_1 (gap 1), S (DNA synthesis), G_2 (gap 2) M (mitosis), and G_0 (resting phase) (American Cancer Society, 2015)

G_0 phase is the resting phase, in which in this phase the cell does not start to divide yet. When the cells at G_0 phase receives signalling for cell division, the G_1 phase starts. In the G_1 phase also known as the first gap phase, the cell prepares for division and undergoes metabolic changes such as increasing its protein amount and growing bigger.

Next, the single cell then moves to the S phase, where DNA synthesis occurs in the nucleus. In DNA synthesis process, chromosomes containing DNA are replicated so that both new cells formed will have matching DNA strands. It also grows centrosomes, a microtubule-organizing structure that helps separate DNA during M phase. The following phase is the second gap, G_2 phase. During this phase, the cell prepares for mitosis by growing more, producing more proteins and organelles and reorganizes its contents. This phase ends when mitosis begins, also called the mitotic, M phase. In this phase the nuclear division occurs, followed by cytokinesis where the cell separates into two new cells. The cell will then circulate back to phase G_1 to divide again or if the standard number of cell is maintained, the cell will move to G_0 phase until signals are received to begin dividing again (Alberts *et al*., 2002)

Since the process of cell division is strictly regulated, at the G_1/S boundary, intra-S, and G_2/M transitions, there is a defence system called checkpoints to resist DNA damage and genotoxic insult (Zhou & Elledge, 2000; Hyun *et al*., 2012). If these cycle regulators are flawed or mutated and do not respond to any signals caused by DNA damage, tumour will develop (Park & Lee, 2003). The cells with damaged DNA and cells which do not successfully complete DNA repair must continue with cell cycle arrest and removed by apoptosis (Bury & Cross, 2003; Hyun *et al*., 2012; Weitzman & Wang, 2013).

2.2.2 Apoptosis

Apoptosis, also known as programmed cell death is a highly-regulated process of cell death in all normal cell where unwanted or abnormal cells are eliminated. Apoptosis is activated by normal cells if a damaged DNA is detected. Cells proceeds to remove itself from the population as damaged DNA can render a cell useless and even harmful to an organism (Letai, 2008). Any defects occur at any point along the apoptosis pathways will

lead to the malignant transformation of the affected cells, tumour metastasis and resistance to anti-cancer drugs (Wong, 2011). Apoptotic cells can be characterized by shrinkage of the cell, condensation and fragmentation of the nucleus, blebbing of the plasma membrane and the formation of apoptotic bodies (Figure 2.2)

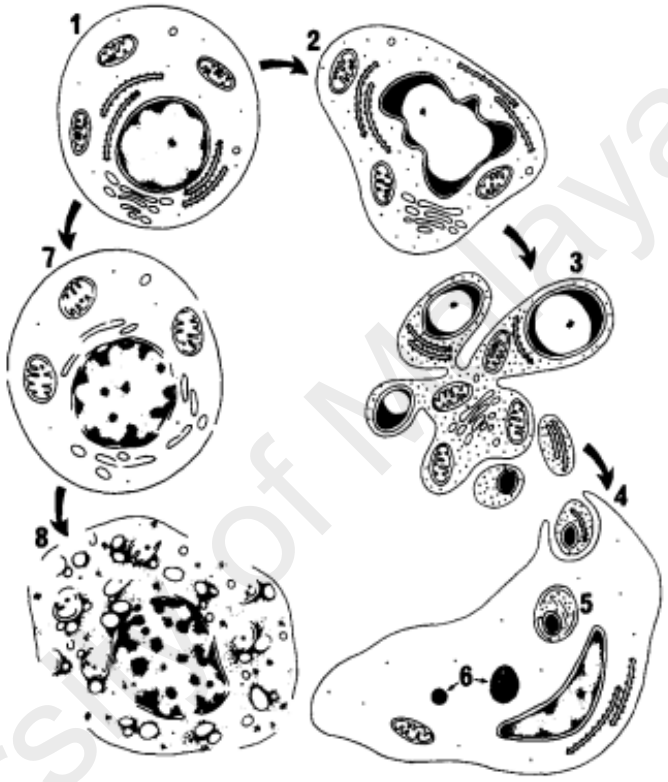


Figure 2.2: Ultrastructural changes in apoptosis (2–3) and necrosis (7–8). (1) shows the normal cell. Early apoptosis in (2) can be characterized by the condensation and margination of the nuclear chromatin, condensation of the cytoplasm and also convolution of nuclear and cell outlines. (3) shows at a later stage where the apoptotic bodies are formed when the nucleus fragments and swellings on the cell surface separates. (4) shows the apoptotic bodies being phagocytosed by nearby cells and (5-6) degraded within the lysosomes. (7) shows the necrosis process characterized by the irregular clumping of chromatin, distension of the organelles, and focal disruption of the membranes. (8) shows the subsequent breakdown of the membrane but the cells usually retain the overall shape until removed by mononuclear phagocytes. (Kerr *et al*., 1994)

Apoptosis mechanism is complex and involves many pathways.The mechanism of apoptosis has to be understood as it is vital and aids the understanding of disease development caused by disrupted apoptosis. There are two major pathways which will

lead to the execution of apoptosis: (i) the extrinsic or death receptor pathway, and (ii) the intrinsic or also known as mitochondrial pathway (Figure 2.3) (Wong, 2011). Caspase is a family of protease enzymes that playing crucial roles in apoptosis as they are the initiators and executioners in both of the apoptosis pathways.

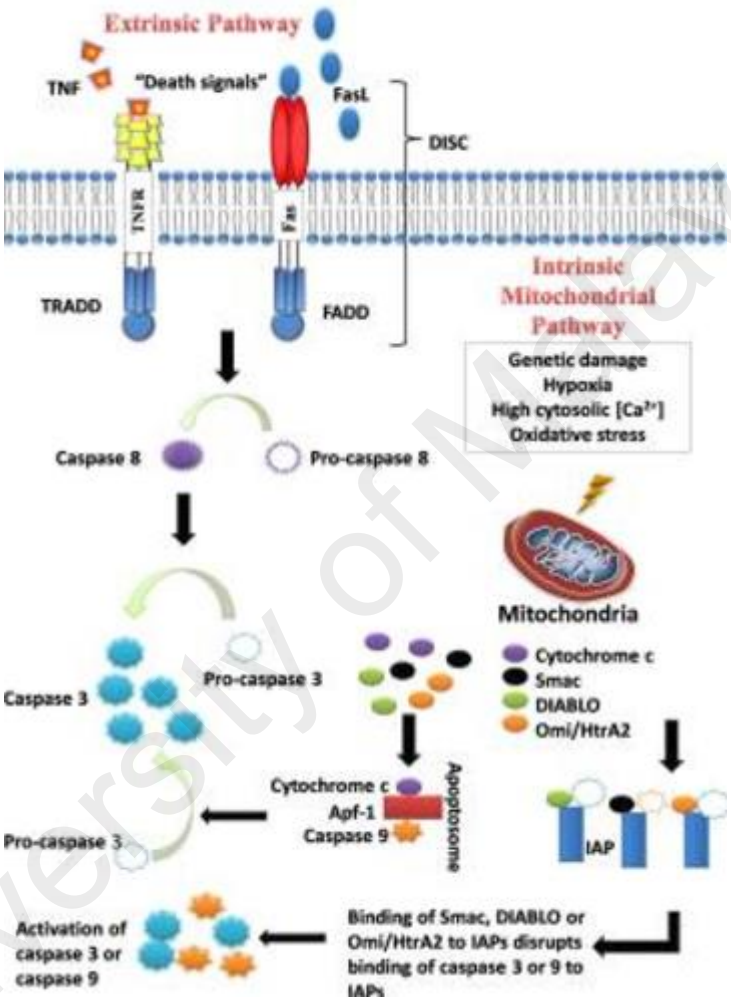


Figure 2.3: The intrinsic and extrinsic pathways of apoptosis (Wong, 2011)

Extrinsic pathway in apoptosis starts when death ligands bind to a death receptor (e.g. TNFR, FAS). The ligand for TNFR are TNF while for FAS is FasL (Hengartner, 2000). Adapter proteins such as TNF receptor-associated death doman (TRADD) and FAS associated death domain (FADD) and caspase-8 are recruited and activated. The binding site for an adaptor protein and the ligand-receptor-adaptor protein complex known as the death-inducing-signalling complex (DISC) is formed when the death ligand

bind to the death receptor (O'Brien & Kirby, 2008) which then starts the formation and activation of pro-caspase 8. Apoptosis starts when activated caspase-8 (initiator caspase) cleave other downstream or executioner caspases (Karp, 2008).

The intrinsic pathway occurs within the cell. The stimuli that activates the apoptotic pathway comprises of genetic damage that is irreparable, oxidative stress that is severe, hypoxia and the extreme concentration of cystolic Ca^{2+} (Karp, 2008; Wong, 2011). In intrinsic pathway, the anti-apoptotic proteins regulate apoptosis by blocking the release of cytochrome-c (hemeprotein that can initiate the activation of caspase), while the pro-apoptotic proteins act by promoting such release. The balance between the pro- and anti-apoptotic proteins determines whether apoptosis would be initiated.

Other apoptotic factors that are released from the mitochondrial intermembrane space into the cytoplasm include apoptosis inducing factor (AIF), direct IAP binding protein with low pI (Diablo), second mitochondria-derived activator of caspase (Smac) and Omi/high temperature requirement protein A (HtrA2). Upon receiving the stress signals, Bcl-2 family protein are activated and subsequently interact with and inactivate the anti-apoptotic Bcl-2 proteins. The interaction results in the increase in membrane permeability and the release of pro-apoptotic molecules such as cytochrome-c into the cytoplasm (Danial, 2004; Wong; 2011). The cytoplasmic release of cytochrome-c activates caspase-3 via the formation of a complex known as apoptosome, which is made up of cytochrome-c Apaf-1 and caspase-9 (LaCasse *et al.*, 2009; Wong, 2011). Smac/Diablo or Omi/HtrA2 promotes caspase activation by binding to inhibitor of apoptosis proteins (IAPs) which then loads to the interaction disruption of IAPs with caspase-3 or -9.

2.2.3 Apoptosis and Cancer

When too little apoptosis occurs, cancer will arise as the abnormal cells continue reproducing and evade death (Wong, 2011). There are several ways a cancerous cell can downregulate or hider apoptosis such as imbalance of the anti-apoptotic and pro-apoptotic proteins, disability of death receptor signalling and reduced caspase function (Wong, 2011). Table 2.1 summarized on the ways apoptosis is reduced and the impacts caused by the reduction.

Table 2.1: The ways apoptosis is reduced and the impacts caused by the reduction (Wong, 2011)

Means of reducing apoptosis	Impact of impaired apoptosis	References
Disrupted balance of pro- and anti-apoptotic proteins	Over expression of Bcl-2 family causes prostate cancer cells to evade apoptosis.	(Raffo *et a*l., 1995; Wong, 2011)
	Resistance of CRC to anti-cancer treatments is due to impaired apoptosis resulting from bax(G)8 frameshift mutation.	(Miquel *et al*., 2005; Wong, 2011)
	Abnormal p53 expressed in melanoma cells causes the continuous propagation of this cell.	(Avery-Kiejda *et al*., 2011; Wong, 2011)
	Abnormal expression of Inhibitor of Apoptosis Protein (IAPs) causes pancreatic cancer cells to become resistant to treatment.	(Lopes *et al*., 2007; Wong, 2011)
Reduced caspase function	Patients suffering from stage II colorectal cancer have been found to have a downregulation of caspase-9 activity.	(Shen *et al*., 2010; Wong, 2011)
	Pathogenesis of choriocarcinoma has been found to be caused by the downregulation of both caspase-8 and -10.	(Fong *et al*., 2006; Wong, 2011)

Table 2.1, continued

Means of reducing apoptosis	Impact of impaired apoptosis	References
Impaired death receptor signalling	Leukaemia that is resistant to treatment have been found to be caused by the reduced expression of CD95, a death receptor signal.	(Friesen *et al*., 1997; Wong, 2011)

However, researchers have also found that apoptosis process plays important role in cancer treatment. New strategies have been developed to restore abnormal apoptotic pathway and stop the proliferation of cancer cells. Several ways cancer can be treated is by targeting the apoptosis factors including Bcl-2 family of proteins, p53, the IAPs, and caspases. Table 2.2 summarized on the apoptosis-based treatment strategy in cancer therapy.

Table 2.2 Summary on the apoptosis-based treatment strategy in cancer therapy (Wong, 2011)

Treatment strategies	Summary of treatment strategy	Example	References
Targeting the Bcl-2 family of proteins	Inhibiting anti-apoptotic proteins by therapeutic agents	Usage of oblimersen sodium when combined with conventional anti-cancer drugs showed chemosensitizing effects in chronic myeloid leukaemia patients.	(Rai et al., 2008; AbouNassar and Brown, 2010; Wong, 2011)
Targeting p53	Gene therapy	Tumour cells of head and neck, prostate cancer and glioma and colorectal cancer are sensitized to ionizing radiation when wild type p53 gene is introduced .	(Chène, 2001; Wong, 2011)

Table 2.2, continued

Treatment strategies	Summary of treatment strategy	Example	References
Targeting caspases	Caspase-based drug therapy	By lowering the activation threshold of caspase, small molecules caspase activators have been reported to increase the sensitivity of cancer cells towards drugs.	(Philchenkov *et al*, 2004, Wong, 2011)

2.3 Drug Combination Strategy in Cancer Treatment

For decades, the standard treatment for most cancers are by drug combination strategy (Chou, 2006). Besides cancer, this strategy is also applied in the treatment of other diseases such as malaria, hypertension and other infectious diseases (Wagner, 2011). Combination chemotherapy is a growing interest among researchers (Pinto *et al*., 2011). One of the main reasons combination chemotherapy is applied is to use drugs that work by different mechanisms, thus reducing the probability of cancer cells developing resistency towards the drugs (Chabner *et al*., 2017). Each drug can also be used at its optimal dose without undesireable side effects when drugs with different effects are combined (Chabner *et al*., 2017). Combining anticancer drugs is also intended to maximize the efficacy of the drugs while minimizing the systemic toxicity through the delivery of lower drug doses (Pinto *et al*., 2011). Throughout the development of combination chemotherapy, the essential principles have remained largely unchanged. According to Pinto *et al.* (2011), the general principles of combination chemotherapy have been to: i) in order for a drug to be administered at a near-maximal dose, drugs with non-overlapping toxicities are used; ii) agents with dissimilar mechanisms of action and minimal cross-resistance are combined in order to hinder the development of broad

spectrum drug resistance; iii) single drugs with proven activity are favourably used; iv) combination is administered at early stage of the disease and at a schedule with a minimal treatment-free period between cycles but still allowing the recovery of sensitive target tissues. Combination chemotherapy has been attributed to improve the compliance of patients due to the number of drug administrations being reduced, capability to overcome or delay MDR, the emergence of additive or synergistic interaction effects, and the reduction of drug concentration with subsequent diminishing of toxicity to healthy tissues (Pinto *et al*., 2011).

2.4 Plant Natural Products

Plants have been used for thousands of years to treat illnesses, health disorders and also to prevent diseases. The information of their therapeutic properties has been conveyed over the centuries within and among societies. In the early 1900s, before synthetic drugs were being widely used as medicine, 80% of all remedies were obtained from leaves, root and barks of plants (McChesney *et al.*, 2007). Nowadays, plant-derived natural products have been used significantly to formulate drugs and therapeutic agents as approximately 60% of anti-cancer drugs and 75% of drugs for infectious diseases are either derived from natural products or are natural product derivatives (McChesney *et al*., 2007).

In contrast to plant's primary metabolites (phytosterols, acyl lipids, amino acids, and nucleotides), plants secondary metabolites do not participate directly in the growth and development of plants (Harborne, 1993) and are unique among other taxonomic groups of the Plant Kingdom (Croteau, 2000). Plant natural products can be divided into three major groups based on their biosynthetic origins, which are the terpenoids, alkaloids and the allied phenolic compounds (Bourgaud *et al*., 2001).

The growing curiosity in plant natural products was due to their great conveniences as polymers, dyes, fibers, oils, waxes, glues, perfumes, drugs and also flavouring agents (Croteau, 2000). The secondary metabolites of plants were also found to have an exceptional supply of new medical coumpunds (Nirmala *et al*., 2011) and have a better patient tolerance and acceptance (Veeresham, 2012). This has fuelled the search for new drugs, herbicides, antibiotics and insecticedes and hopefully would find cures to various diseases. Morphine from opium plant was the first pure natural product that were used as medicine (Veeresham, 2012). Since then, more drugs are being discovered and formulated based on plant's natural product and some are even widely used till this day and age. Table 2.3 summarized on some of plant-derived therapeutics agents and their application. The discovery of natural products have played a crucial part in human health due to their safety and efficacy.

Table 2.3 Plant-derived therapeutic agents and their applications (Veerasham, 2012)

Compound	**Plant Species**	**Therapeutic Application**
Paclitaxel	*Taxus brevifolia*	Treatment of lung, ovarian and breast cancer.
Artemisinin	*Artemisia annua*	Treatment of multidrug resistant malaria
Silymarin	*Silybum marianum*	Treatment of liver diseases
Nitisinone	*Callistemon citrinus*	Treatment of antityrosinaemia
Galantamine	*Galanthus nivalis*	Treatment of Alzheimer's disease

Table 2.3, continued

Compound	Plant Species	Therapeutic Application
Tiotropium	*Atropa belladonna*	Treatment of chronic obstructive pulmonary disease
Dronabinol and Cannabidiol	*Cannabis sativa*	As pain relievers
Capsaicin	*Capsicum annuum*	As pain relievers

2.4.1 Plant as Anti-Cancer Agents

In 1950, the search for anti-cancer agents derived from plants began with the discovery and development of vinca alkaloids isolated from the Madagascar periwinkle (*Catharanthus roseus* synonym *Vinca rosea*) and the isolation of cytotoxic podophyllotoxins from *Podophyllum* species (Cragg & Newman, 2005). Some anti-cancer agents isolated from plants that are currently being used all over the wolrd are taxol, vinblastine, topotecan, irinotecan, derivatives of comptothecin and etoposide derived from epipodophyllotoxin. Table 2.4 summarizes on anti-cancer agents isolated from plants. Higher plants that were selected as candidates in drug development must have the greatest probability of success. Researchers usually depends on the ethnomedicine information to explore its effectiveness prior development of drugs (Manju *et al*., 2012).

Table 2.4: Anti-cancer agents isolated from plants and their therapeutic applications (Manju *et al*., 2012).

Compound	Plant Species	Therapeutic Application	References
Vincristine and Vinblastine	*Catharanthus roseus*	Treatment of leukaemias, lymphomas, advanced testicular cancer, breast and lung cancers, Karposi's sarcoma	(Cragg & Newman, 2005)

Table 2.4, continued

Compound	Plant Species	Therapeutic Application	References
Topotecan and Irinotecan	*Camptotheca acuminate*	Treatmenr of ovarian and small cell lung cancers and colorectal cancers	(Creemers *et al*. 1996; Bertino, 1997)
Epipodophyllotoxin	*Podophyllum peltatum* and *Podophyllum emodi*	Treatment of lymphomas, and bronchial and testicular cancer	(Harvey, 1999)
Homoharringtonine	*Cephalotaxus harringtonia*	Treatment of acute myelogenous leukaemia and chronic myelogenous leukaemia	(Cragg & Newman, 2005)
Combretastatins	*Combretum caffrum*	Treatment of colon and lung cancers and leukaemia	(Itokawa & Wang, 2005)
Elliptinium	*Bleekeria vitensis* A. C. Sm.	Treatment of breast cancer	(Cragg & Newman, 2005)

2.4.2 *Phyllanthus*

Phyllanthus species belongs to the family Phyllanthaceae and is the largest genus in the family. The Phyllanthaceae family consists of about 2,000 species with 60 genera (Samuel *et al*., 2005). *Phyllanthus* species are mostly shrubs, herbs and trees (Burkill, 1966; Ramasamy, 2012) and usually found in most tropical and subtropical countries (Eweka & Enogieru, 2011). The growth forms of *Phyllanthus* species are very diverse, including annual and perennial herbaceous, climbing, aborescent, and phyllocladous (Jagessar *et al*., 2008).

In traditional medicine, *Phyllanthus* have been used as medication to chronic liver disease (Liu *et al*., 2003). *Phyllanthus* has also been used in Ayurvedic medicine to treat gnorrhea, dysentery, jaundice, frequent menstruation, diabetes, skin swellings, ulcers, sores and also itchiness (Tang *et al*., 2013). In addition, in Traditional Chinese Medicine, *Phyllanthus* has been used for the treatment of kidney stones and gallstones (Tang *et al*., 2014). In addition, studies shows that extracts of *Phyllanthus* contain several valuable pharmacological effects, including antiviral activity against hepatitis B, antidiabetic, antibacterial, antihepatotoxic activities (Ramadasan & Harikumar, 2011; Dhongade & Chandewar, 2014; Tang *et al*., 2014) and also anti-cancer properties against different cancer cell lines of different origins (Lee *et al*., 2011; Tang *et al*., 2014). Some of the bioactive compounds reported in *Phyllanthus* species are geraniin, rutin, gallic acid and quercetin (Tang *et al*., 2014).

2.4.3 *Phyllanthus watsonii* Airy Shaw

Belonging to the family Phyllanthaceace, *Phyllanthus watsonii* Airy Shaw is an endemic species to Peninsular Malaysia and only found grown at the riverside of the Endau river of Endau Rompin, Johor (Ramasamy *et al*., 2012). *P. watsonii* is consumed as a tonic by the native people of Jakun of Kampung Peta, Endau Rompin (oral communication). *P. watsonii* was selected in this present study based on the previous reported work that shows the extracts of *P. watsonii* selectively inhibiting the growth of colon HT-29 cells via apoptosis and cell cycle modulation (Ramasamy *et al*., 2012).

CHAPTER 3: MATERIALS AND METHODS

3.1 Plant Materials

Leaves of *Phyllanthus watsonii* were collected from Endau Rompin National Park, Johor (Peninsular Malaysia). The authentication of the plant species was performed by Dr Sugumaran Manickam from Rimba Ilmu Botanical Garden, Institute of Biological Sciences, University of Malaya. The voucher specimen (Reference no. KLU46048) was deposited at the herbarium of Rimba Ilmu Botanical Garden, Institute of Biological Sciences, University of Malaya.

Figure 3.1 : *Phyllanthus watsonii* Airy Shaw (Ramasamy *et al*., 2012)

3.2 Preparation of *P. watsonii* Ethyl Acetate Extract

Organic solvents with analytical grade were used for extraction and fractionation process of the plant sample. The organic solvents such as hexane, ethyl acetate and methanol were purchased from Fisher Scientific, UK. The leaves of *P. watsonii* (PW) were cleaned, sliced, dried, grounded, and extracted three times with methanol (Fisher Scientific, UK) at room temperature for 72 h. The solvent-containing extract was filtered with Whatman No.1 filter paper (Whatman, England) and the excess solvent was

evaporated under reduced pressure using a rotary evaporator (Buchi, Switzerland) resulting in a dark-greenish methanol extract. The extract was then further shaken vigorously with hexane (Fisher Scientific, UK) also until it appeared almost colourless. The chemical compounds that are soluble in hexane were filtered and pooled, followed by concentration under reduced pressure by rotary evaporator to yield hexane extract. Then, the remaining hexane insoluble compound was subjected to solvent-solvent extraction with a mixture of ethyl acetate (Fisher Scientific, UK) and distilled water in a ratio of 1 : 1 followed by vigorous mixing. This mixture was then successively fractionated using a separating funnel in which two distinct layers were formed. The bottom layer (water layer), where the residual water gathered was discarded while the ethyl acetate phase (top layer) was released into a clean beaker. The resultant filtrate was then concentrated under reduced pressure using a rotary evaporator to yield ethyl acetate extract (PW-E) which was used in the bioassay test. For the bioassay test, the PW-E was dissolved in dimethylsulfoxide (DMSO) (Sigma) as a stock solution with a concentration of 20 mg/ml before being further further diluted in 10 % DMSO. Figure 3.2 shows the schematic diagram of the extraction preparation procedure.

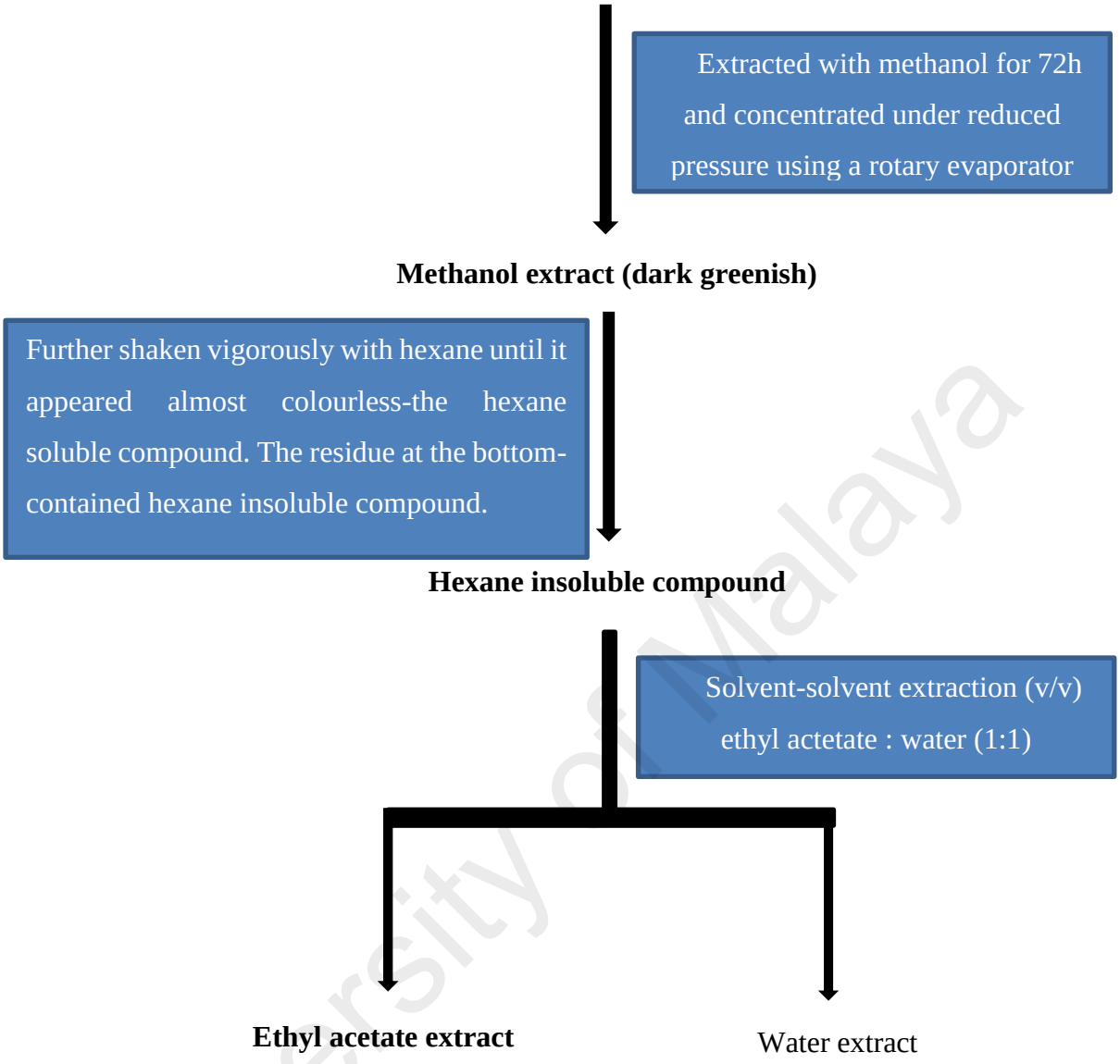


Figure 3.2: Schematic Diagram of Extraction Procedure

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

To determine the phytochemical compounds present in PW-E, the extract were analysed using a AB Sciex 3200Q Trap LC-MS/MS system with a spectrophotometer and an Ultra-high performance liquid chromatography (UHPLC) system (Perkin Elmer FX15) installed according to the method described by Ramasamy *et al*., (2012) with slight modifications. The chromatographic separation were performed on a column eluted with a mobile phase consisting of water (A) and acetonitrile (B) containing 0.1 % formic acid and 5 mM ammonium formate. A gradient elution of different concentration of A and B was used to separate the compounds of interest before the mass spectral analysis. The mass spectrometer analysis were performed in a positive ion mode for the detection of secondary compounds. Identities of the compounds were obtained by matching their molecular ions with reference standards and also by correlation with previoud published data on the chemical compounds of *P. watsonii*.

3.4 Preparation of Culture Media and Other Solutions

McCoy's 5A powder and Minimum Essential Medium (MEM) powder and Foetal Bovine Serum (FBS) were purchased from Sigma-Aldrich, USA. Penicillin-streptomycin (100×) and amphotericin B (250 µg/ml) were obtained from PAA Lab, Austria. Accutase in DPBS, 0.5 mM EDTA was purchased from iCT, CA. N-2-Hydroxylethyl-Piperazine-N-2-Ethane-Sulfonic acid (HEPES) was purchased from Molekula, UK. Neutral Red was purchased from ICN, Ohio. All other chemicals and solvents used were of the highest purity and grade available purchased from BDH AnalaR, UK and Sigma-Aldrich, USA. Cell culture plastic ware was from Nunc (Denmark) and 96-well culture plates were purchased from Orange Scientific (Belgium).

3.4.1 Basic McCoy's 5A Medium

A bottle of McCoy's 5A powder (Sigma-Aldrich, USA), was mixed with 1 L of sterile distilled water. Then, 0.2603 g of N-2-Hydroxylethyl-Peperazine-N-2-Ethane-Sulfonic acid (HEPES) (Molekula, UK) and 2 g of sodium bicarbonate ($NaHCO_3$, R & M Chemicals, UK) were added to the mixture. The pH of the medium was then adjusted to 7.4 using pH meter (Thermo Scientific) and then sterilized by filtration using 0.22 µm filter membranes (Orange Scientific). The sterilized medium was then stored in a sterile bottle at 4 °C for up to 4 months.

3.4.2 10 % Supplemented McCoys Medium

90 ml of the prepared basic McCoy's 5A (Sigma-Aldrich, USA) media were mixed with 10 ml of inactivated FBS (Sigma-Aldrich, USA) to produce 100 ml of 10% supplemented McCoy's 5A (Sigma-Aldrich, USA) medium. The medium was then sterilized by filtration using a 0.22 µm filter membrane (Orange Scientific) and stored at 4 °C for up to two weeks.

3.4.3 Phosphate Buffered Saline (PBS) pH 7.4

1.52 g of sodium phosphate anhydrous (NaH_2PO_4, Merck), 0.58 g of potassium dihydrogen phosphate (KH2PO4, R & M Chemicals) and 8.4 g of sodium chloride (NaCl, R & M Chemicals) were mixed with 1 L of distilled water in the preparation of PBS. The pH of the PBS was then adjusted to 7.4 and then sterilized by filtration using 0.22 µm filter membrane (Orange Scientific). The PBS was then further sterilized by autoclave at 112 °C.

3.4.4 0.4 % Tryphan Blue Solution

0.2 g of tryphan blue powder (Sigma-Aldrich, USA) was dissolved with 50 ml of distilled water in the preparation of 0.4 % tryphan blue solution.

3.4.5 Neutral Red Stock Solution

Neutral Red stock solution was prepared by dissolving 0.4 g of Neutral Red dye (R & M Chemicals) in 100 ml distilled water and then stored at 4 ° C.

3.4.6 Neutral Red Medium

Neutral Red medium was prepared by diluting the Neutral Red stock solution stock solution with 10 % supplemented McCoy's 5A (Sigma-Aldrich, USA) medium at a ratio of 1:80 to produce a final concentration of 50 µg/ml. The prepared Neutral Red medium was incubated overnight in the dark at room temperature and then centrifuged twice at 1,500 rpm for 10 min (Kubota 2010, Tokyo) prior to usage to remove any fine and needle-like precipitate of dye crystals.

3.4.7 Neutral Red Washing Solution

Neutral Red washing solution was prepared by dissolving 1 g of calcium chloride (CaCl, Systerm) in 99.5 ml of distilled water and 500 ml of formaldehyde (R & M Chemicals). The prepared solution was stored at 4 °C.

3.4.8 Neutral Red Resorb Solution

Neutral Red resorb solution was prepared by mixing 50 ml of absolute ethanol (Fisher Scientific, UK) with 1 ml of acetic acid (Fisher Scientific, UK) and 49 ml of distilled water and then kept at 4 °C.

3.5 Cell Lines

The human colorectal carcinoma HCT-116 and human colorectal adenocarcinoma HT-29 cancer cell lines and human normal MRC-5 lung fibroblast cells were purchased from American Type Culture Collection (ATCC, USA). The colorectal cancer cells were cultured in 10 % supplemented McCoy's medium while the normal lung fibroblast cell was cultured in MEM supplemented with 10 % (v/v) FBS. The cells were maintained in 25 cm^3 flasks with 10 ml media and then incubated at 37 °C in an incubator with 5 % CO_2 in a humidified atmosphere. The cells were subcultured every 2 – 3 days and routinely checked under an inverted microscope for any contamination.

The HCT-116 cell line is derived from poorly differentiated (lack of gland-like structure or normal specialized structure) colorectal carcinoma wih Duke's D stage while HT-29 cell line is derived from Duke's stage C colorectal adenocarcinoma (Ahmed *et al*., 2013). HCT-116 cell line has high clonogenicity and lack of differentiation capacity (Yeung *et al*., 2010). It is mainly used to study transfection and development of isogenic cell lines (pair of cell lines with similar genetic background except one targeted gene which is altered by knock-in or knock-out techniques which can lead to insights on the function of a specific gene (Teoh, 2016). Being able to adapt and resist against the cytotoxicity of 5-FU through the activation of survival autophagy and the lack of wild-type p53, HT-29 cell line can act as a model for 5-FU resistance in researches (Sui *et al*., 2014).

3.6 Neutral Red Uptake (NRU) Cytotoxicity Assay

The NRU cytotoxicity assay was modified based on the initial protocol described by Borenfreund & Puerner (1984). The cytotoxicity of the extracts was measured by NRU assay which is based on the uptake and subsequent lysosomal accumulation of the supravital dye, neutral red in the viable and uninjured cells.

Firstly, the cells were detached from the flask with 1.0 ml solution of accutase in 3 ml phosphate buffer solution (PBS) pH 7.4. The cell pellet was then obtained by centrifugation at 1,000 rpm for 5 min and the density of the viable cells were counted by 0.4 % of tryphan blue exclusion method using a haemocytometer. The cells were then seeded in 96-well micro titer plate, at a concentration of 30,000 cells/ml and then incubated in an incubator at 37 ºC for 3 h to allow the cells to adhere before addition of the test agents. The experiment consisted of four groups for each cell line: (i) negative controlled cells only (without addition of any test agents); (ii) positive control (cancer cells treated with different 5-FU concentrations selected based on the sensitivity of the colon cancer cells towards the exposure to 5-FU (concentration ranging from 1.0 to 100.0 µg/ml for human colorectal adenocarcinoma HT-29 cell line and 0.1 to 10 µg/ml for human colorectal carcinoma HCT-116 cell line) ; (iii) cancer cells treated with PW-E (concentration range from 1.0 to 100.0 µg/ml); (iv) cancer cells treated with 5-FU/PW-E combination. Series of PW-E with concentrations which gave 10 % to 90 % cell growth inhibition were combined with different concentrations of 5-FU for the combination index (CI) analysis. In this study, the concentration of PW-E/5-FU combination at ratio 3:1 (constant combination ratio) based on the series of PW-E concentrations that give 10 % to 90% cell growth inhibition were used for the study on HCT-116 cell line while for HT-29, non-constant combination ratio were used whereby the concentration of PW-E remains

constant at 0.03 µg/ml while the concentration of 5-FU were 30.0, 25.0, 19.0, 15.0 and 10.0 µg/ml respectively.

At the end of the incubation period, the medium was replaced with Neutral Red medium and incubated for further 3 h to allow for uptake of the vital dye into the lysosomes of viable and uninjured cells. The medium was then removed and cells were rapidly washed with the Neutral Red washing solution. The dye within viable cells was then eluted from the cells with Neutral Red Resorb solution and incubated for 1 min at room temperature with the plates agitated on a micro titer plate shaker for 30 min and then optical density (OD) against a blank reference was measured at 540 nm using Multiskan Go micro-plate reader (Thermo Fisher Scientific, USA). The experiments were done in three independent experiments for each of the test agents. The percentage of both HCT-116 and HT-29 cell inhibition were calculated according to the following formula.

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

3.7 Cytotoxicity (IC_{50}) Analysis

In the present study, the cytotoxic activities of *P. watsonii* ethyl acetate extract, PW-E and chemotherapeutic drug, 5-Fluorouracil (5-FU) were screened by Neutral Red Uptake (NRU) assay against human colon HCT-116 cancer cell line; and human fetal lung fibroblast MRC-5 cell line. PW-E was tested at concentrations ranging from 0.1 – 10 µg/ml towards HCT-116 cell line, and 1 – 100 µg/ml towards MRC-5 cell line. 5-FU were tested at concentrations ranging from 0.1 – 10 µg/ml towards HCT-116 cell line, and 10 – 1000 µg/ml towards MRC-5 cell line.

A complete dose-response curve and the IC_{50} value were obtained by Compusyn 1.0 Software. IC_{50} value refers to the concentration of extract or drug needed to inhibit 50 % of cell growth. The negative control consisted of the cells without addition of any drug or extract or drug and extract combination. The cytotoxicity data obtained were expressed as percentage of inhibition on the growth of HCT-116 cell line relative to negative control.

The percentage of inhibitions were then converted to effect level, Fa value (0－1.0) before being calculated by CompuSyn 1.0 software (Chou & Martin, 2007) for further analysis. The potency, Dm (IC_{50}, μg/ml) values (concentration required to inhibit cells viability by 50% as compared to the negative control) for PW-E were obtained from antilog of x-intercept of the median-effect plot generated by CompuSyn 1.0 computerized simulation. 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).

3.8 Combination Index (CI) Analysis

The drug combination effect and other related analysis were determined by the Chou and Talalay equation (Chou, 2006): Combination Index, CI = (dA/DA) + (dB/DB). dA and dB are the doses of individual drugs alone, i.e., the concentration of 5-FU or PW-E, respectively, that gives 50 % inhibition. DA and DB are the doses of 5-FU and PW-E in combination that inhibits 50 % of cell growth. CI > 1, CI = 1, and CI < 1 indicate antagonistic, additive, and synergistic effects, respectively.

3.9 Sensitization Factor

The sensitization factor of the drug (5-FU) towards colorectal cancer cells HCT-116 and HT-29 by the PW-E extract was calculated as the ratio of IC_{50} drug alone / IC_{50} drug + PW-E extract.

3.10 Selectivity Index 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 colorectal carcinoma HCT-116 and human colon adenocarcinoma HT-29 cell. Extract with SI value greater than 3 were considered to have a high selectivity towards cancerous cells (Bézivin *et al.*,2003).

3.11 Apoptosis Study

3.11.1 Acridine Orange / Ethidium Bromide (AO/EB) Double Staining Assay

The morphological changes of cells treated with PW-E, 5-FU and PW-E/5-FU combination were assessed using the Acridine Orange (AO, Sigma Aldrich, USA) / Ethidium Bromide, (EB, Sigma Aldrich, USA) double staining technique as previously described by Ribble *et al*. In two different assay, both HCT-116 and HT-29 cells were seeded at a concentration of 30,000 cells per well in a six-well plate and incubated for 24 h at 37 °C before treatment with the PW-E, 5-FU and PW-E/5-FU combination at their respective IC_{50} values. After an incubation period of 72 h, the cells were detached and pelleted. The supernatant was removed and the cells were subsequently stained with the prepared dye mixture (25 µl cold PBS and 2 µl AO/EB dye mixed in a 1:1 ratio). The stained cell suspension was transferred onto a clean glass slide and covered with a coverslip. The morphological changes relative to the untreated control was observed

using the narrow blue excitation filter on Leica fluorescent microscope. The images were then photographed at 200 × magnification.

3.11.2 Determination of Caspase-3 Activation

Caspase-3 activity were determined by using the Caspase-3 DEVD-R110 Fluorometric and Colorimetric assay kit (Biotium, CA) according to the manufacturer's protocol. The assay was based on a spectrophotometric detection of the chromophore, rhodamine 110 (R110), after its cleavage from the labelled substrate (Ac-DEVD) 2-R110. All experiments were carried out in three independent experiments. After treatment with 10 µg/ml of PW-E, 5-FU and PW-E/5-FU combination for 48 h, 2×10^6 cells were pelleted and lysed with 50 µl of chilled cell lyses buffer and incubated on ice for 20 min. After incubation, cell suspension was 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 then transferred 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 and ELISA (enzyme-linked immunosorbent assay) micro-plate reader at absorbance 495 nm. The activity of caspase-3 in treated cells were determined by comparing the results with controls (untreated cells) and presented as percentage.

3.12 Statistical Analysis

The quantitative data for cytotoxic assay study and caspase-3 activation determination assay were presented as mean ± standard deviation. The differences between control, PW-E and PW-E/5-FU combination on treated cells were evaluated by using the Student's t-test. *$p < 0.05$ denotes a statistically significant.

CHAPTER 4: RESULTS

4.1 Liquid Chromatography-Mass Spectrometry Analysis of *Phyllanthus watsonii* Ethyl Acetate Extract (PW-E)

Ethyl acetate extract of *Phyllanthus watsonii*, PW-E was analyzed by LC-MS/MS system allowing the detection of major compound(s). The data obtained were compared with the previous published data of chemical constituents of *P.watsonii*. The LC-MS/MS profiles of compounds in PW-E were obtained using Agilent Zorbax C18 column (150 mm × 4.6 mm × 5 µM) are shown in Figure 4.1. The spectrometric analysis and tentatively identified major compounds are listed in Table 4.1.

Referring to the full chromatogram of PW-E (Figure 4.1), the major peak with a retention time (Rt) of 8.46 min was identified as kaempferol rhamnoside (Appendix F) (MW (molecular weight) m/z (average olecular weight) at 447.1). Other identified compounds are quercetin (Appendix E), kaempferol glucoside (Appendix G), ellagic acid (Appendix K), hyperin (Appendix H), strictinin isomer (Appendix C), galloyl HHDP hexoside, strictinin (Appendix B) and gallic acid (Appendix J). In the analysed sample of PW-E, two compounds were found to have the same MW, which are strictinin and galloyl HHDP (Hexahydroxydiphenic acid) hexoside (Appendix I). Their MW is the same because strictinin is an isomer of galloyl HHDP hexosidase. An isomer is each of two or more compounds with the same formula but a different arrangement of atoms in the molecule and different properties.

Table 4.1: Identification of compounds in PW-E by using LC-MS/MS analysis

Retention Time (Rt)(min)	MW (m/z)	Tentative ID 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
8.0	447.1	Kaempferol glucoside
8.46	431.1	Kaempferol rhamnoside
9.80	301.0	Quercetin

*identification of the compounds were aided by comparison with reference standards where available and by correlation with previus literature reports; PW-E: *P. watsonii* ethyl acetate extract; MW: molecular weight

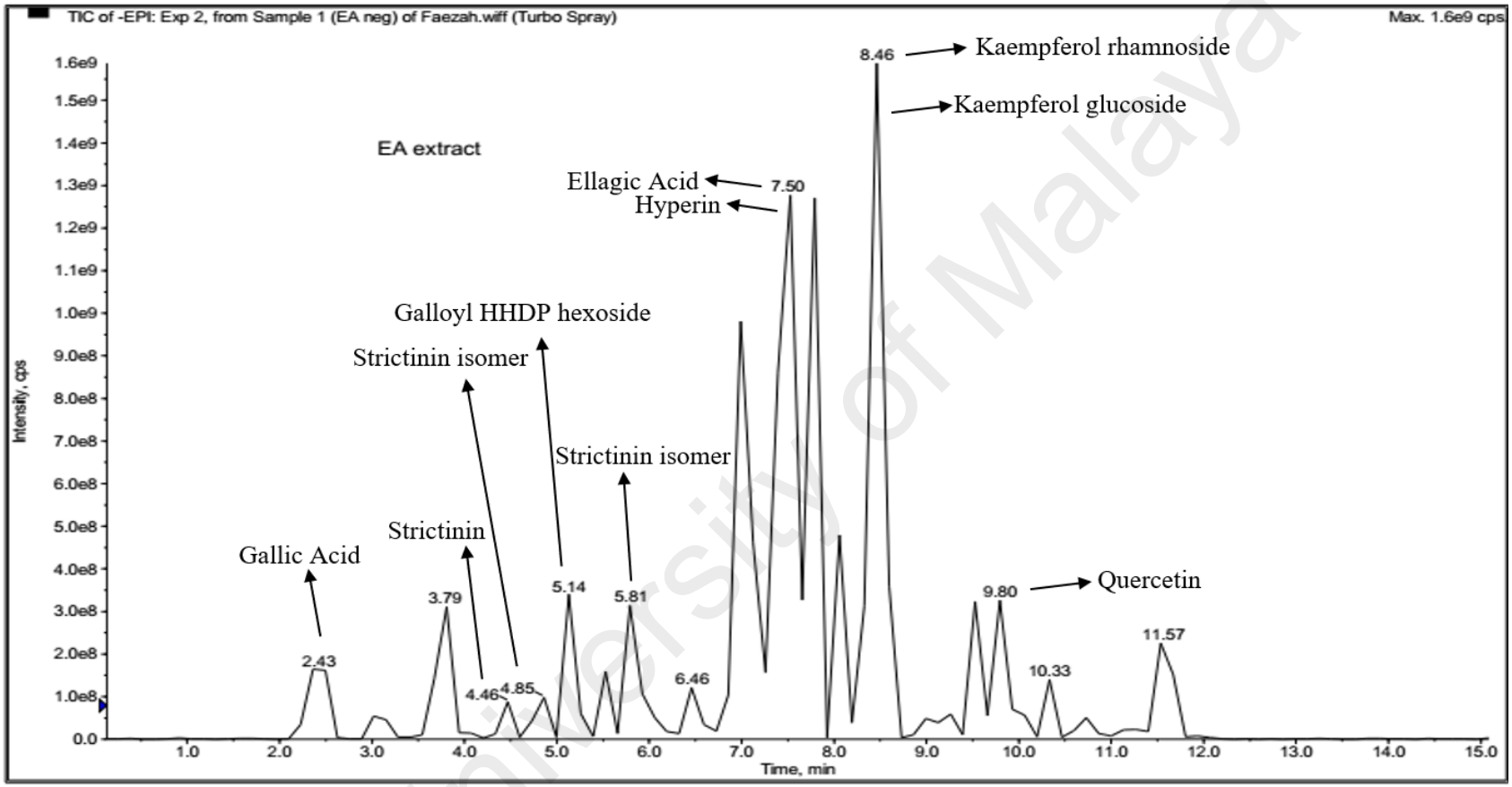


Figure 4.1: LC-MS/MS profiles of chemical compounds in *P. watsonii* ethyl acetate extract.

4.2 Cytotoxic Effects of PW-E, 5-FU and PW-E/5-FU Combo Against Human Colon HCT-116 and HT-29 Cancer Cell Line

In the present study, the cytotoxic activities of *P. watsonii* ethyl acetate extract, PW-E and chemotherapeutic drug, 5-Fluorouracil (5-FU) were screened by Neutral Red Uptake (NRU) assay against human colon HCT-116, HT-29 cancer cell line and human fetal lung fibroblast MRC-5 cell line. Based on the work by Ramasamy *et al*. (2012), PW-E was selected as a test agent as it was reported that PW-E was the best performer in cytotoxicity against colon cancer cells in comparison with PW-E in different solvent. The percentage of inhibition of the HCT-116 and HT-29 colon cancer cells triggered by the test agent used in this study, which is 5-FU, in various concentration in three independent experiment were then entered into CompuSyn1.0 software and the Dm value expressed as IC_{50}, µg/ml (cytotoxicity effect) was generated from the antilog of x-intercept of median-effect plot (Chou & Martin, 2007). A complete dose-response curve and the IC_{50} value were obtained by Compusyn 1.0 Software.

4.2.1 Cytotoxic Activity against HCT-116 Cell Line

Cytotoxicity (IC_{50} value in µg/ml) of PW-E, 5-FU and PW-E/5-FU combination towards HCT-116 cells are summarized in Table 4.2.

5-Fluorouracil (5-FU)

The concentration of 5-FU required to reduce HCT-116 cells viability by 50 % as compared to the negative control cells depicted as IC_{50} value was 1.588 ± 0.46 µg/ml. Based on the US National Cancer Institute guidelines, a crude extract is generally considered to have active cytotoxic activity if the IC_{50} value in carcinoma cells, following incubation between 48 and 72 hours, is ≤ 20 µg/ml, while ≤ 4 µg/ml for pure compounds.

Therefore, based on the result obtained, the chemotherapeutic drug 5-FU possessed cytotoxic activity against HCT-116 cancer cells.

***P. watsonii* ethyl acetate extract (PW-E)**

Concentration of PW-E required to reduce HCT-116 cells viability by 50 % as compared to the control cells depicted as IC_{50} value was 1.522 ± 0.16 µg/ml. Based on the US National Cancer Institute guidelines, PW-E possessed cytotoxic activity towards HCT-116 cancer cells.

PW-E/5-FU Combination

The concentration of PW-E/5-FU combination at ratio 3:1 required to reduce HCT-116 cells viability by 50 % as compared to the control cells depicted as IC_{50} value was 1.482 ± 0.25 µg/ml. PW-E/5-FU combination inhibited 50 % of HCT-116 cells with lower concentration in comparison when the HCT-116 cells was treated with 5-FU and PW-E alone.

Table 4.2: Cytotoxicity (IC_{50}, µg/ml) of 5-FU, PW-E and PW-E/5-FU combo against HCT-116 cell line

Test Agent	Cytotoxicity (IC_{50}, µg/ml) [a] on HCT-116[b]
5-FU[d]	1.588 ± 0.46
PW-E[c]	1.522 ± 0.16
PW-E/5-FU[e]	1.482 ± 0.25

[a]Data are represented as mean±SD from three independent experiments, triplicate each; [b] colon cancer cells; [c] *P. watsonii* ethyl acetate extract; [d]5-Fluorouracil; [e]combination at ratio 3:1.

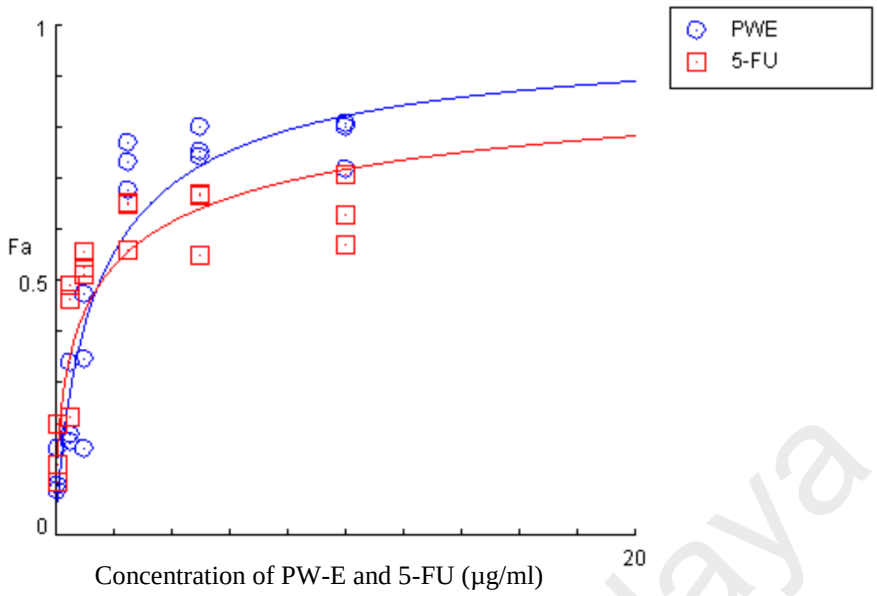


Figure 4.2: Dose-effect curve for PW-E and 5-FU against human colon HCT-116 cancer cell line generated from Compusyn 1.0 software. PW-E: *P. watsonii* ethyl acetate extract; 5-FU: 5-Fluorouracil; Fa: effect level (HCT-116 cells growth inhibition percentages/100).

Figure 4.2 shows the dose-effect curve for PW-E and 5-FU against HCT-116 cell line. Dose-effect relationship shows the changes or effect that an organism goes through when exposed to a stressor after a certain amount of time. Based on the figure above, the effect that both 5-FU and PW-E exerted on the growth of HCT-116 cell line was almost similar, with PW-E exhibited slightly higher growth inhibiton of HCT-116 cells compared to 5-FU.

4.2.2 Cytotoxic Activity against HT-29 Cell Line

Cytotoxicity (IC_{50} value in µg/ml) of the PW-E, 5-FU and PW-E/5-FU combination towards HT-29 cell are summarized in Table 4.3.

5-Fluorouracil (5-FU)

The concentration of 5-FU required to reduce HT-29 cells viability by 50 % as compared to the negative control cells depicted as IC_{50} value was 19.70 ± 1.72 µg/ml. Based on the US National Cancer Institute guidelines the chemotherapeutic drug of 5-FU does not possessed any cytotoxic activity towards HT-29 cancer cells, meaning that the anti-cancer drug is rather ineffective to inhibit the growth of the colon HT-29 cancer cells.

***P. watsonii* Ethyl Acetate Extract (PW-E)**

The concentration of PW-E required to reduce HT-29 cells viability by 50 % as compared to the control cells depicted as IC_{50} value was 0.03 ± 0.003 µg/ml. Based on the US National Cancer Institute guidelines, PW-E has cytotoxic activity on HT-29 cancer cells.

PW-E/5-FU Combination

The concentration of PW-E/5-FU combination required to reduce HT-29 cells viability by 50 % as compared to the negative control cells depicted as IC_{50} values were 0.03 µg/ml of PW-E and 10.0 µg/ml of 5-FU respectively. PW-E/5-FU combination inhibited 50 % of HT-29 cells with lower concentration of 5-FU compared to when the cells was treated with 5-FU and PW-E alone. It can be observed that by combining PW-E with 5-FU, the cytotoxicity effect of 5-FU was increased by 1.9-fold towards HT-29 cells.

Table 4.3: Cytotoxicity (IC_{50}, µg/ml) of 5-FU, PW-E and PW-E/5-FU combo against HT-29 cell line

Test Agents	Cytotoxicity (IC_{50}, µg/ml) [a] on HT-29 cell line[b]
5-FU[d]	19.70 ± 1.72
PW-E[c]	0.03 ± 0.003
PW-E/5-FU[e]	0.03/10.0

[a]Data are represented as mean±SD from three independent experiments, triplicate each; [b] colon cancer cells; [c]*P. watsonii* ethyl acetate extract; [d]5-Fluorouracil; [e]combination at PW-E/5-FU concentration of 0.03 and 10.0 µg/ml.

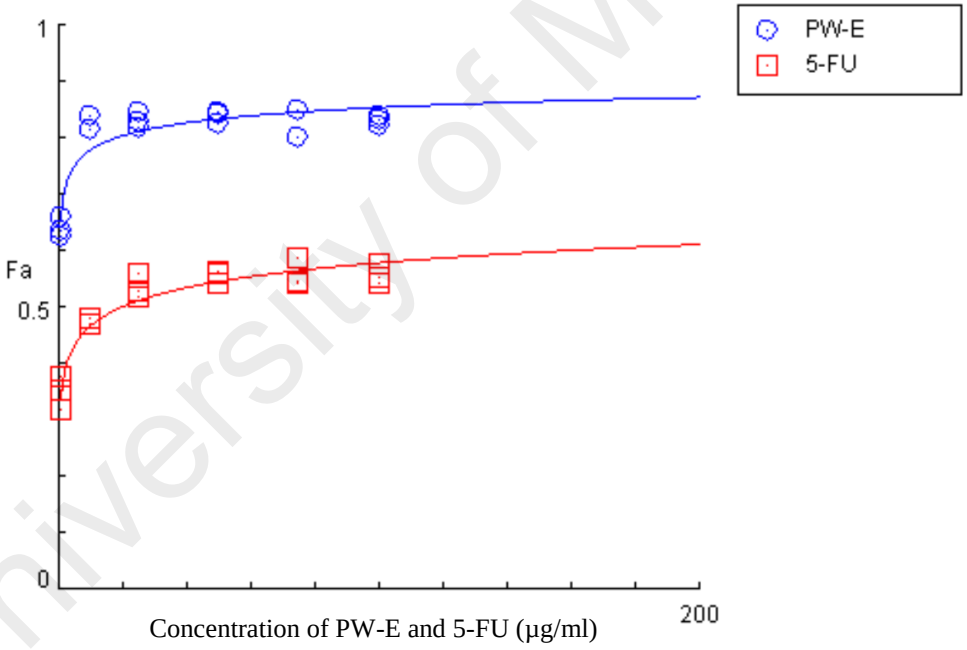


Figure 4.3: Dose-effect curve for PW-E and 5-FU against human colon HT-29 cancer cell line generated from Compusyn 1.0 software. PW-E: *P. watsonii* ethyl acetate extract; 5-FU: 5-Fluorouracil; Fa: effect level (HT-29 cells growth inhibition percentages/100).

Based on Figure 4.3, PW-E has a higher potency in cell inhibition compared to 5-FU as it showed a very steep curve at the lower concentrations and PW-E inhibited higher percentages of the HT-29 cells at much lower concentrations.

4.2.3 Combination Index Analysis on HCT-116 Cell Line

Combination Index (CI) analysis was performed in this study to further explore the effect of PW-E when combined with 5-Fluorouracil (5-FU) on both HCT-116 and HT-29 colon cancer cell lines. Combination Index (CI) analysis was performed and then the CI values, isobologram and Drug Reduction Index were subsequently generated by CompuSyn 1.0 software. According to Chou and Martin (2007), the 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 combined 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.

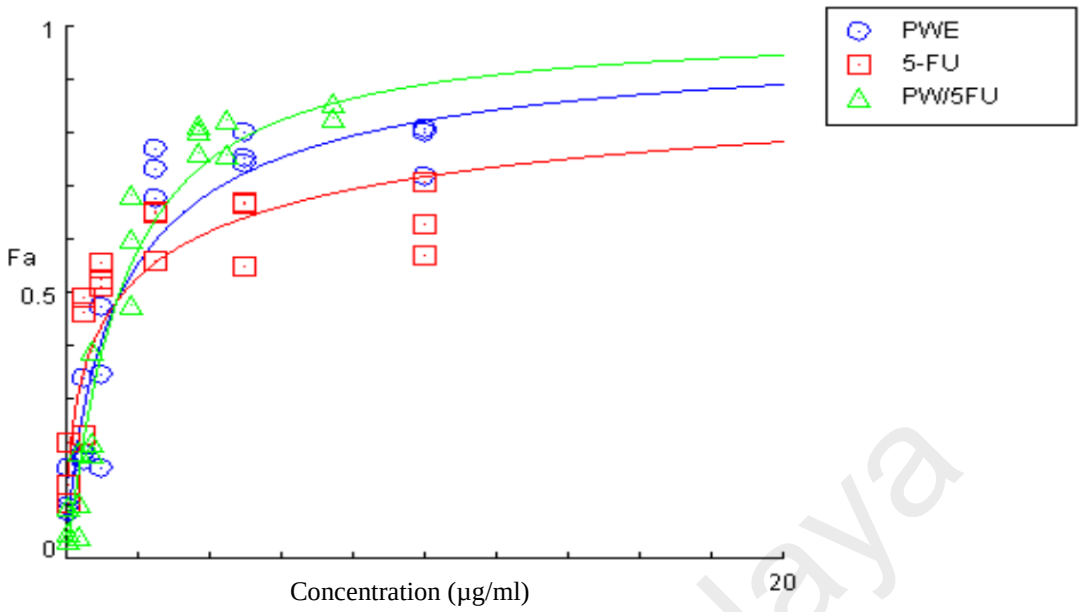


Figure 4.4: Dose-effect curve for PW-E, 5-FU and PW-E/5-FU combination against human colon HCT-116 cancer cells generated from Compusyn 1.0 software. PW-E: *P. watsonii* ethyl acetate extract; 5-FU: 5-Fluorouracil; PW/5-FU: PW-E/5-FU combo; Fa: effect level (HCT-116 cells growth inhibition percentages/100).

Figure 4.4 shows the dose-effect curve generated from Compusyn 1.0 Software. The cytotoxicity data from three independent experiment were entered into the software and the potency, D_m (IC_{50}) and other analysis were summarized in Table 4.4.

Table 4.4: Dose-effect relationship of PW-E/5-FU combination on human colon HCT-116 cancer cell line

Inhibitory Concentration at 72 hours	PW-E[a] (µg/ml)	5-FU[b] (µg/ml)	PWE/5-FU (3:1)	CI[c]	S_f[d]
IC_{50}	1.522 ± 0.16	1.588 ± 0.46	1.482 ± 0.25	0.85982	1.07
IC_{75}	5.823 ± 0.32	13.715 ± 0.69	4.497 ± 0.154	0.61017	3.05
IC_{90}	22.273 ± 1.36	118.389 ± 1.12	13.024 ± 10.34	0.46592	9.09

[a] *P. watsonii* ethyl acetate extract; [b] 5-Fluorouracil; [c] combination index which measure the degree of PW-E/5-FU interaction, CI was measured at the affected fractions of 50 % (IC_{50}), 70 % (IC_{70}) and 90 % (IC_{90}), CI < 1 indicate synergism interaction, CI > 1 indicate antagonism interaction and CI=1 indicate additive interaction.; [d]sensitization factor of the drug (5-FU) towards cancer cell (HCT-116) by extract (PW-E) was calculated as the ratio of $IC_{50/75/90}$ drug alone/ $IC_{50/75/90}$ drug + extract.

Constant ratio design with ratio of 3:1 was selected in combining both PW-E and 5-FU. From the CI analysis, it shows that PW-E/5-FU combination exerted CI values of 0.85982 at IC_{50}, 0.61017 at IC_{75} and 0.46592 at IC_{90} when tested against HCT-116 cells. According to the guidelines given by Chou and Martin (2007), any combination with CI > 1, indicates antagonism interaction, CI = 1, additive interaction and CI < 1 indicate synergistic interaction between the two agents.This data indicate that combination of PW-E and 5-FU able to interact synergistically (CI <1) to inhibit the growth of HCT-116 cells.

The data in Table 4.4 above also summarized the sensitization factor (Sf) of 5-FU towards HCT-116 cell line by PW-E and was calculated as the ratio of $IC_{50/75/90}$ of individual 5-FU and $IC_{50/75/90}$ of PW-E/5-FU combination. Based on the data above, it can be seen clearly that there was an increase in Sf values at higher inhibitory concentration. This indicates that the presence of PW-E in PW-E5-FU combination has sensitizing effect in increasing the inhibitory activity of 5-FU towards HCT-116 cells. In short, the increase in sensitization factor at increasing inhibition concentration indicates that the PW-E extract further sensitizes the HCT-116 cell towards 5-FU.

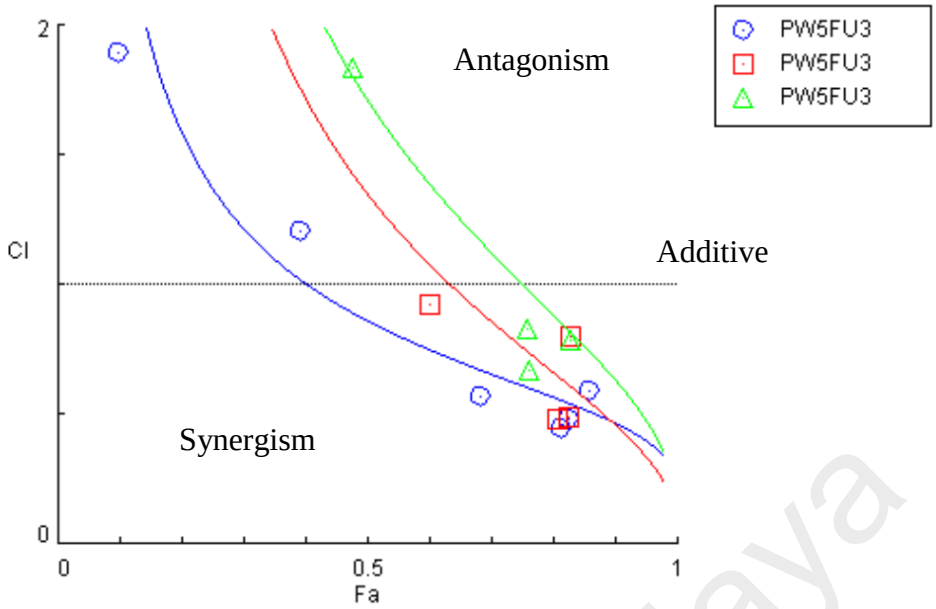


Figure 4.5: Isobologram of Combination Index (CI) of PW-E/5-FU combo towards HCT-116 cells. The lines from three independent replicates indicated the degree of interaction between PW-E and 5-FU. The horizontal line signify additive effect, CI=1. Combination points of PW-E/5-FU situated below and upper level of the line are synergistic and antagonistic respectively. PW-E: *P. watsonii* ethyl acetate extract; 5-FU: 5-Fluorouracil; Fa: effect level (HCT-116 cells growth inhibition percentages/100).

Figure 4.5 depicts the combination effect of PW-E/5-FU at ratio 3:1 on HCT-116 cells. The three lines represented three independent replicates of the combination study. The extract-drug combinations isobologram showcased six dose combinations inhibition for each replicate. The diagonal line indicates additive interaction (between the extract and drug, in which inhibitory effect that fall above each line are antagonism, while inhibitory effect that fall below are synergism. From Figure 4.5, most data points fell under the diagonal line, indicating that the PW-E/5-FU combination exerts synergistic interaction at higher concentration combination dosages at with Fa > 0.5, while at certain low concentration combination, Fa < 0.6, antagonistic interaction can be seen. However, in cancer therapy, the low Fa value (effect level) is of less concern compared to high Fa value as killing cancer in a small fraction is not useful in cancer therapy (Zhang *et al.*, 2016).

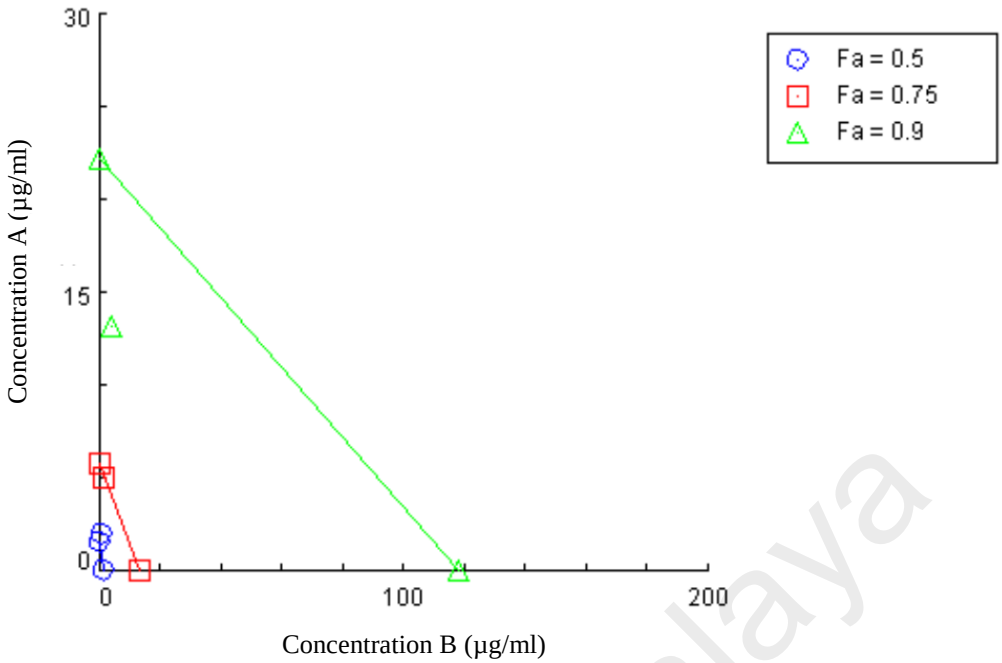


Figure 4.6: Isobologram for PW-E/5-FU combination at ratio 3:1 against HCT-116 cell line. The lines indicated the degree of interaction between PW-E (Concentration A) and 5-FU (Concentration B). Effect level that fall on the diagonal lines of its colour indicated additive, below the lines synergism and above the lines, antagonism interaction. PW-E: *P. watsonii* ethyl acetate extract; 5-FU: 5-Fluorouracil; Fa: effect level (HCT-116 cells growth inhibition percentages/100).

Figure 4.6 shows the isobologram of simultaneous and sequential exposure of HCT-16 cells to PW-E/5-FU combination at three cell inhibitory effect level of Fa, 50 % (Fa = 0.5), 70 % (Fa = 0.70) and 90 % (Fa = 90). The line indicates alignment of theoretical value of an additive interaction between concentration A (PW-E) and concentration B (5-FU). Values above the diagonal line of additive effects in the isobologram suggest antagonism and below line suggests synergism interaction. It can be seen that all the values fall below the diagonal line indicating that the PW-E/5-FU combination lead to synergism interaction upon treatment on HCT-116 cells.

Table 4.5: Drug Reduction Index (DRI) values for PW-E, 5-FU and PW-E/5-FU combo at ratio 3:1 against HCT-116 cells

	Concentration (µg/ml)		DRI [e]	
Fa [a]	PW-E [c]	5-FU[d]	PW-E [c]	5-FU[d]
0.828	10.345	34.527	1.379	13.811
0.825	10.138	33.425	2.253	22.283
0.806	8.688	26.086	2.317	20.869
0.601	2.508	3.543	1.337	5.669
0.217	0.319	0.129	0.425	0.515
0.106	0.112	0.024	0.300	0.193
0.035	0.026	0.002	0.351	0.094
0.500 [b]	1.522	1.588	1.534	4.803

[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; [c] *P. watsonii* ethyl acetate extract; [d] 5-Fluorouracil; [e] a measure on how many folds the dose of 5-FU in a synergistic combination with PW-E may be reduced at a given level when compared with the doses of each alone. DRI > 1 indicate dose reduction, DRI < 1 indicate not-favourable reduction and DRI =1 indicates no dose reduction.

Table 4.5 shows the Drug Reduction Index (DRI) data for each PW-E and 5-FU prior PW-E/5-FU combination at ratio of 3:1 against HCT-116 cells. The DRI for drug-agent combination is measured on how much (-fold) the dose of a drug or agent (e.g. PW-E or 5-FU) in synergistic combination may be reduced at a given effect level compared with the dose of each drug alone. The DRI is another mathematical interpretation of the CI, and CI= $1/(DRI)_1 + 1(DRI)_2$. The value of DRI >1 shows that the combination is favourable in terms of clinical therapy (Chou, 2010). Based on Table 4.5, it can be inferred that to achieve 50 % HCT-116 cells inhibition, it requires 1.522 µg/ml of PW-E, and 1.588 µg/ml of 5-FU of each individual extract and drug. However, it requires 1.53452-fold less PW-E and 4.80342-fold less 5-FU to achieve the same 50 % inhibition if both the drug and extract when they are combined.

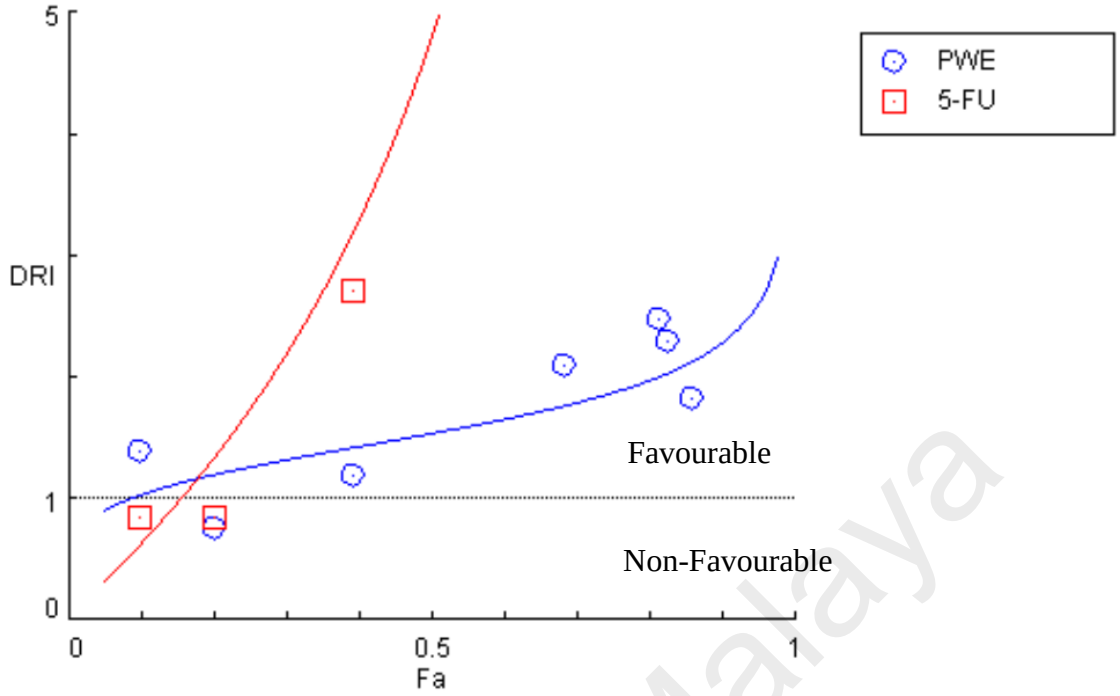


Figure 4.7: Drug Reduction Index (Fa-DRI) plot for PW-E, 5-FU and PW-E/5-FU combo at 3:1 ratio on HCT-116 and HT-29 cell lines. DRI >1 indicated favourable dose reduction and the combination is favourable in terms of clinical therapy (Chou, 2010). PW-E: *P. watsonii* ethyl acetate extract; 5-FU: 5-Fluorouracil. Fa: effect level (HCT-116 cells growth inhibition percentages/100).

Figure 4.7 depicts the DRI for PW-E/5-FU combination at ratio of 3:1 on HCT-116 cells. DRI > 1 indicate favourable dose reduction, DRI < 1 indicate not favourable reduction and DRI = 1 indicates no dose reduction (Chou, 2010). It can be seen in the graph most of the plots fall in a favourable dose reduction region (DRI > 1).

4.2.4 Combination Index Analysis of HT-29 Cell Line

Drug combination analysis was determined by the Chou and Talalay equation (Chou, 2006): Combination Index, CI = (*d*A/*D*A) + (*d*B/*D*B). *d*A and *d*B are the doses of individual drugs alone, i.e., the concentration of 5-FU or PW-E, respectively, that gives 50 % inhibition. 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.

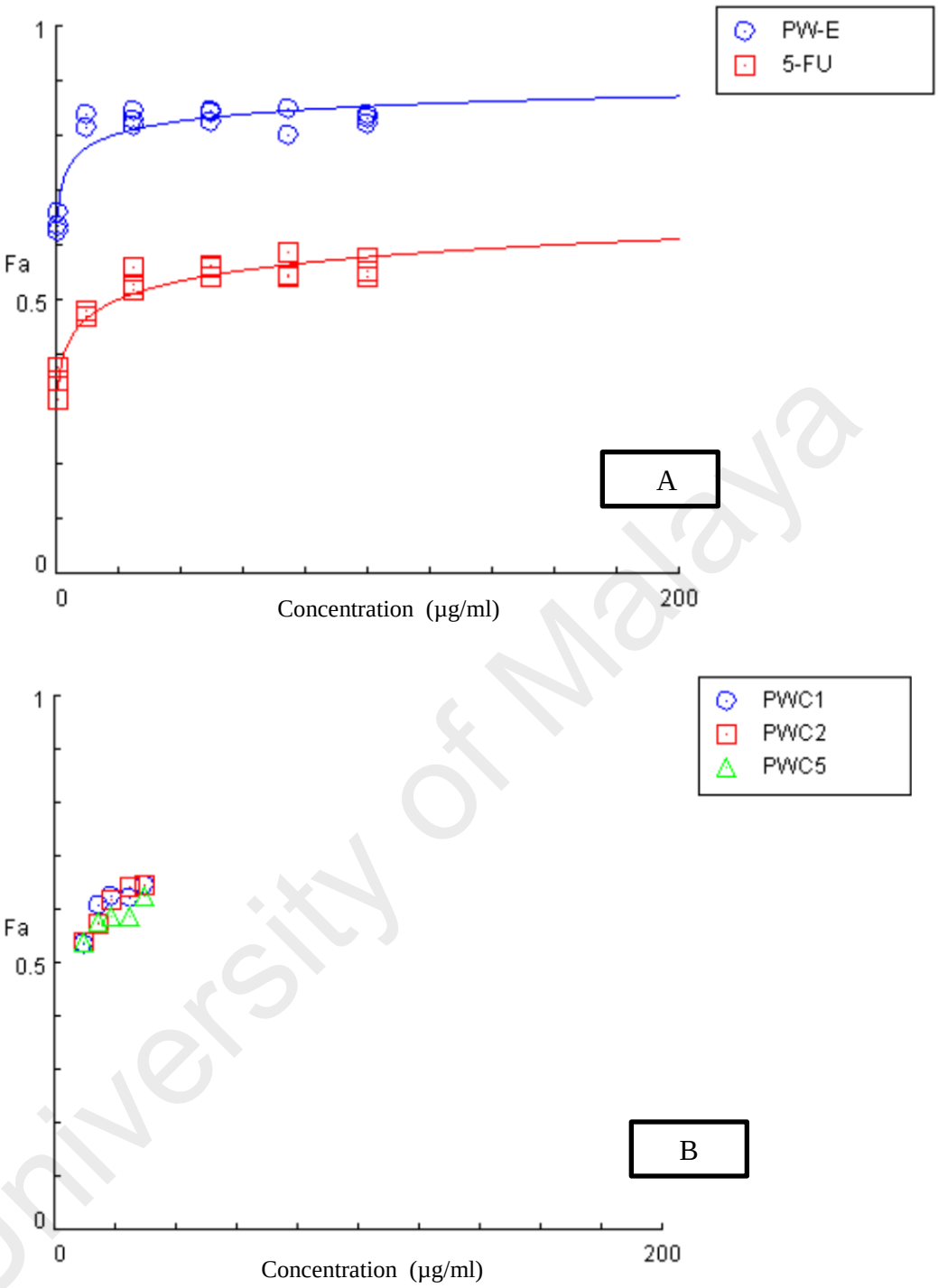


Figure 4.8: Dose-effect curve for PW-E, 5-FU and PW-E/5-FU combination against human colon HT-29 cancer cells generated from Compusyn 1.0 software. A: Dose-Effect curve for 5-FU and PW-E tested alone against HT-29 cells; B: Dose-Effect curve for PW-E and 5-FU combined tested against HT-29 cells. PW-E: *P. watsonii* ethyl acetate extract; 5-FU: 5-Fluorouracil; PWC1, PWC2, PWC5: three independent experiments of PW-E tested alone against HT-29 cells; Fa: effect level (HT-29 cells growth inhibition percentages/100).

Figure 4.8 shows the dose-effect curve generated from Compusyn 1.0 Software. (A) Dose-Effect curve for 5-FU and PW-E tested alone against HT-29 cells. (B) Dose-Effect curve for PW-E and 5-FU combined tested against HT-29 cells. The cytotoxicity data from three independent experiment were entered into the software and the potency, D_m (IC_{50}) and other analysis are summarized in Table 4.7.

Table 4.6: Dose-effect relationship of PW-E/5-FU combination on human colon HT-29 cancer cell line

Extract/Drug	Dm[c] (µg/ml)	M[d]	r[e]	CI [f]
PW-E[a]	0.03 ± 0.003	0.22029	0.87789	
5-FU[b]	19.70 ±1.72	0.19469	0.95914	
PWE/5-FU (0.03:19.0)				0.67947

[a] *P. watsonii* ethyl acetate extract; [b] 5-Fluorouracil; [c] median-effect dose that produces 50% cell death; [d] shape of dose-effect curve; [e] conformity parameter for goodness of fit, for *in vitro* experiment r > 0.95 are considered good; [f] combination index which measure the degree of PW-E-/5-FU interaction, CI was measured at the affected fractions of 50 % (IC_{50}), 70 % (IC_{70}) and 90 % (IC_{90}), CI < 1 indicate synergism interaction, CI > 1 indicate antagonism interaction and CI=1 indicate additive interaction.

The concentrations to inhibit 50 % of HT-29 colon cancer cell by both 5-FU and PW-E were 19.70 ±1.72 µg/ml and 0.03 ± 0.003 µg/ml respectively and the concentration value was selected based on the IC_{50} values of the drug and extract. Non-constant ratio design was selected for this experiment and the ratio used was a constant 0.03 µg/ml for PW-E extract while the concentration of 5-FU used was 30.0, 25.0, 19.0, 15.0 and 10.0 µg/ml respectively. The CI analysis shows that PW-E/5-FU combination exhibited IC_{50} value of 10.0 µg ml of 5-FU with 0.03 µg/ml of PW-E when tested against HT-29 cells. This data indicated lower concentration of 5-FU is needed to be combined with PW-E in order to achieve 50 % inhibition of HT-29 cells.

Table 4.6 shows CI analysis for HT-29 cell line tested with the combination of 5-FU and PW-E at a non-constant ratio where the concentration of PW-E was kept constant. The CI analysis indicated that PW-E and 5-FU interacted synergistically as shown by CI < 1 in the Table 4.6. The sensitization factor for HT-29 cell line could not be calculated because since using non-constant combination ratio concept, the Compusyn 1.0 software does not calculate the IC_{70} and IC_{90}.

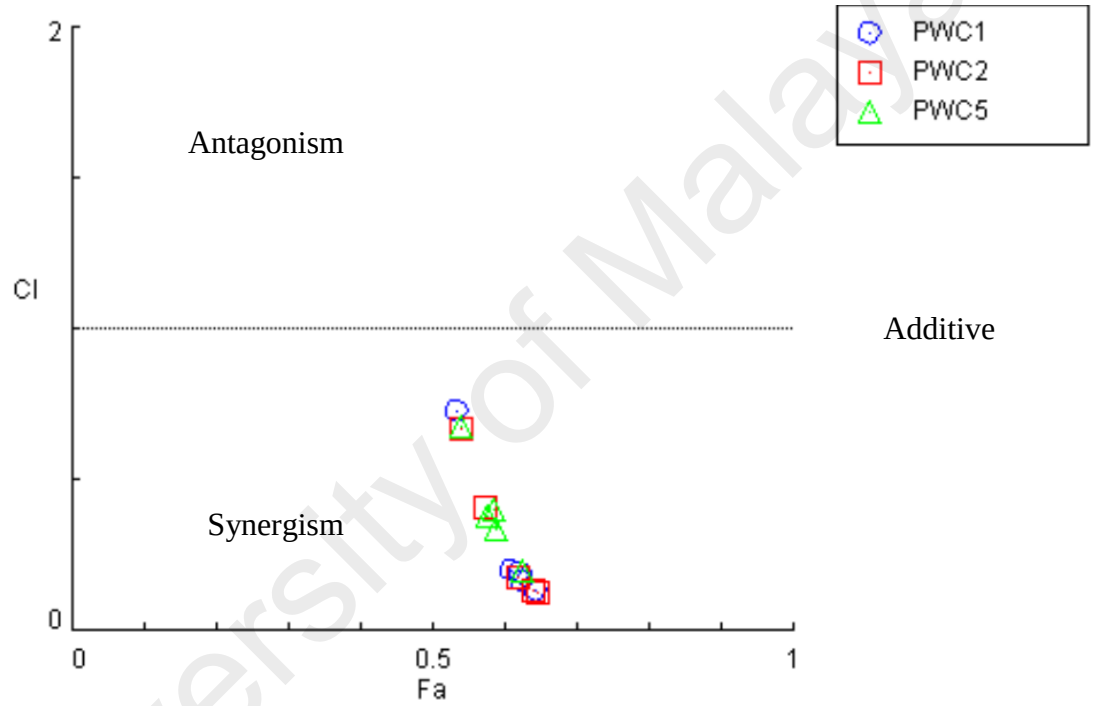


Figure 4.9: Isobologram of Combination Index (CI) of PW-E/5-FU towards HT-29 cells. The lines indicated the degree of interaction between PW-E and 5-FU. Effect level that fall on the diagonal lines of its colour indicated additive, below the lines synergism and above the lines, antagonism interaction. PW-E: *P. watsonii* ethyl acetate extract; 5-FU: 5-Fluorouracil; PWC1, PWC2, PWC5: three independent experiments of PW-E tested alone against HT-29 cells; Fa: effect level (HT-29 cells growth inhibition percentages/100).

Figure 4.9 depicts the combination effect of PW-E/5-FU at concentration of 0.03 and 10.0 µg/ml for PW-E and 5-FU respectively on HT-29 cells. The three different colours of data points represented three independent replicates of the combination study. The extract-drug combinations isobologram, showcased five dose combinations

inhibition for each replicate. The combination ratio was at a constant 0.03 µg/ml for PW-E while the concentrations of 5-FU are 30.0, 25.0, 19.0, 15.0 and 10.0 µg/ml respectively as non-constant ratio concept were used. The diagonal line indicated additive interaction between the extract and drug, in which inhibitory effect that fall above each line are antagonism, while inhibitory effect that fell below the diagonal line is synergism. From the figure, all data points fell under the diagonal line, indicating that the PW-E/5-FU combination exerts synergistic interaction at higher dosages. In cancer therapy, the low Fa value (effect level) is of less concern compared to high Fa value as killing cancer in a small fraction is not useful in cancer therapy (Zhang *et al*., 2016).

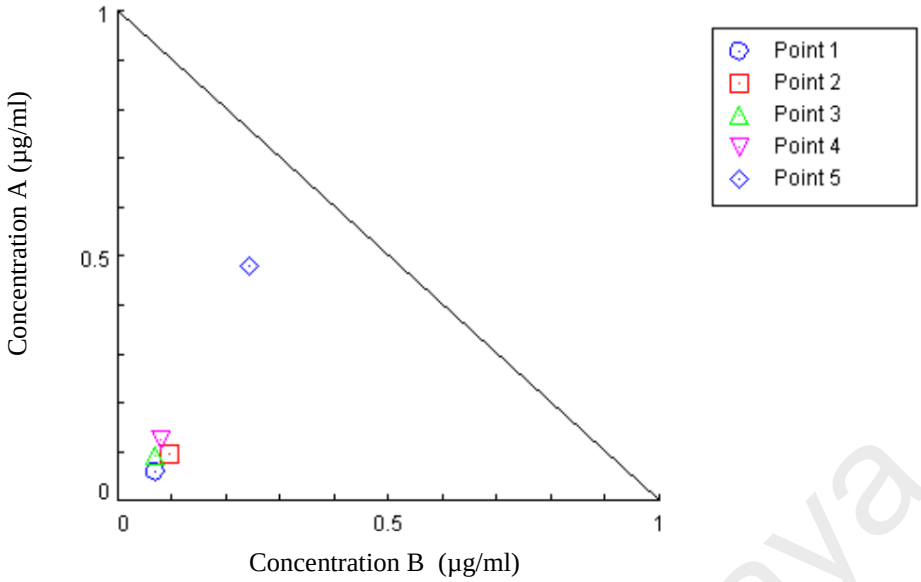


Figure 4.10: Isobologram for PW-E/5-FU combination at concentration of 0.03 and 10.0 µg/ml concentration for PW-E and 5-FU respectively against HT-29 cell line. The lines indicated the degree of interaction between PW-E (Concentration A) and 5-FU (Concentration B). Effect level that fall on the diagonal line indicated additive, below the line synergism and above the line, antagonism interaction. PW-E: *P. watsonii* ethyl acetate extract; 5-FU: 5-Fluorouracil; Fa: effect level (HT-29 cells growth inhibition percentages/100); Point 1-5: The five different colours of data points represented fve independent replicates of the combination study.

Figure 4.10 shows the isobologram of simultaneous and sequential exposure of HT-29 cells to PW-E/5-FU combination at 50 % (Fa = 0.5), 70 % (Fa = 0.70) and 90% (Fa = 90) Fa. The line indicates alignment of theoretical value of an additive interaction between concentration A (PW-E) and concentration B (5-FU). The five different colours of data points represented five independent replicates of the combination study. Values above the diagonal line of additive effects in the isobol suggest antagonism and below line suggests synergism interaction. It can be seen that all the values fall below the diagonal line indicating that the PW-E/5-FU combination lead to synergism interaction upon treatment on HT-29 cells.

Table 4.7: Drug Reduction Index (DRI) values for PW-E, 5-FU and PW-E/5-FU combo (0.03:10.0) against HT-29 cells

Fa [a]	Concentration (µg/ml)		DRI [e]	
	PW-E [c]	5-FU[d]	PW-E [c]	5-FU[d]
0.645	0.488	418.658	16.268	13.955
0.623	0.316	256.508	10.551	10.260
0.625	0.331	269.690	11.029	14.194
0.609	0.243	190.358	8.1063	12.690
0.536	0.062	40.8030	2.078	4.080
0.500 [b]	0.003	19.0000		

[a] Fa: Fractional inhibition; [b] Fa=0.500 is Compusyn computerized simulation of the DRI at 50 % inhibition based on the IC_{50} of individual test agents; [c] *P. watsonii* ethyl acetate extract; [d] 5-Fluorouracil; [e] a measure on how many folds the dose of 5-Fu 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 favorable dose reduction, DRI<1 indicate not-favourable reduction and DRI = 1 no dose reduction (Chou, 2010).

Table 4.7 shows the Drug Reduction Index (DRI) data for each PW-E and 5-FU prior PW-E/5-FU combination at concentration of 0.03 and 10.0 µg.ml against HT-29 cells. The DRI for drug-agent combination is measured on how much (-fold) the dose of a drug or agent (e.g. PW-E) in synergistic combination may be reduced at a given effect level compared with the dose of each drug alone. The DRI is another mathematical interpretation of the CI, and $CI = 1/(DRI)_1 + 1(DRI)_2$. The value of DRI > 1 shows that the combination is favourable in terms of clinical therapy (Chou, 2010).

Based on the Table 4.7, it can be inferred that to achieve 50 % inhibition HT-29 cells inhibition, it requires 0.03 µg/ml of PW-E, and requires 19.0 µg/ml of 5-FU of each individual extract and drug. However, it requires 2.078-fold less PW-E and 4.080-fold less 5-FU to achieve the same 53 % inhibition if both the drug and extract were combined.

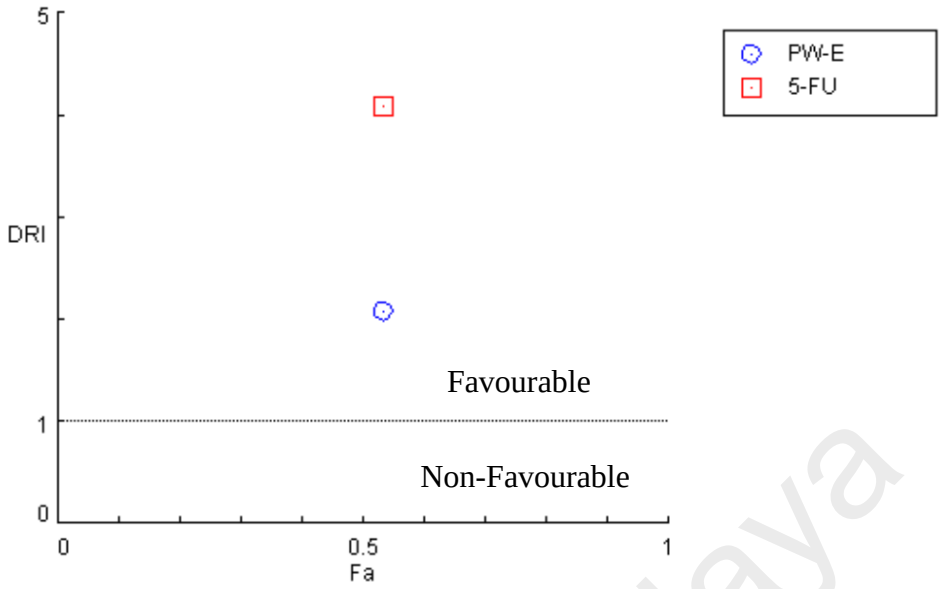


Figure 4.11: Drug Reduction Index (Fa-DRI) plot for PW-E, 5-FU and PW-E/5-FU combo against HT29 cell line. DRI > 1 indicated favourable dose reduction and the combination is favourable in terms of clinical therapy (Chou, 2010). PW-E: *P. watsonii* ethyl acetate extract; 5-FU: 5-Fluorouracil; Fa: effect level (HT-29 cells growth inhibition percentages/100).

Figure 4.11 depicted the DRI for PW-E/5-FU combination at ratio of 0.03:10.0 on HT-29 cells. DRI > 1 indicates favourable dose reduction, DRI < 1 indicate not favourable reduction and DRI = 1 indicate no dose reductions. It can be seen from the graph that all of the plots fell in a favourable dose reduction region (DRI > 1).

4.2.5 Selectivity Index Analysis of HCT-116 Cell Line

PW-E, 5-FU and PW-E/5-FU combo were further tested against human fetal lung MRC-5 fibroblast cells in order to determine the Selectivity Index (SI) and the analysis data were tabulated in Table 4.8.

Table 4.8: Cytotoxicity (IC_{50}, µg/ml) of 5-FU, PW-E and PW-E/5-FU against HCT-116 Cell Line

Cell lines [b]	IC_{50} (µg/ml) [a]			SI [f]
	PW-E [c]	5-FU [d]	PW-E/5-FU [e]	
HCT-116	1.522 ± 0.16	1.588 ± 0.46	1.482 ± 0.25	
MRC-5	3.640 ± 0.14	1687.884 ± 958	103.64	69.932

[a] Data are represented as mean ± SD from three independent experiments, triplicate each; [b] HCT-116 (colorectal cancer) and MRC-5 (normal lung fibroblast); [c] *P. watsonii* ethyl acetate extract; [d] 5-Fluorouracil; [e] combination at ratio 3:1 for HCT-116 cell; [f] SI, selectivity index is the ratio of the IC_{50} values of extract/drug on MRC5 cells to HCT-116. Samples with SI greater than 3 were considered to have high selectivity towards the cancer cells (Mahavorasirikul *et al*., 2010).

The SI values were determined by comparing the cytotoxic activity of PW-E/5-FU combo on normal human lung MRC-5 fibroblast cells to colon HCT-116 cancer cell line. According to Mahavorasirikul *et al*. (2010), if the SI value is more than 3, the combo is said to be highly selective towards the cancer cells. The SI values calculated for the extract-drug combo tested against MRC-5 showed that the combo was more selective towards the cancer cells compared to normal cells as the value was more than 3.

4.2.6 Selectivity Index Analysis of HT-29 Cell Line

PW-E, 5-FU and PW-E/5-FU combo were further tested against human fetal lung MRC-5 fibroblast cells in order to determine the Selectivity Index (SI) and the analysis data were tabulated in Table 4.9.

Table 4.9: Cytotoxicity (IC_{50}, µg/ml) of 5-FU, PW-E and PW-E/5-FU against HT-29 cell line

Cell lines [b]	IC_{50} (µg/ml) [a]			SI [f]
	PW-E [c]	5-FU[d]	PW-E/5-FU[e]	
HT-29	0.030 ± 0.003	19.000 ± 1.8	10.030	10.33
MRC-5	3.640 ± 0.14	1687.884 ± 958	103.64	

[a] Data are represented as mean ± SD from three independent experiments, triplicate each; [b] HT-29 (colorectal cancer) and MRC5 (normal lung fibroblast); [c] *P. watsonii* ethyl acetate extract; [d] 5-Fluorouracil; [e] combination at IC_{50} of both PW-E and 5-FU against HT-29 cells which are 0.03 µg/ml and 19.0 µg/ml respectively. [f] SI, selectivity index is the ratio of the IC_{50} values of extract/drug on MRC-5 cells to HT-29. Samples with SI greater than 3 were considered to have high selectivity towards cancer cells (Mahavorasirikul *et al*., 2010).

The SI values were determined by comparing the cytotoxic activity of PW-E/5-FU combo on normal human lung MRC-5 fibroblast cells to colon HT-29 cancer cell lines. According to Mahavorasirikul *et al*. (2010), if the SI value is more than 3, the combo is said to be highly selective towards the cancer cells. The SI values calculated for the extract-drug combo tested against MRC-5 showed that the combo was more selective towards the cancer cells compared to cancer cells as the values were more than 3.

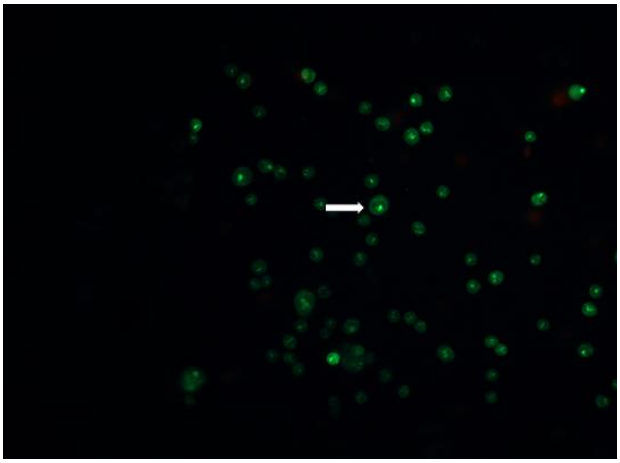
4.3 Apoptosis Study

4.3.1 Morphological Assessment of Apoptotic Cells byAcridine Orange (AO)-Ethidium Bromide (EB) Double Staining

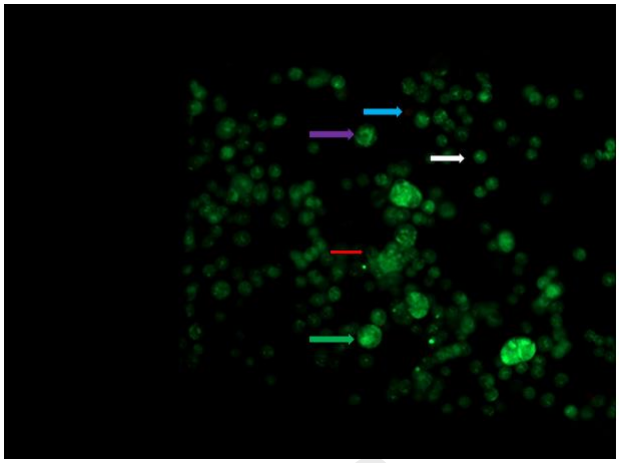
The morphological changes of the HCT-116 and HT-29 cells treated with PW-E, 5-FU and their combination (at IC_{50} concentration) for 24 hours were observed by AO/EB staining and depicted in Figure 4.12 and Figure 4.13. The colon cancer cell HCT-116 were treated at PW-E concentration of 1.5 µg/ml and 5-Fu concentration of 1.5 µg/ml respectively and PW-E/5-FU combination concentration of 1.875+0.625 µg/ml respectively. For HT-29 cell line,the concentration of PW-E used was 0.03 µg/ml, concentration of 5-Fu were at 19.0 µg/ml while PW-E/5-Fu combination were 0.03+10.0 µg/ml respectively. The cells were then classified as apoptotic, necrotic, or live (viable). Apoptotic cells will have condensed chromatin which gives several green coloured nuclei while the necrotic cells would be stained red. Cells that are still alive will have a round and green coloured nuclei with intact DNA and nucleus and will not stain as brightly as apoptotic cells.

As shown in Figure 4.12 and Figure 4.13, the control cells (cells without addition of any test agent) have cytoplasm and nucleus that are smoother and uniformly bright green. Cells treated with extract, drug and drug-extract combo demonstrated some signs of apoptotic cells. Cells underwent apotosis showing morphological changes include shrinkage of cells and nuclear condensation and fragmentation. The treated cells showed brightly stained nucleus with highly condensed chromatin and were uniformly fluorescent. The condensed chromatin were observed to be either in the form of crescents around the periphery of the nucleus or the entire chromatin present as one or a group of featureless, bright spherical beads. Less red stained cells were observed in the treated cells, which means that the cell death occurs primarily through apoptosis instead of necrosis.

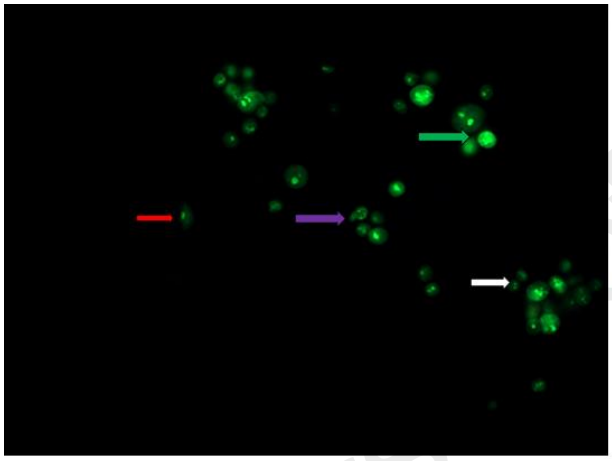
White arrows in Figure 4.12 and 4.13 indicated live cells where it was observed that the nuclei of the cells are uniformly stained green. The green arrows indicated brightly green stained condensed chromatin. The purple arrows indicated the blebbing of the cell membrane, also an indicator of apoptosis. The red arrows showed the loss of membrane shape while the blue arrow showed necrotic cells.



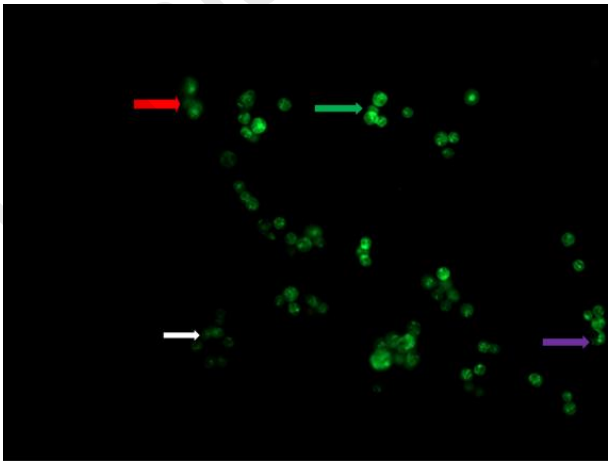
(A) Untreated HCT-116 cells



(B) HCT-116 cells treated with PW-E (1.5 µg/ml)

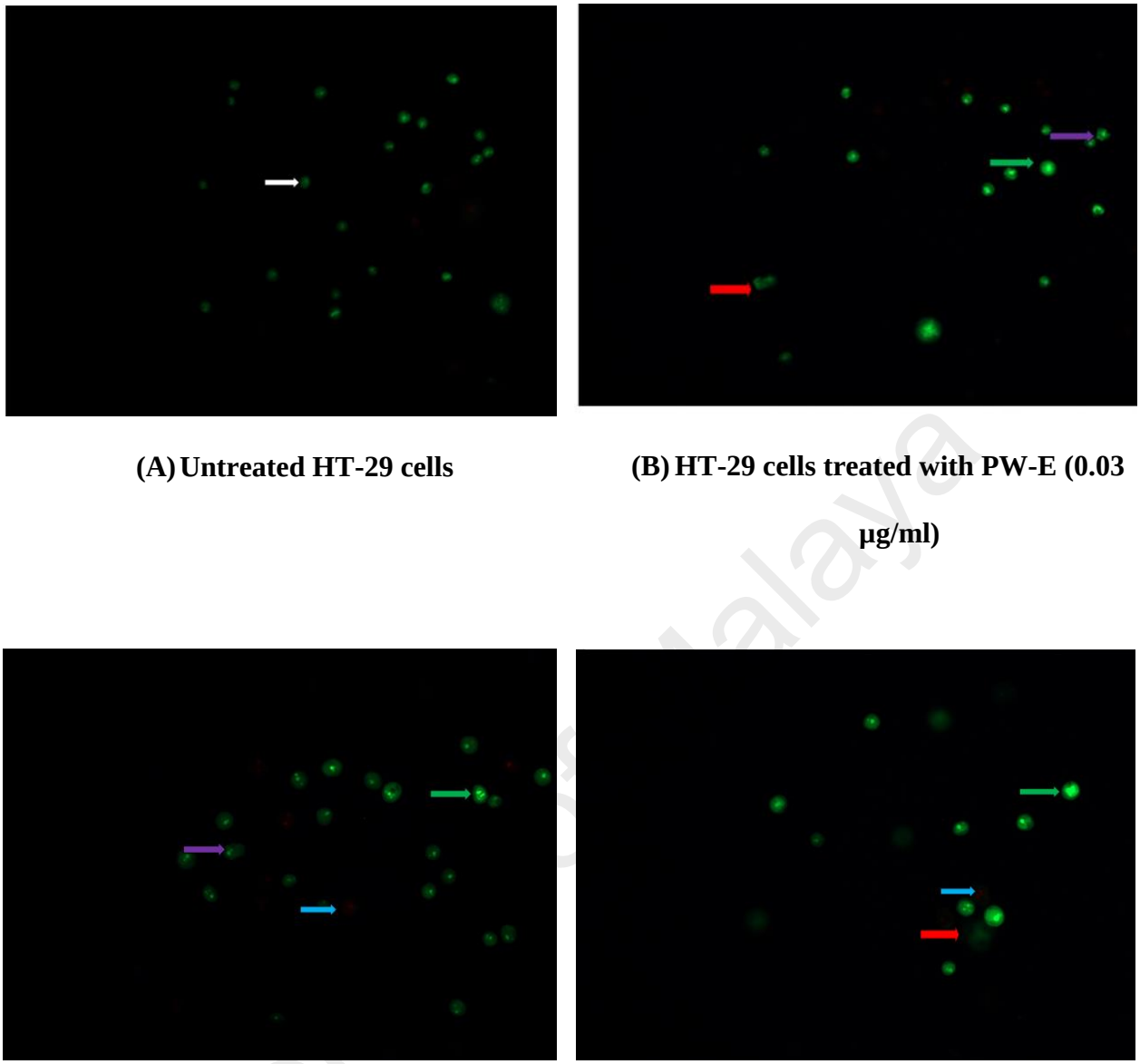


(C) HCT-116 cells treated with 5-FU (1.5 µg/ml)



(D) HCT-116 cells treated with PW-E/5-FU combo (1.875+0.625 µg/ml)

Figure 4.12: Morphological observation of HCT-116 treated with PW-E, 5-FU and PW-E/5-FU combo after stained with AO/EB (magnifications 200 ×). White arrows in indicated live cells where it was observed that the nuclei of the cells are uniformly stained green. The green arrows indicated brightly green stained condensed chromatin. The purple arrows indicated the blebbing of the cell membrane, also an indicator of apoptosis. The red arrows showed the loss of membrane shape while the blue arrow showed necrotic cells. Images are representatives from three independent experiments.



(A) Untreated HT-29 cells

(B) HT-29 cells treated with PW-E (0.03 µg/ml)

(C) HT-29 cells treated with 5-FU (19.0 µg/ml)

(D) HT-29 cells treated with PW-E/5-FU combo (0.03+10.0µg/ml)

Figure 4.13: Morphological observation of HT-29 treated with PW-E, 5-FU and PW-E/5-FU combo after stained with AO/EB (magnifications 200 ×). White arrows in indicated live cells where it was observed that the nuclei of the cells are uniformly stained green. The green arrows indicated brightly green stained condensed chromatin. The purple arrows indicated the blebbing of the cell membrane, also an indicator of apoptosis. The red arrows showed the loss of membrane shape while the blue arrow showed necrotic cells. Images are representatives from three independent experiments.

4.3.2 Caspase-3 Activation Determination

The intracellular levels of caspase-3, which plays a central role in mediating apoptotic responses (Nieves-Neira & Pommier, 1999) were measured to ascertain whether the cytotoxic activity could be dependent by the activation of the caspase-3 protein. The intracellular levels of caspase-3 in HCT-116 and HT-29 cells were measured after being induced with PW-E, 5-FU and PW-E/5-FU combination. Following 48 hours treatment of HCT-116 and HT-29 cells with the respective IC_{50} concentrations of PW-E, 5-FU and PW-E/5-FU combination, caspase-3 activities were then measured and compared with control cells (without addition of any test agent). The colon cancer cell HCT-116 were treated at PW-E concentration of 1.5 µg/ml and 5-Fu concentration of 1.5 µg/ml respectively and PW-E/5-FU combination concentration of 1.875+0.625 µg/ml respectively. For HT-29 cell line,the concentration of PW-E used was 0.03 µg/ml, concentration of 5-Fu were at 19.0 µg/ml while PW-E/5-Fu combination were 0.03+10.0 µg/ml respectively.

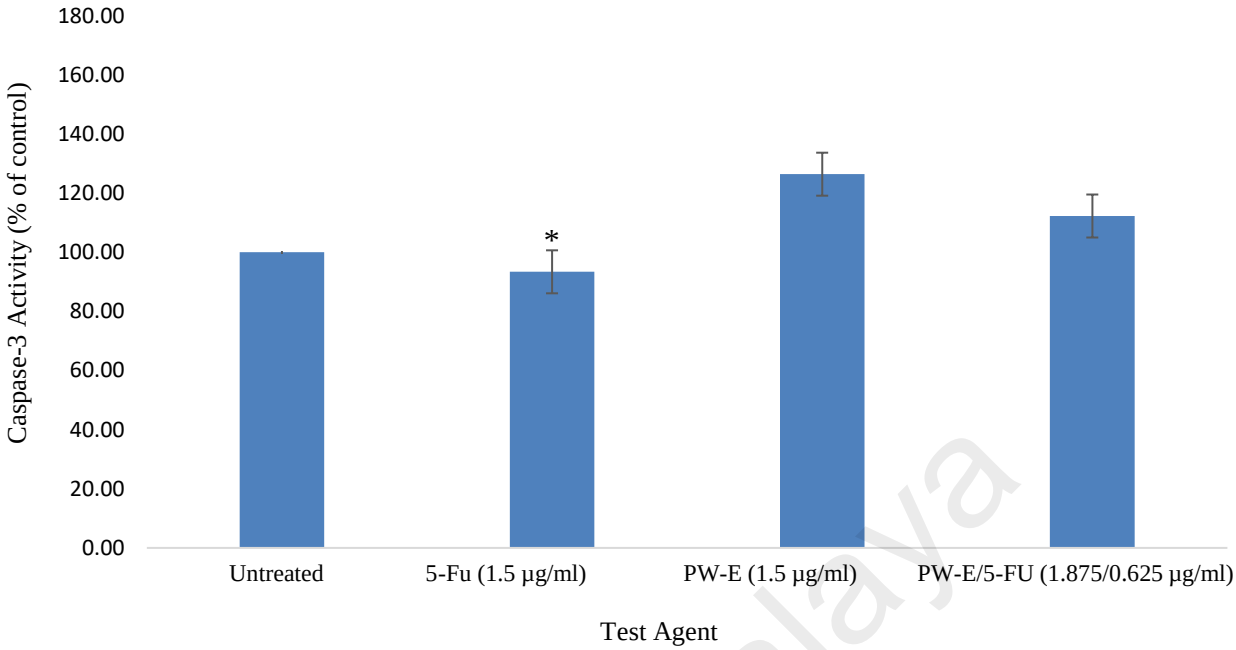


Figure 4.14: Caspase-3 activation in HCT-116 cancer cells with and without addition of different test agents and analysed using Caspase-3 DEVD-R110 Fluorometric and Colorimetric assay kit. The values are expressed as percentage mean ± S.D compared with control from three independent experiments. PW-E: P. watsonii ethyl acetate extract; 5-FU: 5-Fluorouracil, PW-E/5-FU combo at ratio 3:1. 5-Fu (5-Fluorouracil) = 93 ± 7 %, PW-E (*P. watsonii* ethyl acetate extract) = 126 ± 4 % and PW-E+5-Fu = 112 ± 8 %. (* indicates a significant difference from the control ($p < 0.05$).

As shown on Figure 4.14, HCT-116 cells when treated with PW-E alone exhibited increment in caspase-3 activity ranging from 1.23 to 1.51-folds higher compared to untreated cells. The caspase-3 activity in cell-treated with 5-FU alone ranges from 1.04 and 1.26-folds higher than untreated cells. Caspase-3 activation are even higher and increased in HCT-116 cells-treated with PW-E/5-FU combo, where the increment ranging from 1.12-folds to 1.61 folds.

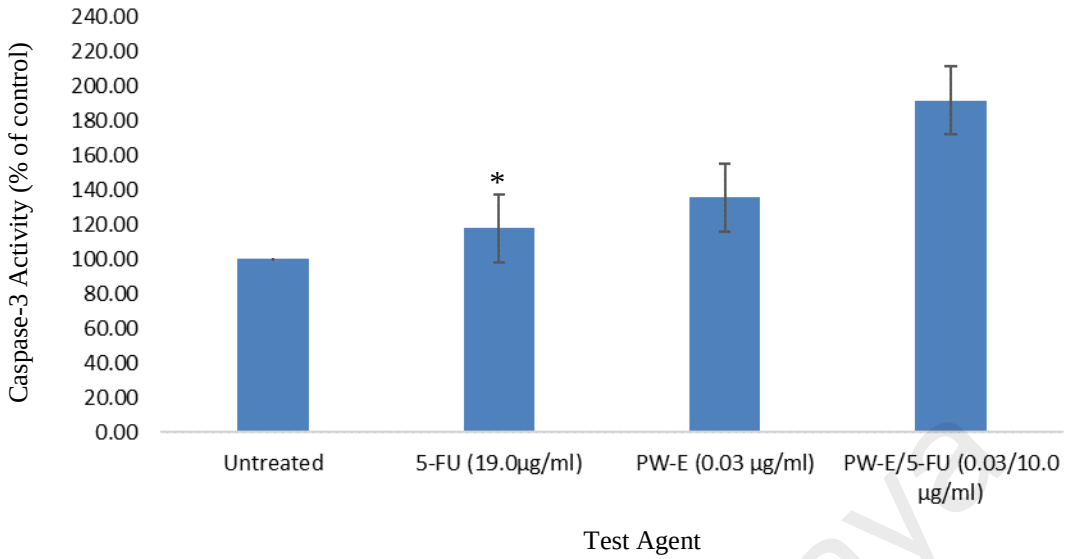


Figure 4.15:Caspase-3 activation in HT-29 cancer cells with and without addition of different test agents and analysed using Caspase-3 DEVD-R110 Fluorometric and Colorimetric assay kit. The values are expressed as percentage mean ± S.D compared with control from three independent experiments. PW-E: *P. watsonii* ethyl acetate extract; 5-FU: 5-Fluorouracil, PW-E/5-FU combo at PW-E and 5-FU concentration of 0.03:10.0. 5-Fu = 118 ± 23%, PW-E (*P. watsonii* ethyl acetate extract) = 135 ± 25 % and PW-E+5-Fu = 191 ± 9%. (* indicates a significant difference from the control ($p < 0.05$).

As can be seen in Figure 4.15, when HT-29 cells were treated with 5-FU alone, the caspase-3 activity increased by about 1.09-folds to1.17-folds, while when treated with PW-E, the caspase-3 activity increased by about 1.29-folds to 1.43-folds when compared to untreated cells. However, the highest increment of caspase-3 activity can be observed when HT-29 cells were treated with the combination of PW-E and 5-FU, where the increment were ranging from 1.52-folds to 1.91-folds when compared to untreated cells.

CHAPTER 5: DISCUSSION

5.1 LC-MS/MS Analysis of *P. watsonii* Ethyl Acetate Extract

In this study, LC-MS/MS analysis was done to identify the major compounds present in *P. watsonii* ethyl acetate extract. LC-MS/MS was chosen for the compound analysis due to the technique's ability to analyse a wide range of plant metabolites including secondary metabolites such as benzoids, alkaloids, flavonoids, isoprenes, terpenes and glucosinolates (Hill & Roessner, 2015). It can also detect highly polar and/or higher molecular weight molecules. In this present study, it was indentified that the *P. watsonii* ethyl acetate extract contains quercetin, kaempferol rhamnoside, kaempferol glucoside, ellagic acid, hyperin, strictinin isomer, strictinin, galloyl HHDP hexoside and also gallic acid. These compounds detected may be responsible for the observed cytotoxic activity of *P. watsonii* in this present work.

Flavonoid is one of the major compounds identified in *P. watsonii* ethyl acetate extract. The flavonoids identified are quercetin, kaempferol rhamnoside and kaempferol glucoside and hyperin. Kuo, (1997) stated that flavonoids can be further classified into flavonols, flavones, flavanes, isoflavanes and flavanols. Depending on their biological activities, flavonoids can play a role in human cancer prevention. Among flavonoids, quercetin is considered an excellent free-radical scavenger, even if such an activity strongly depends on the intracellular availability of reduced glutathione (Gibellini *et al*., 2011). Besides its potent antioxidant activity, quercetin also exerts a direct, pro-apoptotic effect in cancer cells, and blocks the growth of several human cancer cell lines at different phases of the cell cycle and this findings have been recorded in cellular and animal models (Gibellini *et al*., 2011). Quercetin has also been found to exert no damage towards normal, non transformed cells but exert high toxicity on cancer cells (Gibellini *et al*., 2011) which

could explain *P. watsonii* ethyl acetate extract selectivity towards cancer cells compared to normal cells. Kaempferol, which was also identified to be present in *P. watsonii* ethyl acetate extract is also known to have antioxidant activity and used for cyto-protection, where it protects cells from harmful agents (Huang *et al*., 2010). Studies have also reported that kaempferol has anti-proliferation activity and induces apoptosis in various human cancer cell lines *in vitro* such as prostate cancer (De Leo *et al*., 2006), colon cancer (Mutoh *et al*., 2000), oral cavity cancer (Kim *et al*., 2005) and leukaemia (Marfe *et al*., 2009).

Besides flavonoids, other phenolic compounds identified in *P. watsonii* ethyl acetate extract are ellagic acid, galloyl HHDP hexoside and gallic acid. Various studies had shown that phenolic compounds exerted antitumour properties by inhibiting the growth of the cells, inducing cell-cycle arrest, and exerting pro-apoptotic effects on colon cancer cells (Larrosa *et al*., 2006; Kern *et al*., 2007; Gonzalez *et al*., 2009). Ellagic acid derived from plants had also been proveen to be a potent anti-cancer agent (Das *et al*., 1984). Ellagic acid has been hypothesized to target NF-kB, cyclin D1, p21cip1/waf1 and p53 (Aggarwal & Shishoida, 2006). Ca Ski cervival carcinoma cells, Caco-2 colon cells and MCF-7 breast cells are some of the cancer cells that have been reported to be susceptible to ellagic acid as it possesses growth inhibiting properties and promotes apoptosis in the cancer cells (Ramasamy *et al*., 2012).

5.2 Cytotoxic Activity of *P. watsonii*, 5-FU and *P. watsonii*-5-FU Combination

The search for an effective treatment for colorectal cancer (CRC) is a vital matter as it is the third most common cancer that causes death in the world. Despite early treatment by surgery, about 25 % experience post-operative relapse (Galizia *et al*., 2013). 5-Fluorouracil (5-FU) is currently considered a key drug in clinical

chemotherapeutic of CRC. However, several issues arise with the use of 5-FU in CRC treatment such as short half-life of 5-FU and its resistance and severe side effects to the patients whose taking this drug (Li *et al*., 2009). Many studies had been carried out in improvement of 5-FU in CRC treatment and overcome the problems associated with the use of 5-FU.

New strategies are needed to overcome the disadvantages of 5-FU and other chemotherapy drugs in clinical application. One of the means is by combination therapy which combines the chemotherapy drug with natural product agents, such as plant extract or compound. In this approach, natural product-derived extract or compound is combined with the chemotherapy conventional drug to enhance the cytotoxicity and improve the effectiveness of these drugs. This combination may enhance or lessen the cytotoxicity effects compared to when being used in a single dosage form. Combinations between plant active ingredients with conventional anti-cancer drug are becoming a new target in drug delivery (Ng *et al*., 2014).

Plant extracts have fewer side effects compared to chemotherapy drugs as it is loaded with natural secondary metabolites and other phytochemicals. In addition, minimum cytotoxicity towards normal cells and synergistic interaction between anti-cancer drug-plant extract combination have been shown in laboratory studies (Ng *et al*., 2014). Li *et al*. (2009) also reported that chemo-adjuvant compounds that have the potential to inhibit the growth of cancer cells, also can reduce chemotherapy-induced toxicity. By identifying potential non-toxic plant extracts, and further combining it with 5-FU, the cytotoxicity effects of the combination towards cancer cells can be analysed and this will be an important step in enhancing and improving CRC chemotherapy (Li *et al*., 2009).

In this present study, the Neutral Red Uptake Cytotoxic Assay was used to evaluate the cytotoxic activity of *P. watsonii* ethyl acetate extract, 5-FU and their combination on human colon cancer cells, HCT-116 and HT-29 cells. This is one of the most commonly used cytotoxicity assays and it provides a quantitative estimation of the number of viable cells in a culture based on the ability of viable cells to incorporate and bind the supravital dye neutral red in the lysosomes (Repetto *et al*., 2008).

P. watsonii ethyl acetate extract showed a good cytotoxicity against both colon cancer cells compared to 5-FU. When both extract and 5-FU were combined, it can be clearly seen that the extract-drug combination synergistically inhibits the growth of both colon cancer with less concentration of 5-FU was needed for an effective cells growth inhibition. A literature survey indicated that there are no study have been reported on the combination effect of *P. watsonii* and 5-FU on cytotoxicity. Therefore, the current information studies were compared with other work reported on the combination effects of plant-derived natural product with 5-FU in on colon cancer cells.

In this present study, the cytotoxicity of PW-E/5-Fu combination against HCT-116 and HT-29 cell lines and human lung fibroblast MRC-5 were also compared and stimulating outcome were found. When 5-Fu were combined with PW-E, the Selectivity Index (SI), the SI values calculated for the extract-drug combo tested against MRC-5 showed that the combo was more selective towards the cancer cells compared to normal cells as the value was more than 3. SI value is and indicator to measure the safety of extract/drug on non-cancerous cells. The result showed that the drug-extract combination was more selective towards cancer cells compared to normal cells. Since 5-Fu is known to cause undesireable side effects towards normal cells and also towards patients receiving treatment (Li *et al*., 2009), the combination of PW-E/5-Fu may offer more bearable treatment to patients under treatment with 5-Fu. *P.*

watsonii extracts have also been reported to be more selective toward cancer cells compared to normal cells (Ramasamy *et al*., 2012; Ramasamy *et al*., 2013). Thus, more study could be done to find out the effectiveness of drug-extract combination in killing cancer cells with less impact towards normal cells.

Clinical applications of 5-FU have been greatly limited due to drug resistance (Zhang *et al*., 2008). Resistance to 5-FU is a multifactorial event (Zhang *et al*., 2008). There are reports stating that 5-FU depends on the p53 tumour suppressor protein to trigger apoptosis (Pritchard *et al*., 1998; Bunz *et al*., 1999; Petak *et al.*, 2000; Borralho *et al*., 2007). p53 is a tumour suppressor protein that aids in regulating feedback towards 5-FU. If p53 protein is disrupted, it will cause the colon cancer cells to be resistant to 5-FU and cancer cells will fail to undergo apoptosis and cell cycle arrest (Mader *et al*., 1998; Bunz *et al*., 1999; Borralho *et al*., 2007; Ng *et al*., 2014). The colon cancer cell line used in this study are HCT-116 and HT-29 cell lines. HT-29 cell lines, as tested with 5-FU in this study requires higher concentration of 5-FU to be able to achieve 50 % inhibition compared to HCT-116. This was because HT-29 was able to adapt and resist against the cytotoxicity of 5-FU through the activation of survival autophagy and the lack of wild-type p53. However, when 5-FU was combined with PW-E, the concentration of 5-FU needed to achieve the same 50 % inhibition was reduced. By understanding more on the reduced resistance of HT-29 towards 5-FU and PW-E combination, CRC chemotherapy can further be enhanced and improved.

Carnesecchi *et al*. (2004) reported that geraniol, an acyclic dietary monoterpene found in aromatic herb oils, increases the cytotoxicity of 5-FU against TC-118 human tumours transplanted in Swiss nu/nu mice. The combination also enhances the drug uptake of the transplanted cell. In the present study, the cytotoxicity of 5-FU against

HCT-116 and HT-29 colon cancer cell lines were increased when the drug was combined with *P. watsonii* ethyl acetate extract. Study by Fishbein *et al*. (2009) also showed that combination of extract of *Panax ginseng* C. A. Meyer (Asian ginseng) with 5-FU increased the anti-proliferative effect towards human colon HCT-116 cancer cells, and the effect was significantly higher when compared with 5-FU and extract tested individually on the same colon cancer cells. Similarly, in a study done by Wang *et al*. (2007), anti-proliferative effects on HCT-116 cell were also significantly enhanced by the combination of flower extract of *Panax notoginseng* with 5-FU, when compared to cells treated with single dosage of the extract and 5-FU. A study by Tang *et al*. (2015) also showed that when 5-FU was combined with triptolide, a diterpenoid tripoxide derived from the herb *Tripterygium wilfordii*, the proliferation of HT-29 cell lines was significantly inhibited. The current results on the cytotoxic activity of *P. watsonii* and 5-FU combination had also shown that it enhanced the anti-proliferative effect of 5-FU on both HCT-116 and HT-29 cells compared to that of cells treated with the drug alone.

Combination of plant-derived extract and drug also had been proven to reverse the resistance of colon cancer cells towards 5-FU. In a study done by Gonzàlez-Vallinas *et al*. (2013), supercritical fluid rosemary extract (SFRE) from *Rosmarinus officinalis* could sensitize the 5-FU resistant towards SW620 colon cancer cells by downregulating the expressions of enzymes related to 5-FU resistancy. When Real-Time Polymerase Chain Reaction (RT-PCR) was performed on the RNA extracted from the colon cancer cells after treatment with (Supuercritical fluid rosemary extract (SFRE) of *R. officinalis* , it showed that there is a downregulation of thymidylate synthetase and tropomyosin 1 genes indicating that the modulation of these genes might be involved in the enhancement of the effect of 5-FU in resistant SW620 colon cancer cells. Similarly, in this present study, the sensitization factor values showed that the combination of *P. watsonii* and 5-FU

increased the sensitivity of 5-FU towards both colon cancer cells. Further studies to determine the mechanism of this resistance reversal by the *P. watsonii* and 5-FU combination is warranted.

Cytotoxicity effect towards the colon cancer cells triggered by the combination between *P. watsonii* and 5-FU has also been shown by induction of apoptosis. The apoptotic activity in human gastric carcinoma cells was induced when gambogic acid, a resin exuded from the tree of *Garcinia hanburyi* tree was combined with 5-FU (Wang *et al*., 2009). Study done by Xu *et al*. (2013) also proved that the effect of apoptosis is induced when 5-FU is combined with extract of *Scutellaria barbata* D. Don (SBE). SBE also increase the apoptosis inducing effect when combined with low dose of 5-FU in both human hepatocellular Bel-7402 carcinoma cell and human colon HCT-8 cancer cells (Xu *et al*., 2013). In addition, Kodach *et al*. (2006) showed that violacin, a pigmnt isolated from *Chromobacterium violaceum*, when combined with 5-FU, also induces apoptosis and potentiates the cytotxic effect of 5-FU in HCT-116 colon cancer cell lines. The combinaton also increases the chemosensitivity of HCT-116 cells towards 5-FU. 5-FU alone cannot increase the chemosensitivity of the cell. In another study by Hwang *et al.* (2005) showed that genistein, a soy derived phytoestrogen belonging to the 'isoflavone' family, when combined with 5-FU, causes the reduction of survival signal Glut-1 and the elevation of pro-apoptotic p53 and p21 in HT-29 colon cancer cell lines. In the present study, combination of *P. watsonii* and 5-FU triggered the cell death by apoptosis induction in both HCT-116 and HT-29 cancer cells.

More research supports on effectiveness of combined therapy between natural products and 5-FU in which the combination significantly synergizing the cytotoxic effect of 5-FU. The concept of dual therapy by combining the plant-derived extract and anti-cancer drug might decrease the dose-related toxicities such as nausea vomiting and frequently impede effective treatment (Forman, 1994; Wu *et al*., 2007).

5.3 *P.* watsonii-5-FU Combination Analysis

Combination between anti-cancer drug and extract/compound-derived natural product could yield three types of interaction, which are synergistic, antagonistic or additive. Synergism interaction is caused when the agents combination is more effective than each agent reacted individually, meaning one of the agents enhances the actions of the second drug. Antagonism is defined as the combination being less effective than the single agents, meaning that one of the agents counteracts the actions of the other (Bijnsdorp *et al*., 2011). Additive effect is the total effect caused by two substances in combination is the same as the sum of individual effects (Drees *et al*., 2005). There are various ways a combination and their interaction can be studied, which are, firstly by combining simultaneously or secondly by a sequential combination schedule (Bijnsdorp *et al*., 2011). In this present study, the first method is used, where combination and its interaction is studied by combining *P. watsonii* and 5-FU simulatenously. It is vital to test the potency of a combination in *in vitro* model, before clinical trials are done and to avert antagonistic actions. Nevertheless, it should be understood that sometimes, antagonistic interaction is actually desired, especially when one drug decreases the unwanted side effects of another drug (Bijnsdorp *et al*., 2011).

One of the most widely used methods to effectively measure quantitatively the dose–effect relationship of each drug alone and its combinations and the interaction occur is the median-drug effect analysis. By using this method, based on the drug cytotoxicity or growth inhibition curve, a combination index (CI) is then calculated. In this present study, the Compusyn 1.0 software was used to calculate the CI by taking the entire shape of the growth inhibition curve into account to determine whether the combination exert synergistic, antagonistic or additive interaction.

According to the Compusyn 1.0 software CI < 1 indicates that the combination is able to react synergistically with each other. If CI = 1, the combination results in additive interaction and if CI > 1, the interaction is antagonistic (Zhang *et al*., 2016). Based on this analysis, CI values obtained when *P. watsonii* and 5-FU combination tested on HCT-116 and HT-29 colon cancer cells were both less than 1, meaning that the combination exerts synergistic interaction in inhibiting the growth of the cells. When 5-FU was combined with *P. watsonii* in the treatment of both HCT-116 and HT-29 colon cancer cell, the total dose needed to reduce cell viability by 50 % was lower than that of 5-FU or *P. watsonii* alone, and this indicate a synergistic interaction occurred between both combination. Based on the Drug Reduction Index (DRI) analysis, which measure on how much (-fold) dose of the drug or agent in a synergistic combination may be reduced at a given effect level when compared to the dose of each drug or agent alone, the DRI values in both cancer cells is more than 1. This indicates that the dose of *P. watsoni* and 5-FU combination is favourable as the value of DRI > 1 shows that the combination is favourable in terms of clinical therapy (Chou, 2010).

The CI value is the natural law–based general expression of pharmacologic drug interactions and is revealed to be the simplest probable way for calculating synergism or antagonism. It featues efficiency in its equation simplicity, experimental designs analysis of data and reduces the experimental size of animals used or the number of patients needed for drug combination clinical trials. The future of drug combination studies have been made simpler and more efficient based on the general theory of the median-effect principle of the mass-action law, its CI algorithm, and its computerized simulation and have been broadly accepted in scientific application and the swift increase in citation numbers (Chou, 2010).

5.4 Apoptosis Assessment in Cells Treated with *P. watsonii*, 5-FU and *P. watsonii*-5-FU Combination

One of the hallmarks of cancer is the ability of cancer cells to evade apoptosis (Hanahan & Weinberg, 2011). New strategies have been developed to restore abnormal apoptotic pathway and stop the proliferation of cancer cells. Some of the strategies are by targeting the BCl-2 family of proteins, p53, the IAPs and targeting caspases. The ability to induce apoptosis, or programmed cell death in cancer cells or malignant tissues, is recognized as one of the effective approaches in cancer chemotherapy and a very vital characteristic of a possible anti-cancer drug (Panchal, 1998; Ramasamy *et al*., 2012).

The morphological assessment of apoptotic cells was done via acridine orange/ ethidium bromide (AO/EB) double staining in order to investigate whether the cytotoxic effects of *P. watsonii* and 5-FU combination are exerted via apoptotic pathway in both HCT-116 and HT-29 colon cancer cells. In this present study, the apoptotic cells were identified based on the cell morphological changes and differentiated between necrotic and viable cells by dye staining which was observed under fluorescence microscope.

Distinct apoptotic morphological changes in treated HCT-116 and HT-29 cells included the rounding up of cells, blebbing of the cell membrane, brightly green stained condensed chromatin, and loss of membrane shape. The viable cells performed circular nuclei which were uniformly distributed in the center of the cell. The intact membranes and DNA of the viable and early apoptotic cells were stained with fluorescence green by acridine orange (Liu *et al*., 2015).

Ethidium bromide only enters the damaged membranes of cells such as late apoptotic and dead cells, releasing orange-red fluorescence when bound to concentrated DNA fragments or apoptotic bodies (Li *et al*., 2013). In addition, AO/EB double staining is also able to detect mild DNA injuries (Ghergi *et al*., 2003). In the past, fluorescent staining using AO alone has been used while the apoptosis detection using AO/EB double staining is a relatively new strategy (Lecoeur, 2002; Liu *et al*., 2015). The AO/EB double staining has further improved apoptosis detection and is able to differentiate between late apoptotic and dead cells compared to AO single staining (Liu *et al*., 2015).

Apoptosis induction is frequently associated with the activation of caspases (Thornberry & Lazebnik, 1998; Nhan *et al*., 2006; Ramasamy *et al.*, 2012). Caspases are a family of protease enzymes playing crucial roles in apoptosis as they are the initiators and executioners in the apoptotic pathway. In the present study, following the treatment of HCT-116 and HT-29 cells with *P. watsonii* and 5-FU combination, showed an increment in the activity of caspase-3 compared to when the cells without addition of any drug or extract and cells treated with the drug and extract individually. Extracts from different *Phyllanthus* species have been reported to show apoptosis induction by increasing caspase-3 activity in various cancer cell lines of different origins (Huang *et al.*, 2003; Liu *et al.*, 2006; Abu Bakar *et al*., 2010).

The capability to control or modulate the life or death of a cell is recognized for its enormous potential in cancer therapy (Elmore, 2007). Studies are now continued to emphasize on the elucidation and analysis of the machinery of cell cycle and signaling pathways that control cell cycle arrest and apoptosis since abnormal machinery of the cell cycle has been associated with the beginning and the progression of cancer (Ramasamy *et al*., 2012). Extensive work needs to be done to find out on at which stage of the cell cycle does *P. watsonii* and 5-FU combination induces apoptosis. Further studies should be carried out in order to understand in depth the mechanism of action of *P. watsonii* and 5-FU combination on inhibiting the growth of the colon cancer cells.

CHAPTER 6: CONCLUSION

Nowadays, researchers have been focusing on combination chemotherapy to overcome the drawbacks of conventional chemotherapy. One of the main reasons combination chemotherapy is applied is to reduce the probability of cancer cells developing resistency towards the drugs . Besides that, combination chemotherapy is also intended to maximize the efficacy of the drug while minimizing undesireable side effects through the delivery of lower drug doses. Combination chemotherapy has been linked to the improvement in patient's compliance to treatments, overcome or delay multi-drug resistance, the development of additive or synergistic interaction effects between drugs and the reduction of drug concentration with subsequent diminishing of toxicity to healthy tissues.

The combination of drug and plant extract is the current potential approach in combination chemotherapy for the treatment of cancer. For thousands of years, plants have been used to treat illnesses, health disorders and prevent diseases. Plants extracts have fewer side effects compared to chemotherapy drugs as it is loaded with natural secondary metabolites and other phytochemicals and the usage of plant extract for many years had proven that they were relatively safe and may even improve the effectiveness of single dosage form of chemotherapeutic drugs. Due to its strong antioxidant activity, the synergistic combination of the plant-derived extract/compound with anticancer drugs is expected diminish toxicities towards normal and healthy cells, subsequently permitting the usage of anticancer drug at high doses with minimal side effects; maintaining the efficacy of the anticancer drug while reducing its concentration; and improving the sensitivity of anticancer drugs towards the cancer cells.

In this study, the cytotoxic effect of *Phyllanthus watsonii* ethyl acetate extract in combination with 5-Fluorouracil on two types of colon cancer cell lines which are HCT-116 and the more 5-FU resistant HT-29 were investigated. This study revealed that the individual treatment of *P. watsonii* and 5-FU inhibited the growth of both HCT-116 and HT-29 cell lines in dose dependent manner while combination of 5-Fluorouracil with extract of *Phyllanthus watsonii* had a potential to enhance the cytotoxicity effect on both human colon cancer cell lines. The combination also has a potential to reduce the resistency of HT-29 cell line towards 5-FU as less concentration of 5-FU was needed to achive the same inhibitory effect when 5-FU was combined with PW-E in this study. In addition, the combination index analysis through median-effect method showed that PW-E-5-FU combination was able to reduce the dosage of individual 5-FU, in turn reducing toxicity while still gives out the same inhibitory efficacy on HCT-116 and HT-29 cell lines. The combination also showed greater selectivity towards colon cancer cells as opposed to normal cells. The result also indicates that both *P. watsonii* and 5-FU combination interacts synergistically towards inhibiting the growth of HCT-116 and HT-29 and this is also a good indication of improvements on the effectiveness of the cells-treated with 5-FU. The study also revealed that the potential cell death mechanism triggered by the *P. watsonii* and 5-FU combination is via apoptosis initiation through caspase-3 activation.

Additional studies are warranted to determine at which cell cycle stage does the drug-extract combination induce apoptosis, and also to understand the interaction mechanism being involved in the apoptotic signaling pathway are induced by this combination. Studies should also be done whether the combination of 5-FU and PW-E could lessen the side effects caused by the use of 5-FU in cancer therapy. Testing using animal models (*in vivo* study) should also be considered as testing *in vitro* and *in vivo* are

under distinct difference. This is to learn if the dose reduction can be sustained in animal study or even true synergism can be attained.

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