UNIVERSITI PUTRA MALAYSIA

SCREENING OF AQUEOUS EXTRACT OF A COMBINATION OF MELASTOMA MALABATHRICUM, MUNTINGIA CALABURA, BAUHINIA PURPUREA AND DICRANOPTERIS LINEARIS LEAVES AGAINST HT-29

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By

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ABSTRACT

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Colorectal cancer is one of the most diagnosed and leading causes of cancer death worldwide. Despite the advancement in the field of drug-mediated cancer treatment, their effectiveness has been overshadowed by the development of various side effects. In an attempt to contribute towards the advancement of new anticancer drug discovery from natural products, this study evaluate the cytotoxic potential of several medicinal plants, namely a combination of aqueous extract of *Melastoma malabathricum* L. (family *Melastomataceae*) and *Muntingia calabura* L. (family *Muntigiaceae*), AEMMMC as well as the combination of aqueous extract of *Bauhinia purpurea* L. (family *Fabaceae*) and *Dicranopteris linearis L.* (family *Gleicheniaceae*), AEBPDL against human colorectal adenocarcinoma cell line (HT29). All these plants have been reported to exert various medicinal properties which are known to play a role in the mechanism of anticancer action. The leaves were mixed in a ratio of 1:1 for the combination of AEMMMC and AEBPDL and were extracted using ultrasonication with water as solvent and further freeze dried. Serial dilutions of different concentrations (7.8125 - 1000 µg/mL) were prepared to evaluate their anticancer activity using the *in vitro* MTT assay. The combination of AEMMMC demonstrated potential cytotoxic effect in reducing the cell viability of the HT-29 cancer cell line for 24, 48 and 72 hours post treatment. The cytotoxic effect of the extract increases in a dose-dependent manner which it shows a dramatic cytotoxic effect starting from concentration of 250µg/mL. However, the combination of AEBPDL showed no effect in reducing the cell viability of the HT-29 cancer cell line. In conclusion, the combination of AEMMMC has the potential as an anticancer agent against colorectal cancer. More comprehensive studies need to be done to evaluate its activity as an anticancer agent.

Keywords: Natural product; *Melastoma malabathricum, Muntingia calabura; Bauhinia purpurea; Dicranopteris linearis; in vitro* anticancer; colon cancer

ABSTRAK

SARINGAN EKSTRAK AKUEUS GABUNGAN DAUN *MELASTOMA MALABATHRICUM, MUNTINGIA CALABURA, BAUHINIA PURPUREA* DAN *DICRANOPTERIS LINEARIS* TERHADAP HT-29

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Kanser kolorektal adalah salah satu penyakit yang paling didiagnosis dan penyebab utama kematian kanser diseluruh dunia. Walaupun terdapat kemajuan dalam bidang rawatan kanser, keberkesanan mereka telah dibayangi oleh pelbagai kesan sampingan. Dalam usaha untuk menyumbang ke arah kemajuan penemuan ubat baharu anti kanser daripada produk semulajadi, kajian ini menyiasat potensi sitotoksik beberapa tanaman perubatan, iaitu gabungan yang dinamakan *Melastoma malabathricum* L. (famili *Melastomataceae*) and *Muntingia calabura* L. (famili *Muntigiaceae*), AEMMMC serta gabungan *Bauhinia purpurea* L. (famili *Fabaceae*) and *Dicranopteris linearis L.* (famili *Gleicheniaceae*), AEBPDL terhadap sel kanser kolorektal (HT-29). Semua tumbuhan ini telah dilaporkan untuk mempunyai pelbagai ciri perubatan yang memainkan peranan dalam mekanisme anti kanser. Daun-daun tersebut digabungkan dengan nisbah 1:1 untuk gabungan AEMMMC dan AEBPDL dan diekstrak menggunakan ultrasonik dengan air sebagai pelarut dan seterusnya dibeku kering. Pencairan bersiri dengan kepekatan yang berbeza (7.8125 - 1000 µg/mL) telah disediakan untuk menilai aktiviti anti kanser mereka dengan menggunakan kaedah MTT. Gabungan AEMMMC menunjukkan potensi kesan sitotoksik dalam mengurangkan sel hidup HT-29 untuk 24, 48 dan 72 jam selepas rawatan. Kesan sitotoksik ekstrak meningkat bergantung kepada dos dan ia menunjukkan kesan sitotoksik yang dramatik bermula daripada kepekatan 250µg/mL. Walaubagaimanapun, gabungan AEBPDL tidak menunjukkan kesan dalam mengurangkan sel hidup HT-29. Kesimpulannya, gabungan AEMMMC mempunyai potensi sebagai agen antikanser terhadap kanser kolorektal. Kajian yang lebih komprehensif perlu dilakukan untuk menilai aktivitinya sebagai agen anti kanser.

Kata kunci: Produk semulajadi; *Melastoma malabathricum, Muntingia calabura; Bauhinia purpurea; Dicranopteris linearis; in vitro* anti kanser; kanser kolorektal

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cm	Centimetre
cm^2	Centimetre square
°C	Degree Celsius
gm	Gram
mg	Milligram
kg	Kilogram
mL	Millilitre
µl	Microlitre
%	Percentage
AEMMMC	Aqueous extract of *Melastoma malabathricum* and *Muntingia calabura*
AEBPDL	Aqueous extract of *Bauhinia purpurea* and *Dicranopteris linearis*
CRC	Colorectal cancer
HT-29	Human colorectal adenocarcinoma cell line
HL-60	Human promyelocytic leukemia cell line
Caov-3	Human ovarian cancer cell line
MCF-7	Human breast adenocarcinoma cell line
MDA-MB-231	Human breast adenocarcinoma cell line
HeLa	Immortal cell line
K-562	Human myelogenous leukemia cell line

WiDr	Human colon adenocarcinoma cell line
A549	Human lung cancer cell line
5-FU	5-Fluorouracil
BRAF	BRAF gene
MEK	Mitogen-activated extracellular signal-regulated kinase
PD1	Programmed cell death protein 1
PDL1	Programmed death ligand 1
IC_{50}	Half maximal inhibitory concentration
CO_2	Carbon dioxide
NO	Nitric oxide
cGMP	Cyclic 3'-5' guanosine monophosphate
RPMI	Roswell Park Memorial Institute
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
DMSO	Dimethyl sulfoxide
PBS	Phosphate-buffered saline
FBS	Fetal bovine serum
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay

CHAPTER 1

INTRODUCTION

Cancer is defined as the excessive growth of abnormal cells. Cancer has been a major cause of death among humans worldwide with about 9.6 million death in 2018. Based on GLOBOCAN 2018 data, colorectal cancer is one of the most common cancer, being the third most diagnosed and the second leading cause of cancer death worldwide (WHO, 2018). It has been estimated that cancer causes the highest death rate among the world's human population which affects around 6 million lives annually (Koul, 2019). As cancer is one of the major burden of diseases, studies on the early prevention or treatment for cancer is highly needed to reduce the incidence of this disease.

Every culture has been extensively using plants as traditional medicine for treatment of diseases. In 1950, U.S. Natural Cancer Institute has recognized natural products as a potential source of anticancer drugs. A variety of medicinal plants are widely studied for their natural antioxidants content which are believed to exert medicinal activities such as flavonoids, phenolics and tannins (Aboshoufa & Elgubbi, 2019). Furthermore, plant-derived compounds are considered to have less toxic effect, which has drawn many interests for the development of new clinical cancer drug (Greenwell and Rahman, 2015). Four major classes of plant-derived compounds have been found to play a critical role as anticancer agents namely bisindole (vinca) alkaloid, podophyllotoxins, taxanes and camptothecins (Safe and Kasiappan, 2016).

Despite the advancement in the field of drug-mediated cancer treatment, their effectiveness has been overshadowed by the development of various side effects. The drawbacks of colorectal cancer drugs include the recurrence of cancer, associated with drug resistance, expensive, adverse effects, ineffective response rate and poor tumor-specific selectivity that can impair patients' quality of life (Xie et al., 2020). Due to this matter, scientists have turned to natural products as a new source of lead for the development of new anticancer drugs which is well tolerated with diverse bioactivities, wide range of sources and less side effects.

To contribute towards the advancement of new anticancer drug discovery from natural products, there are many studies investigating the medicinal plants for their anticancer activity. Hence, the aim of this study is to evaluate the cytotoxic potential of the combination of *Melastoma malabathricum* L. (family *Melastomataceae*) and *Muntingia calabura* L. (family *Muntigiaceae*), and *Bauhinia purpurea* L. (family *Fabaceae*) and *Dicranopteris linearis L.* (family *Gleicheniaceae*) as anticancer agent against the human colorectal adenocarcinoma cell line (HT-29). All of these plants have been reported to exert various medicinal properties including antioxidant and anti-inflammatory activities, which are known to play a role in the mechanisms of anticancer action (Sari et al., 2018; Suhaimy et al., 2017; Rahmawati et al., 2018; Rajesh et al., 2016; Sarkar et al., 2017). It is noteworthy to highlight on the link between oxidative stress and inflammation with the development of cancer (Thanan, 2015; Mileo & Miccadei, 2016). Thus, it is plausible to suggest that any compounds that can attenuate oxidative stress and/or inflammation might also inhibit cancer development.

General objective

To evaluate the cytotoxic potential of aqueous extract of a combination of *M. malabathricum* and *M. calabura* (AEMMMC) and aqueous extract of a combination of *B. purpurea* and *D. linearis* (AEBPDL) as anticancer agent against the human colorectal adenocarcinoma cell line (HT-29).

Specific objectives

1. To determine the cells viability of HT-29 after treatment with AEMMMC and AEBPDL for 24, 48 and 72 hours.

It is hypothesized that the aqueous combination of these leaves possesses synergistic effect as anticancer agent to attenuate the growth of HT-29 cancer cell line.

CHAPTER 2

LITERATURE REVIEW

2.1 Cancer

Cancer is characterized by the development of abnormal cells in the body which can be defined as benign (non-cancerous) or malignant (cancerous). Cancer is named by where the cancer originates from, for instance tumour originates from colon or rectum is defined as colorectal cancer (Kumari, 2020). Carcinogenesis is a multistep process which involve initiation, promotion and progression of cancer. Initiation involve a series of irreversible changes such as mutation or modification of the gene which leads to dysregulation of normal cell proliferation, differentiation and survival. The promotion stage is a reversible and lengthy process, involving the proliferation of the preneoplastic cells containing the mutation from initiation. Progression is a rapid, irreversible stage where the neoplastic cells become malignant, having the potential to metastasize and become invasive (Fig. 2.1).

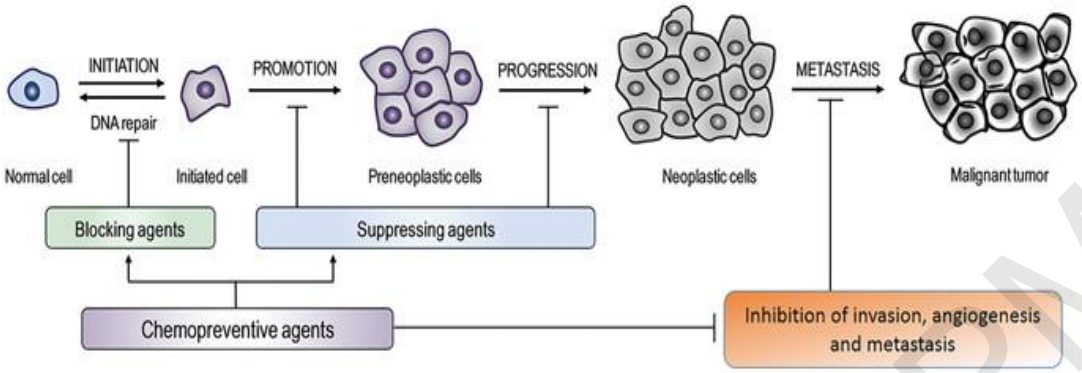

Figure 2.1. Carcinogenesis

(Source: Siddiqui et al., 2015)

2.1.1 Epidemiology of cancer

In 2020, about 600 000 Americans are estimated to die of cancer and more than 1.8 million new cases of cancer will be diagnosed in the United States. The most common cancer for men and women is lung, breast, colorectal, prostate and stomach cancer. Among men, (40.1%) the average lifetime risk of being diagnosed with invasive cancer is marginally higher than for women (38.7%). The gender difference can vary according to sex. For instance, prevalence of cancer during childhood (birth until 14 years) is around 10 % higher in males than in females, while it is 77% higher for females in early adulthood (20 to 49 years), primarily due to incidence of breast cancer in young females (Siegel et al., 2020a). In Malaysia, cancer is ranked as second most diagnosed for both sexes (GLOBOCAN, 2018).

2.2 Colorectal cancer

Colorectal cancer is a type of cancer than begins as tumour or tissue growth called as polyps in the lining of the colon or the rectum. Just around 10% of the polyps develop into invasive cancer and invasive cancer that develops in the colorectal region is classified as colorectal adenocarcinoma which responsible for 96% of colorectal cancer (Rawla et al., 2019).

2.2.1 Epidemiology of colorectal cancer

The incidence of colorectal cancer is predicted to increase to 2.5 million new cases worldwide in 2035 (Dekker et al., 2019). According to Siegel et al., (2020b), about 147,950 individuals are estimated to be diagnosed with colorectal cancer in 2020, and 53,200 will die from the condition, including 17,930 cases and 3,640 deaths of people younger than 50 years old. Although the lifetime risk of colorectal cancer is comparable for both men (4.4%) and women (4.1%) aged 45 years and below, the incidence rate is 30% to 40% higher risk in men than women ages 55 to 74 years old. The incidence of colorectal cancer in United States for old age group (50 years old and above) have decreases while the incidence of colorectal cancer in middle age group (25 to 49 years old) have increases over the last few decades. The cases are found to be higher in developed countries (Rawla et al., 2019).

In Malaysia, incidence and mortality of colorectal cancer (based on 2008 to 2013 data) was higher in Chinese ethnicity followed by Malay and India. Moreover, males have 1.33 times higher risk of developing colorectal cancer than female (Abu Hassan et al., 2016). The incidence of colorectal cancer is higher in developed countries such as Singapore, South Korea and Japan compared to other developing countries such as Malaysia. Initially, more than 90 % of the cases of colorectal cancer in Malaysia arise in people over 40 years of age which are often diagnosed in advanced stage (Veettil et al., 2017).

2.2.2 Risks of colorectal cancer

Most colorectal cancers are sporadic cases, which genetic changes is not due to inherited gene mutation. Nevertheless, both genetic and environmental factors play a role in colorectal cancer. Inherited colorectal cancers are less common as it accounts for 7-10% of the overall cases. Inherited mutations of colorectal cancer can be subdivided as non-polyposis syndromes (familial colorectal cancer and Lynch syndrome) and hamartomatous and adenomatous polyposis syndromes (Dekker et al., 2019; Rawla et al., 2019). Besides genetic factors, studies found that environmental and dietary factors such as the 'Western pattern diet', tobacco smoking and alcohol consumption can attribute to increased risk of colorectal cancer (Marley & Nan, 2016). Furthermore, the are several groups that have higher risk to develop this cancer than the general population which are those treated with adenoma or colorectal cancer, those with family background having benign and malignant tumour of the intestine,

and women prior to age 45 with epidemiologically related cancer of the breast, ovary and uterine (Arvelo et al., 2015).

2.2.3 Colorectal cancer screening and diagnosis

There are 5 stages of colorectal cancer which are 0, I, II, III, and IV, indicating the progression of the cancer (Table 2.1).

Table 2.1. Stages of colorectal cancer

Stages	Explanation
Stage 0	It is a non-invasive stage wherein the cancer has not grown beyond the first layer of the colon wall
Stage I	It indicates that the cancer has grown into either the second or third layer of the colon wall. There is no cancer in nearby or distant sites
Stage II	It indicates that the cancer has grown into the fourth layer of the colon wall. There is no cancer in nearby or distant sites
Stage III	It indicates that the cancer has reached the neighbouring lymph nodes or presence of tumor deposits (small secondary tumors within the colon)
Stage IV	It indicates that the cancer has spread to the other organs like the liver and lungs

Reprinted from Koul, B. (2020).

Colorectal cancer is mostly asymptomatic until it reaches advanced stage where the clinical signs are broad such as change in bowel habits, occult or overt rectal bleeding, mass in the abdomen or rectum, abdominal pain or anaemia. The screening and diagnostic tests for colorectal cancer are stool based tests or visual examination of the colon or rectum such as colonoscopy or sigmoidoscopy, double-contrast barium enema and biopsy (Koul, 2020; Dekker et al., 2019).

2.3 Currently available colorectal cancer drugs

Surgery, targeted therapy, neoadjuvant radiotherapy and adjuvant chemotherapy are the standard treatment for colorectal cancer (Van Der Jeught, 2018). For early stages of non-metastatic CRC (stages I – III), it is typically treated with surgical resection followed by chemotherapy and/or radiotherapy, while patients with metastatic colorectal cancer usually receive systemic chemotherapy (Cassidy & Syed, 2017). Chemotherapeutics act in cancer cell death by inducing DNA damage and/or initiating multiple signalling pathways, such as cell cycle arrest, DNA repair and inhibition of global translation (Huang et al., 2019). There are two main types of chemopreventive agents, which are suppressing agents and blocking agents. Suppressing agents inhibit the initiated cells from progressing to malignant by interfering the promotion and progression stage. On the other hand, blocking agents act by preventing the initiation of cancer, by interfering with important macromolecules or metabolic activation (Siddiqui et al., 2015).

The standard treatment for cancer depends on the stages of the cancer, which includes single-drug or multiple/combination of drugs for effective treatment. Drugs commonly used to treat colorectal cancer include 5-Fluorouracil (5-FU), Irinotecan (Camptosar), Capecitabine (Xeloda), Oxaliplatin (Eloxatin) and in combination of Trifluridine and Tipiracil (Lonsurf). In addition, monoclonal antibodies such as Bevacimuvab (Avastin) and Cetuximab (Erbitux) has made a huge progress for colorectal cancer treatment (Van Der Jeught, 2018). For the treatment of late stages colorectal cancer patients, targeted therapies such as Nintedanib and Fruquintinib are

used as anti-angiogenic. Targeted therapies will function on cancer cells by specifically inhibiting the growth, differentiation, and migration of the cells (Xie et al., 2020). Other drugs act by inhibiting other pathways such as BRAF, MEK, PDL1, and PD1 (Table 2).

Table 2.2. Selected products in late-stage development for colorectal cancer

Drug	Key developers	Mode of action	Highest phase
Nintedanib*	Boehringer Ingelheim	TKI targeting VEGFR	Phase III
Fruquintinib	Hutchison MediPharma/Lilly	TKI targeting VEGFR	Phase III
Masitinib	AB Science	TKI targeting KIT	Phase II/III
Encorafenib	Array BioPharma	BRAF inhibitor	Phase III
Binimetinib	Array BioPharma	MEK inhibitor	Phase III
Cobimetinib*	Genentech/Roche	MEK inhibitor	Phase III
Atezolizumab*	Genentech/Roche	PDL1 inhibitor	Phase III
Pembrolizumab*	Merck & Co.	PD1 inhibitor	Phase III
Nivolumab*	Bristol-Myers Squibb	PD1 inhibitor	Phase II
Lefitolimod	Mologen	TLR9 agonist	Phase III
OncoVax	Vaccinogen	Autologous tumour cell vaccine	Phase III
Napabucasin	Sumitomo Dainippon	Cancer stemness inhibitor targeting STAT3 pathway	Phase III

MEK, MAPK/ERK kinase; PD1, programmed cell death 1; PDL1, PD1 ligand 1; STAT3, signal transducer and activator of transcription 3; TKI, tyrosine kinase inhibitor; TLR9, Toll-like receptor 9; VEGF, vascular endothelial growth factor. *Already marketed for other cancers.

Reprinted from Cassidy, S., & Syed, B. A. (2017).

2.3.1 Limitations of current anti colorectal cancer drug

However, there are several limitations of the treatment for instance, drug resistance, expensive, cytotoxicity, adverse reactions, ineffective response rate, poor tumour-specific selectivity and relapse of cancer (Xie et al., 2020; Huang et al., 2019). Studies found that about 50% of colorectal cancer patients develop resistance towards 5-fluorouracil-based chemotherapies (Van der Jeught et al, 2018). The typical adverse reactions of cancer drugs are nausea, loss of appetite, hair loss, fatigue, diarrhea, constipation, depression. rashes, bruises and bleeding (Wong, 2018). Other than that, certain chemo drugs can lead to a more serious complications such as menstrual and infertility issues, heart damage, nerve damage, hand-foot syndrome, muscle and bone problems, brain function and increased risk of leukemia (National Cancer Institute US, 2017).

2.4 Natural products

Since decades, cancer patients commonly opted for medicinal plants to find cure, as complementary or alternative therapy for cancer treatment (Jacobo-Herrera et al., 2016). Drugs derived from plants are desired as they are natural and readily available. Traditionally, the methods to obtain plant-derived medicines are inexpensive and simple, which is by preparation of crude extracts or direct consumption of the plants. Moreover, the non-invasive administration of the medicines is painless and safe for the patients (Buyel., 2018). The antioxidants properties of natural products have been widely studied for their anti-proliferative and pro-apoptotic properties for them to be

introduce as preventive agents or therapeutic drug candidates. It is reported about 74% of cancer drugs are derived from natural products, which these molecules are found to exert anti-inflammatory and antioxidant properties (Pucci et al., 2019; Al-Asmari et al., 2015).

For instance, vinca alkaloid, taxanes, podophilotoxins, polyphenols, brassinosteroids and camptotoxins are plant-derived compounds that has been identified and extracted for their anticancer properties (Mohammadi et al., 2016; Grenwell & Rahman., 2015). In addition, nutraceutical natural compounds such as anthocyanidins, terpenoids, carotenoids, and flavonoids has been studied for their anticancer potential as there are much evidence of moderate consumption of the compounds can decrease the risk of colorectal cancer (Fernández et al., 2016). Natural-product anticancer agents act by activating or inhibiting multiple pathways that lead to tumor growth suppression, apoptosis activation, and antimetastatic and/or antiangiogenic activity (Safe & Kasiappan, 2016).

2.5 Melastoma malabathricum

Melastoma malabathricum Linn (family *Melastomaceae*) is a shrub that grows in the wild, and locally known as '*Senduduk*'or '*Keduduk'* to the Malay. It is found abundantly throughout South and Southeast Asia, Taiwan, China, the South Pacific Ocean and Australia on the Indian Ocean Islands. The plant of *M. malabathricum* is 2 to 5 metres tall and the leaves are elliptical to lanceolate shaped. The flower has 5 petals, purplish pink coloured with fine orange seeds (Edianto et al., 2020).

Figure 2.2 *Melastoma malabathricum* plant

Traditionally, the leaves, barks, roots, stems and whole plants are used for various diseases such as dysentery, diarrhea, haemorrhoids, wounds, toothache, skin problems and gastric ulcers (Samad et al., 2018; Zakaria et al., 2015). Findings found that *M. malabathricum* has anticancer, antimicrobial, anti-inflammatory, antipyretic, antinociceptive, antioxidant, antiviral, antifungal, anti-obesity, anticoagulant, antidiarrheal, antiulcer, antarthritic and hepatoprotective activities (Idris et al., 2017; Kumar et al., 2016; Zakaria et al., 2016a).

There are various constituents isolated from *M. malabathricum* leaves such as malabathrins (A, B, C, D, E and F), nobotanin (B, D, G, H and J), phenols, strictinin, flavonoids, saponins, casuarictin, pterocarinin C, anthocyanins, pedunculagin, 1,2,4,6-tetra-O-galloyl-b-D-glucose, 1,4,6-tri-Ogalloyl-b-D-glucose, 8,11-octadecadienoic acid methyl ester, stearic acid methyl ester, tocopherol, α-tocopherol-β-D-mannoside, kaempferol, kaempferol-3-O-(2″,6″ di-*O*-*p*-*trans*-coumaroyl)-*β*-glucoside, 2-

hydroursolic acid, ursolic acid, aceatic acid, procyanidin A, cathecin, epigallocathecin, gallocathecin, triterpenes, oligomers, b-sitosterol, uvaol, a-amyrin, sitosterol 3-O-b-dglucopyranoside, p-coumaric acid, hesperidin, rutin, steroids, chlorogenic acid, gylcosides, caffeic acid, quercetin, and gallic acid, which were initiated to show antioxidant and anticancer activity in vitro (Kamsani et al., 2019; Isnaini et al., 2018; Samad et al., 2018; Verma et al., 2016).

The yield of *M. malabathricum* leaves were found to be the highest by using water extraction (Awang et al., 2016). Aqueous extract of *M. malabathricum* was found to inhibit the proliferation HL-60 and Caov-3 cancer cell lines with IC_{50} of 50 µg/mL and 11 µg/mL, respectively (Samad et al., 2018). Besides, methanol extract of *M. malabathricum* leaves was found to be cytotoxic to HT-29 and HeLa cancer cell lines, but not to 3T3 cell line, which indicate it is not toxic to normal cells. The IC_{50} of the extract against HT-29 cell line after 72 hours incubation was approximately 100 µg/mL. The study also mentioned that the plant extract is safe at 5000 mg/kg for oral administration, without nephrotoxicity or hepatotoxicity reported (Edianto et al., 2020; Kamsani et al., 2019).

According to Idris et al., (2017), *M. malabathricum* leaves extract shows promising anticancer properties as it was found to induce secondary necrosis or late apoptosis in MCF-7 and A549 cells with IC_{50} of more than 400 µg/mL at 24 hours after treatment. It was also reported that ethanol extract of *M. malabathricum* exhibit cytotoxic effect towards PC-3 cancer cell line, with IC_{50} of 630.57 µg/mL (Sophiya et al., 2019). Besides, the plant showed non-opioid induced antinociceptive function at

the peripheral and central levels in part by modulating the vanilloid receptors, the glutamatergic network, and the NO-mediated/cGMP-independent pathway (Zakaria et al., 2016).

2.6 Muntingia calabura

Muntingia calabura Linn (family *Muntingiaciae*) is known worldwide as Jamaican cherry or '*kerukup siam*' in Malaysia. It is a shrub widely cultivated in Southeast Asia, particularly along the roadsite. The tree reaches a height of 25 to 40 feet while its leaves are 2.5 to 15 cm and 1 to 6.5 cm wide. The leaves are evergreen, lanceolate or ovate, long pointed at the apex, oblique at the base and the flowers are small, white-coloured and quite odourless (Vijayanand & Thomas, 2016).

Figure 2.3 *Muntingia calabura* plant

It is traditionally used to treat swelling, gastric ulcers, measles, headache, stomach ache, incipient cold and as antiseptics (Nirmala et al., 2020; Buhian et al., 2016; Balan et al., 2015). Several findings reported that *M. calabura* exhibited significant pharmacological effects such as gastroprotective, antiproliferative, antipyretic, antiulcer, anticancer, antioxidant, antidiabetic, anti-inflammatory, antipyretic, antibacterial, antitryrosinase and cardioprotective activities (Nirmala et al., 2020; Ragasa et al., 2015).

Several scientific studies have been conducted to identify the phytoconstituents of *M. calabura* leaves which include chalcones (i.e., 2′,4′-dihydroxychalcone and 2′,4′-dihydroxy-3′-methoxychalcone) , flavonoids [i.e., (2S)-5′-hydroxy-7,3′,4′-trimethoxyflavanone and 4′-hydroxy-7-methoxyflavanone], sterols, phenolics, quinones, terpenoids, alkaloids, glycosides, rutin, ferulic acid, pinocembrin, gallic acid, carbohydrates, fixed oils, amino acids, saponins and tannins (Nirmala et al., 2020; Nasir et al., 2017; Buhian et al., 2016).

Findings reported that aqueous, chloroform and methanol extract of *M. calabura* exert cytotoxic effects towards MCF-7, HeLa, K-562 and WiDr (methanol extract only). Aqueous and methanol extracts of the plants were also reported to inhibit HT-29 cell proliferation while chloroform and methanol extracts reported to have cytotoxic effect on HL-60 cancer cell line (Nirmala et al., 2020; Desrini & Purnamasari, 2017). However, the methanolic extract of *M. calabura* was reported to have no significant cytotoxic effect against the cell line of A549 human lung adenocarcinoma (Ampil et al., 2018). A study by Nasir and Zakaria (2017) reported

that ethanol extract of *M. calabura* exert highest cytotoxicity effect on HT-29 at IC_{50} of 12.9 µg/mL. This can be supported by Jisha et al., (2020) which revealed that ethyl acetate fraction of *M. calabura* leaves exert anti-colorectal cancer activity due to abundance of phytoconstituents which may reduce damage to colon tissue caused by oxidative stress.

2.7 Bauhinia purpurea

Bauhinia purpurea Linn (family *Fabaceae*) is known as '*Tapak Kuda'* in Malaysia and known as purple orchid-tree or butterfly tree. It is 7 to 10 metres tall erect shrub, semi-evergreen leaves with broad, long, rounded, bilobed at the base. The pink to purple flowers looks like orchids, with around 5 inch wide. It is also an ornamental plant and widely cultivated throughout Southeast Asia to South China.

Figure 2.4 *Bauhinia purpurea* plant

(Source: https://garden.org/plants/view/87781/Orchid-Tree-Bauhinia-purpurea/)

It is traditionally used to treat ulcer, diarrhea, dropsy, goiter, enlarge cervical glands, cuts, pain, nausea, rheumatism, swelling of the legs, hallucinations, delirium febris and tongue or lips blackness. The plant demonstrated the anticancer, antioxidant, antimicrobial, analgesic, antidiarrheal, anti-inflammatory, antipyretic, antimalarial, antiarthritic and thrombolytic activities. (Vijayan et al., 2018; Das et al., 2018; Kiranmayi et al., 2018; Sarkar et al., 2016a). In addition, studies reported anticonvulsant, nephroprotective and hepatoprotective activities of the plant. Phytochemical analysis of *B. purpurea* leaves identified the presence of flavonoids, glycosides, phenolic compounds, fixed oils, fats, alkaloids, steroids, triterpenes, phytosterols, lignin's, saponins, condensed tannins, proteins and carbohydrates (Kaliyaperumal et al., 2016; Rana et al., 2016; Zakaria et al., 2016b)

Findings from Vijayan et al., (2018) reported that *B. purpurea* aqueous leaf extract shows remarkable antioxidant and anticancer activity. It was revealed to have significant cytotoxicity activity against A549 adenocarcinoma cell line with IC_{50} of 69.87µg/mL. Moreover, ethylacetate, petroleum ether, and aqueous partitions of methanol extract of *B. purpurea* leaves was discovered to possess a strong antioxidant activity due to its flavonoids, phenolic compounds, saponins and tannins content (Zakaria et al., 2016b). Sarkar et al., (2016b) mentioned in his study that the yield for aqueous extracts of *B. purpurea* leaves was found to be higher than hydroalcoholic extracts.

2.8 Dicranopteris linearis

Dicranopteris linearis Linn (family *Gleicheniaceae*) is locally known as *'resam'* or false staghorn fern. It is a terrestrial pteridophyte found widespread in subtropical and tropical regions in the world (Mai et al., 2019). It has large leaves, forking stems and compound stalked fronds that grow along the ground.

Figure 2.5 *Dicranopteris linearis* plant

Various uses of *D. linearis* in traditional practices include fever, ulcer, asthma, cough, reproductive problems, boils, skin problems, wound, intestinal worm infection and as insect repellent (Aboshoufa & Elbuggi, 2019). A growing body of literature has evaluated the antioxidant, hepatoprotective, antinociceptive, antipyretic, anti-inflammatory, gastroprotective, antimicrobial, antiproliferative, chemopreventive, anthelmintic and wound healing activities of the plant (Zakaria et al., 2020; Zakaria et al., 2017; Rajesh et al., 2016b; Ponnusamy et al., 2015).

The phytoconstituents analysis of *D. linearis* leaves revealed the presence of tannins, saponins, quinones, triterpenes, phenols, flavonoids, protocatechuic acid, ferulic acid, caffeic acid, gallic acid, p-coumaric acid, rutin, astragalin, alkaloids, glycosides, coumarins, betacyanin, catechin, quercetin, kaempferol, apigenin, steroids and reducing sugars (Zakaria et al., 2020; Aboshoufa & Elbuggi, 2019; Rajesh et al, 2016a). The phytoconstituents are identified for their radical scavenging activity. Ethyl acetate extract of *D. linearis* was found to exert prominent antioxidant activity. However, the extract was found to exhibit low anti-inflammatory effect on in vitro xanthine oxidase and lipoxygenase mediated inflammatory assays (Zakaria et al., 2020). Other than that, Baharuddin et al., (2018) in his study revealed that methanol extract of *D. linearis* was found to be effective against MDA-MB-231, MCF-7 and HeLa cancer cell lines but did not cause toxicity on normal cells, which indicate its safety. The study reported that *D. linearis* inhibit MDA-MB-231 cancer cell line proliferation through S-phase arrest and apoptosis induction.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Preparation of plant

The leaves of *M. malabathrum, M. calabura, B. purpurea and D. linearis* were collected from Serdang district, Selangor between July to September 2019. Instruments for the extraction of the plants include Erlenmayer flask (DURAN, Germany), cotton wool, Whatman no. 1 filter paper (Sigma-Aldrich, USA), grinding machine from Longer machinery (China), Bransonic ultrasonic bath (Thomas Scientific, USA) and freeze dryer (Labconco, Kansas City).

3.1.2 Cell culture

The cell line of human colorectal adenocarcinoma (HT-29) was purchased from American Type Culture Collection, ATCC (LA, USA). Roswell Park Memorial Institute (RPMI)1640 medium was purchased from Invitrogen Corporation (California, USA). Trypsin-EDTA (10x) and Fetal Bovine Serum (FBS) were purchased from PAA Laboratories GmBH (Pasching, Austria). Phosphate buffered saline (PBS) was obtained from Sigma-Aldrich (Steinheim, Germany). T25 flask, 96-

well microplate, 2, 5 and 10 mL serological pipette were purchased from Techno Plastic products, TPP (AG, Switzerland). Falcon 50 mL conical centrifuge tube was purchased from Fisher Scientific, (Pittsburgh, USA). Instruments that have been used in this experiment include Biosafety cabinet Class II (Labconco, Kansas City), CO_2 incubator (Shelab, Germany), Axiovert 25 inverted phase-contrast microscope (ZEISS, Germany), haemocytometer (La Fortain, France) and multi-channel pipettte (RAININ, USA).

3.1.3 MTT Assay

The tetrazolium salt, 3-4,5 dimethylthiazol-2,5 diphenyl tetrazolium bromide (MTT) and 0.1% dimethyl sulfoxide (DMSO) was obtained from Sigma-Aldrich (USA). All measurements were carried out with the samples contained in 96-well microplate (Palcon-plastics, USA). Optical density was measured by using enzyme-linked immunosorbent assay, ELISA reader (Anthos Zynith 340S).

3.2 Methods

3.2.1 Preparation of plant

3.2.1.1 Plant collection

The leaves of *M. malabathrum, M. calabura, B. purpurea and D. linearis* were collected from Serdang district, Selangor between July to September 2019. The leaves were rinsed with water and dried indoors at room temperature for 2 weeks. The leaves were further dried in the oven before grinded into coarse powder.

3.2.1.2 Extraction of plant

The coarse powder leaves were mix in a ratio of 1:1, 50 g of *M. malabathricum* and 50 g of *M. calabura* were mixed for the combination AEMMMC. For the combination of AEBPDL, 50 g of *B. purpurea* were mixed with 50 g of *D. linearis.* The combination of the leaves was suspended in ultrapure water and ultrasonicated at 80°C for 30 minutes. Then, the supernatant was filtered using cotton wool and Whatman no.1 filter paper. The residue was subjected for the same procedure twice. It was further freeze dried to obtain dry powder, resulting in a yield 7.5% for AEMMMC and 13.8% for AEBPDL. All the crude dried extracts collected were stored at 4°C and dissolved in distilled water before treatment.

3.2.2 Cell culture

3.2.2.1 Cell line and culture conditions

Human colorectal adenocarcinoma cell line (HT-29) was obtained from American Type Cell Culture (ATCC). They were cultured in Roswell Park Memorial

Institute (RPMI) 1640 medium supplemented with 10% Fetal Bovine Serum (FBS). The cells were maintained in incubator with 5% CO_2 at 37°C. HT-29 were grown as a monolayer culture in 25 cm^2 tissue culture flasks. The cells were subcultured once they have reached approximately 80% confluence. The medium was discarded, and the cells were rinsed using sterile 2 mL PBS at pH 7.4. The PBS was discarded and 1ml of trypsin-EDTA was added into the flasks. Then, the flask was incubated in CO_2 incubator for 5 minutes. After that, the flask was observed under inverted microscope to look for detachment of the cells. 5 mL of new fresh medium was added into the flask and the cells were resuspended using the sterile pipettes. The cells suspension was then transferred into a new T25 flask. Cell counting was done using haemocytometer under inverted microscope before seeding. 1 x 10^5 cells/mL were seeded into 96 well plates and allowed to grow in CO_2 incubator for 24 hours (37°C, 5% CO_2) to allow adhesion of cells after seeding.

3.2.2.2 Cell treatment with extracts

Stock of 1 mL of AEMMMC and AEBPDL was diluted into eight different concentration of 7.8125, 15.625, 31.25, 62.5, 125, 250, 500 and 1000 µg/mL. Upon 24 hour incubation, the medium was discarded and replaced with µl of fresh complete medium. Then, 100 µl of each concentration extract was added into the wells with 3 replicates. The last row of the plate is used as control which contain the medium alone. The treatment was applied on 3 different treatment plates at 24, 48 and 72 hours respectively. The plate was incubated at 37°C, 5% CO_2 atmosphere with their respective incubation time.

3.2.2.3 MTT Assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is a colometric assay to determine the cells viability, cytotoxicity and proliferation. The MTT assay measures the reduction of yellow MTT to an insoluble purple formazan product by mitochondrial succinate dehydrogenase. At the end of the treatment, 50 µl of MTT reagent (2 mg/mL) was added into each well and the plate was incubated for 4 hours (37°C, 5% CO_2 atmosphere). After incubation, the MTT reagent was discarded and 100 µl of DMSO was added into each well to solubilize the purple formazan crystals formed. The absorbance of the formazan product was read at 570nm using ELISA reader. The percentage of viable cells was calculated using the formula:

$$\text{Cell viability (\%)} = \frac{\text{Sample absorbance}}{\text{Control absorbance}} \times 100$$

Graph of percentage of cell viability against sample concentrations were plotted.

CHAPTER 4

RESULTS

4.1 Cytotoxic effect of AEMMMC and AEBPDL on HT-29 cancer cell line

Human colorectal adenocarcinoma cell line (HT-29) was treated with different concentration of AEMMMC and AEBPDL ranging from 7.8125 to 1000 µg/mL for 24, 48 and 72 hours incubation period. After the incubation period, the percentage of cell viability of the HT-29 cells was determined by MTT assay. The plate was then read using the ELISA reader at absorbance of 570nm. The data is from one replication data of triplicates. A graph of cell viability against concentration was plotted.

Results by MTT assay were presented in Figure (4.1 – 4.6). After certain period of incubation of cells treated with AEMMMC, Figure (4.1 – 4.3) showed that the percentage of cell viability decreases in increased time. This indicate the cytotoxic effect of AEMMMC is in dose dependent manner. The cell viability was dramatically decrease at concentration of 250 µg/mL for all 24, 48 and 72 hours post incubation.

For cell viability after treatment with AEBPDL, the results (Figure 4.4 – 4.6) showed an unstable data with low cytotoxicity effect. The lowest cell viability (67.94%) was at concentration of 250 µg/mL at 24 hours post incubation. For both AEMMMC and AEBPDL, the cytotoxic effect was time independent.

4.1.1 Effect of AEMMMC on colorectal adenocarcinoma cell line (HT-29) after 24 hours treatment

The percentages of cytotoxicity of AEMMMC against HT-29 cancer cell line after 24 hours incubation was illustrated in Figure 4.1. AEMMMC gave cytotoxic effect on HT-29 by reducing the cell viability with increased concentration.

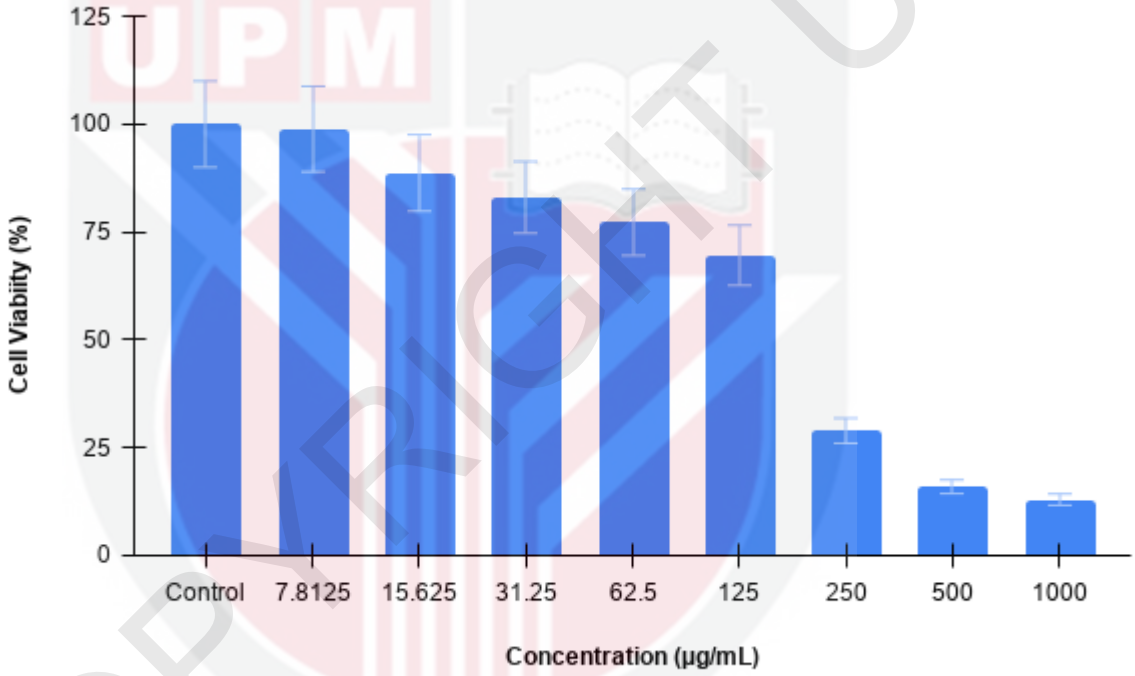

Figure 4.1 The effect of different concentrations of AEMMMC on HT-29 for 24 hours post-incubation. HT-29 cells (1×10^5 cell/well) were seeded into 96-well plate and treated with different concentration of AEMMMC (7.8125 – 1000 µg/mL) for 24 h and MTT assay were done to determine the cell viability of the cells. The data is from one replication data.

4.1.2 Effect of AEMMMC on colorectal adenocarcinoma cell line (HT-29) after 48 hours treatment

The percentages of cytotoxicity of AEMMMC against HT-29 cancer cell line after 48 hours incubation was illustrated in Figure 4.2. AEMMMC gave cytotoxic effect on HT-29 by reducing the cell viability with increased concentration.

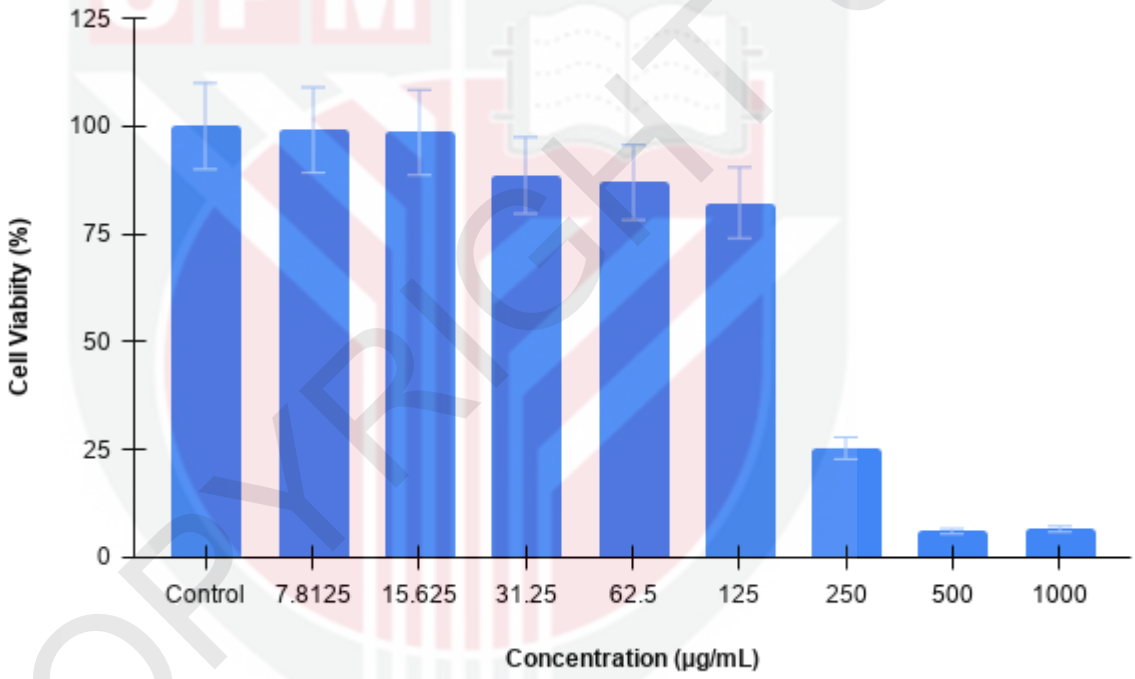

Figure 4.2 The effect of different concentrations of AEMMMC on HT-29 for 48 hours post-incubation. HT-29 cells (1×10^5 cell/well) were seeded into 96-well plate and treated with different concentration of AEMMMC (7.8125 – 1000 µg/mL) for 48 h and MTT assay were done to determine the cell viability of the cells. The data is from one replication data.

4.1.3 Effect of AEMMMC on colorectal adenocarcinoma cell line (HT-29) after 72 hours treatment

The percentages of cytotoxicity of AEMMMC against HT-29 cancer cell line after 72 hours incubation was illustrated in Figure 4.3. AEMMMC gave cytotoxic effect on HT-29 by reducing the cell viability with increased concentration.

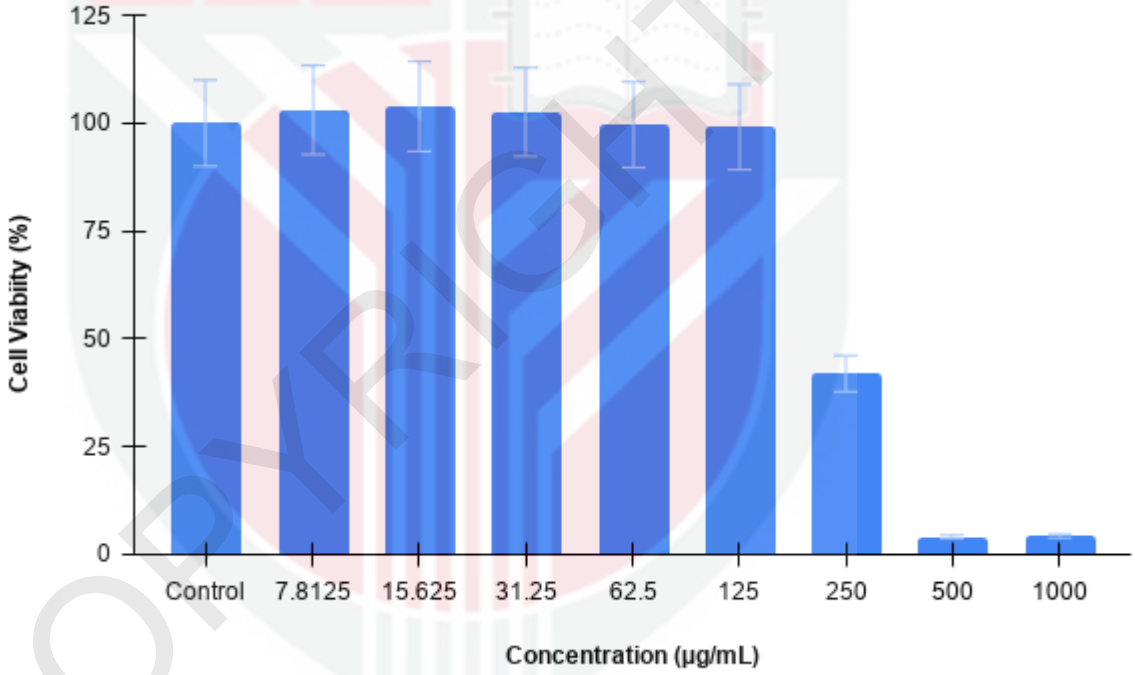

Figure 4.3 The effect of different concentrations of AEMMMC on HT-29 for 72 hours post-incubation. HT-29 cells (1×10^5 cell/well) were seeded into 96-well plate and treated with different concentration of AEMMMC (7.8125 – 1000 µg/mL) for 72 h and MTT assay were done to determine the cell viability of the cells. The data is from one replication data.

4.1.4 Effect of AEBPDL on colorectal adenocarcinoma cell line (HT-29) after 24 hours treatment

The percentages of cytotoxicity of AEBPDL against HT-29 cancer cell line after 24 hours incubation was illustrated in Figure 4.4. AEBPDL showed low cytotoxicity against HT-29 and there is no correlation between dose and cell viability.

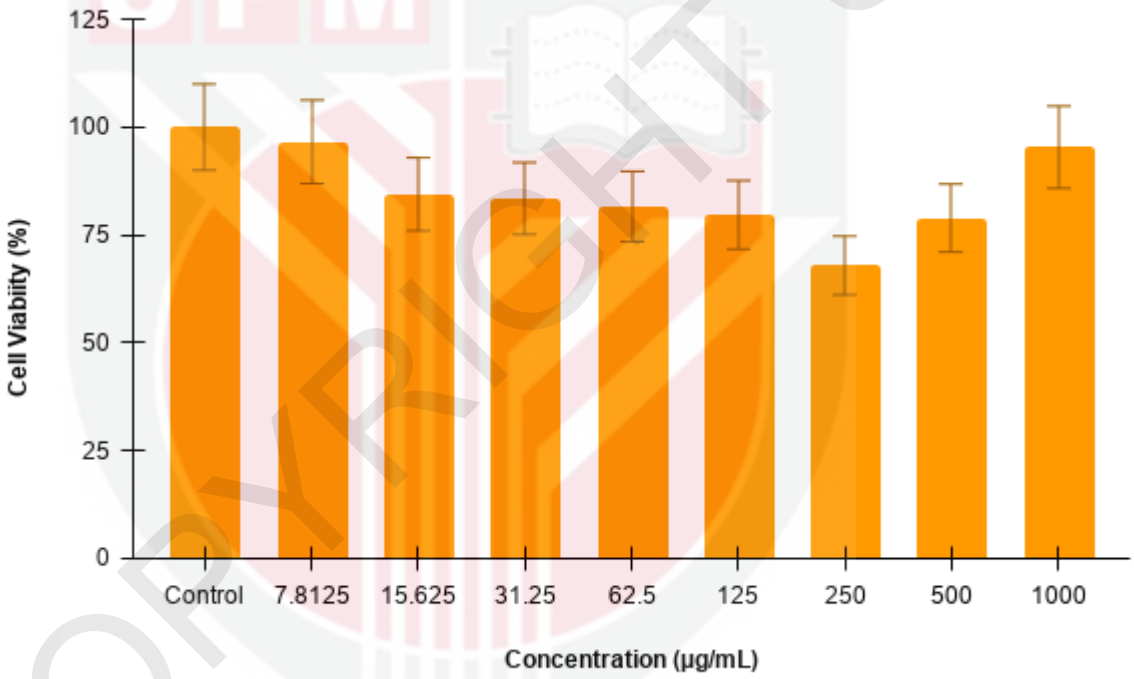

Figure 4.4 The effect of different concentrations of AEBPDL on HT-29 for 24 hours post-incubation. HT-29 cells (1×10^5 cell/well) were seeded into 96-well plate and treated with different concentration of AEBPDL (7.8125 – 1000 µg/mL) for 24 h and MTT assay were done to determine the cell viability of the cells. The data is from one replication data.

4.1.5 Effect of AEBPDL on colorectal adenocarcinoma cell line (HT-29) after 48 hours treatment

The percentages of cytotoxicity of AEBPDL against HT-29 cancer cell line after 48 hours incubation was illustrated in Figure 4.5. AEBPDL showed low cytotoxicity against HT-29 and there is no correlation between dose and cell viability.

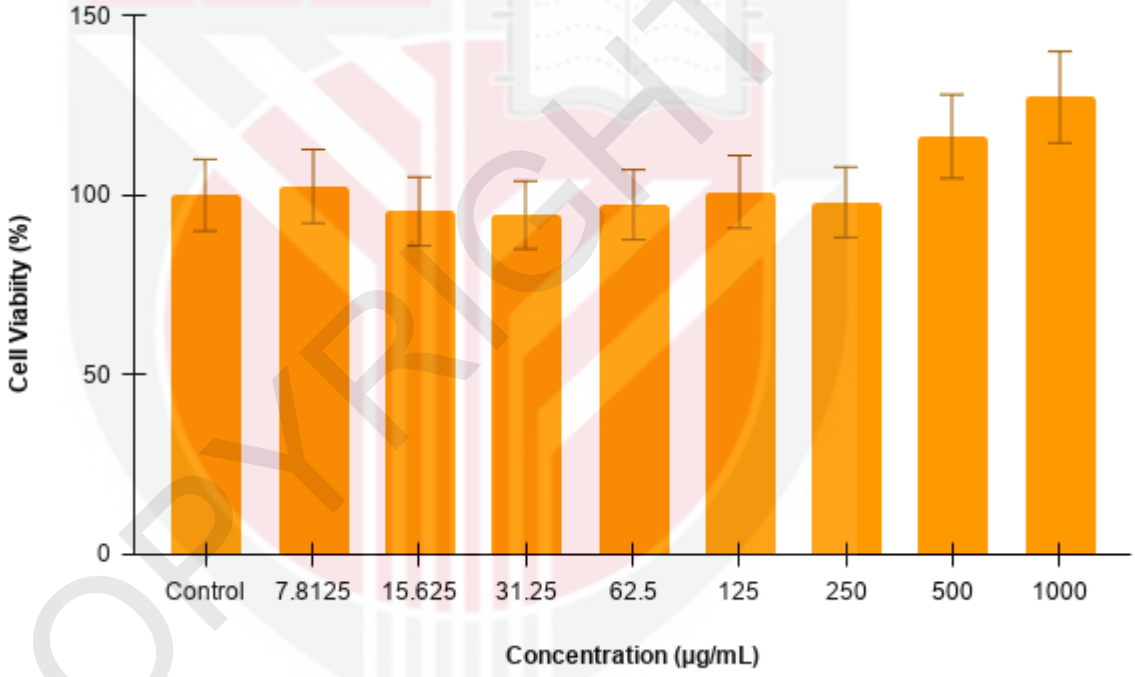

Figure 4.5 The effect of different concentrations of AEBPDL on HT-29 for 48 hours post-incubation. HT-29 cells (1×10^5 cell/well) were seeded into 96-well plate and treated with different concentration of AEBPDL (7.8125 – 1000 µg/mL) for 48 h and MTT assay were done to determine the cell viability of the cells. The data is from one replication data.

4.1.6 Effect of AEBPDL on colorectal adenocarcinoma cell line (HT-29) after 72 hours treatment

The percentages of cytotoxicity of AEBPDL against HT-29 cancer cell line after 72 hours incubation was illustrated in Figure 4.6. AEBPDL showed low cytotoxicity against HT-29 and there is no correlation between dose and cell viability.

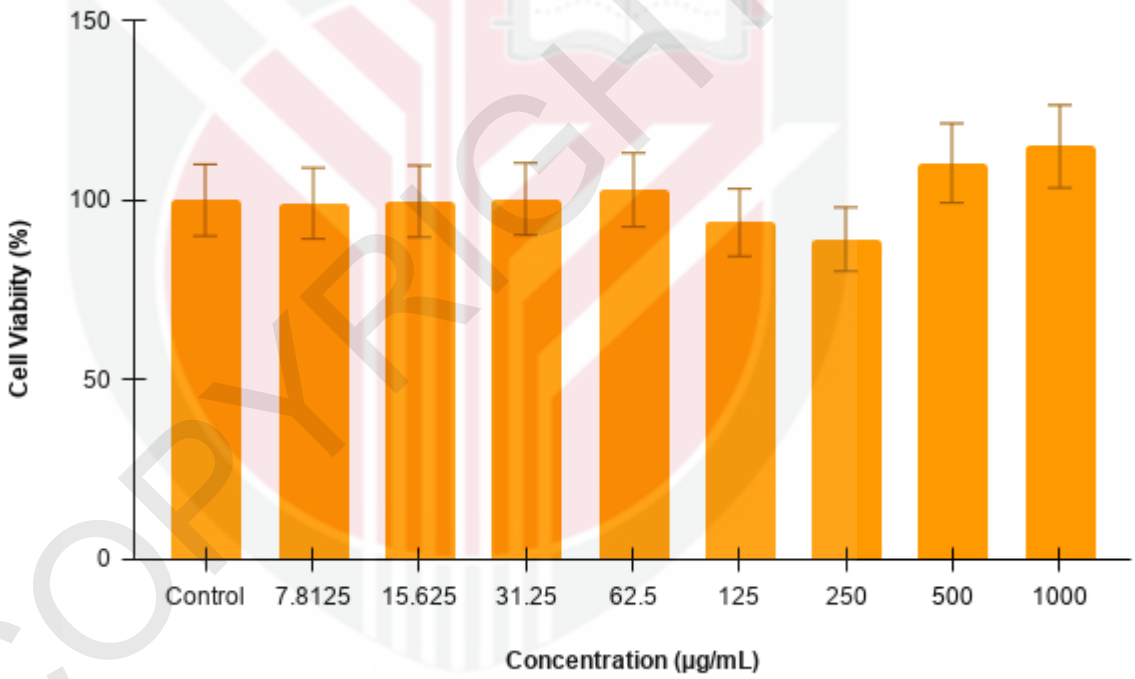

Figure 4.6 The effect of different concentrations of AEBPDL on HT-29 for 72 hours post-incubation. HT-29 cells (1×10^5 cell/well) were seeded into 96-well plate and treated with different concentration of AEBPDL (7.8125 – 1000 µg/mL) for 72 h and MTT assay were done to determine the cell viability of the cells. The data is from one replication data.

4.1.7 Effect of treatment period of AEMMMC on HT-29

The percentages of cytotoxicity of AEMMMC against HT-29 cancer cell line after 24, 48 and 72 hours incubation was illustrated in Figure 4.7. The graph showed the relationship between time and concentration. From the graph, it can be concluded that the effects of AEMMMC on HT-29 cell line was time independent.

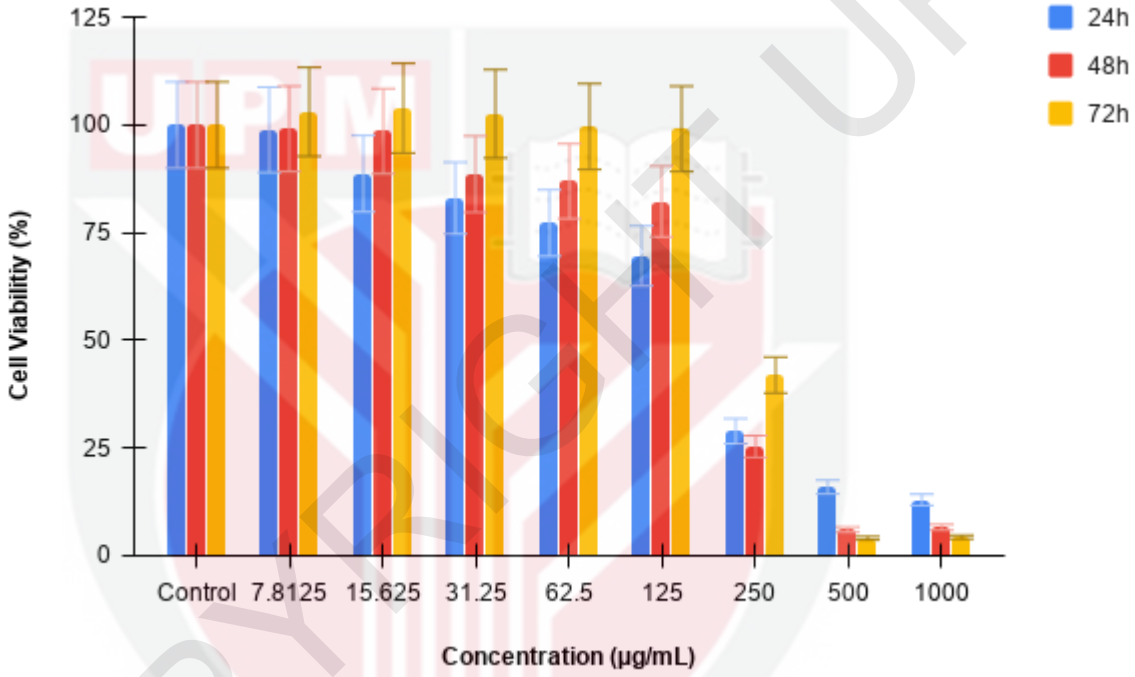

Figure 4.7 The effect of different concentrations of AEMMMC on HT-29 for 24, 48 and 72 hours post-incubation. Results obtained in Figure 4.1 – 4.3 were combined to observe the cytotoxic effect of AEMMMC on HT-29. The results demonstrated that the cytotoxic effect was time independent.

4.1.8 Effect of treatment period of AEBPDL on HT-29

The percentages of cytotoxicity of AEBPDL against HT-29 cell line after 24, 48 and 72 hours incubation were illustrated in Figure 4.8. The graph showed the relationship between time and concentration. From the graph, it can be concluded that the effects of AEBPDL on HT-29 cell line was dose- and time-independent.

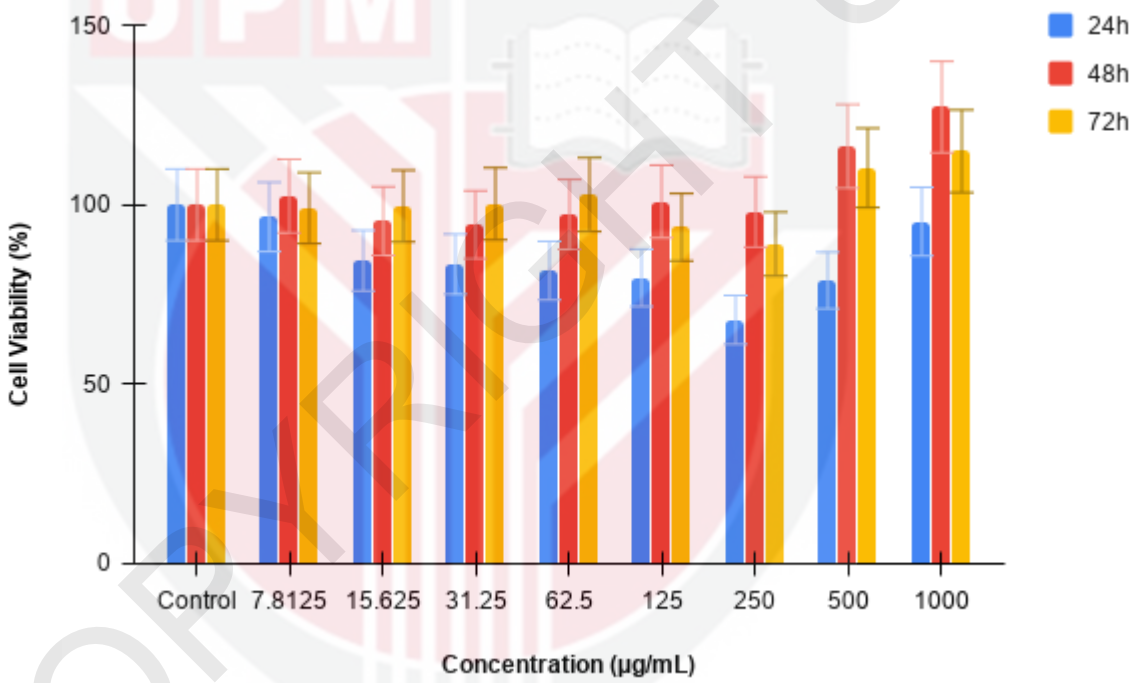

Figure 4.8 The effect of different concentrations of AEBPDL on HT-29 for 24, 48 and 72 hours post-incubation. Results obtained in Figure 4.4 – 4.6 were combined to observe the cytotoxic effect of AEBPDL on HT-29. The results demonstrated that the cytotoxic effect was time independent.

CHAPTER 5

DISCUSSION

Natural products have gained interest for the development of new anticancer drugs. Our study screened the anticancer activity of the combination of 4 aqueous plant extracts which are *M. malabathricum, M. calabura, B. purpurea* and *D. linearis* against human colorectal adenocarcinoma cell line (HT-29). The plants exhibit various medicinal properties such as antioxidant and anti-inflammatory which plays an important role in the mechanism of cancer. Our findings demonstrated that aqueous extract of the combination of *M. malabathricum* and *M. calabura* (AEMMMC) exhibit potential cytotoxic effect towards HT-29 cancer cell line compared to untreated control group. From the MTT assay result, the cytotoxic effect exhibited by AEMMMC was in a dose dependent manner, which the cell viability of HT-29 cancer cell line reduces in increased concentration. However, the cytotoxic effect of the plants was not correlated with time. In contrast, aqueous extract of the combination of *B. purpurea* and *D. linearis* (AEBPDL) showed low and unstable cytotoxicity effect. The extracts were also not associated with the incubation time. The polar compounds of the plants might not have the anti colorectal cancer properties. The failure of AEPBDL in inhibiting HT-29 cancer growth could not be taken to indicate their ineffectiveness, as only one replication data was used. Before a final decision can be made on the anticancer potential of AEBPDL, it is suggested that the plants are studied on other types of cancer cells and/or using other types of extracts.

The combination of the plants has several active compounds which might function synergistically to provide beneficial benefits and reduce adverse effects (Shukla & Mehta, 2015). In general, plants also contain a wide range of free radical scavenging molecules such as flavonoids, phenols, carotenoids, tannins, alkaloids, saponins and terpenoids with high antioxidant activity (Aboshoufa & Elgubbi, 2019). These phytoconstituents play an important role in the medicinal properties of the plants, which contribute towards the prevention and suppression of chronic diseases such as cancer. Aqueous extracts were found to have higher yield with less toxicity and resulted in high concentration of polar compounds such as flavonoids, saponin and tannin (Nirmala et al., 2020).

The possible anticancer mechanisms exerted by the phytoconstituents identified in the plant might be due to its ability in preventing or interfering with cancer metabolisms such as inhibiting hormones or enzymes that activate cancer, alteration of cell cycle, promote DNA repair, stimulate the body to produce protective enzymes, induce antioxidant activity, improve immunity trigger apoptosis, target abnormally expressed molecular factors, modulate cell growth factors, and prevent cancer tissue angiogenesis and metastasis (Singh et al., 2016; Shukla & Mehta, 2015). They are rich in flavonoids, which plays a role in inflammation inhibition through apoptotic mechanism or cyclin dependent kinase inhibitors and alter the cell cycle capture during G1/S phase. Furthermore, flavonoid is responsible for down regulating antiapoptotic gene products, suppressing inflammatory transcription factors and cell survival kinase (Kumar et al., 2016). Phytoconstituents identified in *M. malabathricum* such as asiatic acid, caffeic acid, ursolic acid, chlorogenic acid, p-coumaric acid, quercetin, rutin and

kaempferol was reported to have anti colorectal cancer activity against HT-29 cancer cell line (Kamsani et al., 2019).

Natural products have become a major interest for cancer drug candidates with desired therapeutic effect and less side effects. However, even if the effect of plant crude extract is genuine, the safety and efficacy of crude mixtures need to be studied as there might be metabolites with undesirable effects in addition to the active pharmaceutical ingredients (APIs), and the relative concentrations of the APIs and other metabolites might be difficult to control. APIs with anticancer activity are usually isolated to prevent deleterious side effects, thus allowing specific mode of action to be assessed at defined concentrations (Buyel., 2018).

The data from this study demonstrated the combination of aqueous extract of *M. malabathricum* and *M. calabura* (AEMMMC) has cytotoxic activity against human adenocarcinoma cell line (HT-29). The cytotoxic activity increases in increased concentration.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

In conclusion, the combination of AEMMMC has the potential as an anticancer agent against colorectal cancer in a dose dependent manner. There is a dramatic decrease in cell viability of the HT-29 cell line starting from concentration 250 µg/mL. Although the combination of AEBPDL showed low and unstable cytotoxic effect, the potential of the combination of plants was not fully discovered as only one replication data was used. There is no correlation between treatment of both AEMMMC and AEBPDL with the incubation time.

Further analyses need to be carried out for at least 3 independent experiments, to prove the anti colorectal potential of the plants. Moreover, comprehensive studies on cytotoxicity and antiproliferative activities of the plants such as apoptotic and cell cycle analyses of the plants need to be done to determine the mode of cell death induced by the plants. It is suggested that the plants are studied on other types of cancer cells and/or using other types of extracts to discover the anticancer potential of the plants.

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