CELL CYCLE ARREST AND APOPTOSIS- INDUCING EFFECT OF Dicranopteris linearis (Burm.f.) Underw. LEAF CRUDE EXTRACT IN MDA-MB-231 CELL

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Dicranopteris linearis is a common species of fern locally known to the Malays as 'Resam'. Scientifically, the plant has been reported to have antinociceptive, anti-inflammatory, antipyretic, chemopreventive and antioxidant properties which can be linked to its potential to treat various kinds of ailments including inflammatory-related diseases and cancer. Nevertheless, its anticancer potential has not been extensively investigated. This study was done to evaluate the cytotoxic activities of *D. linearis* extracts against several cancer cell lines, to observe the morphological changes on MDA-MB-231 cells, to determine the cell cycle arrest induction in MDA-MB-231 cells and to analyze the mode of cell death in MDA-MB-231 cells. MTT assay was used to determine the cytotoxic effects of Methanol (MEDL) and petroleum ether (PEEDL) extracts of *D. linearis* at concentration of 100, 50, 25, 12.5, 6.25 and 3.125 µg/mL against a panel of cancer cell lines namely breast adenocarcinomas (MCF-7 and MDA-MB-231), cervical adenocarcinoma (HeLa), colon carcinoma (HT-29), hepatocellular carcinoma (HepG2) and lung carcinoma (A549) for 72 hours period of incubation. MTT results were compared with cancer cells that were not pretreated with MEDL and PEEDL and cancer cells that were treated with 5-fluorouracil (5-FU) as the positive drug control group. Mouse fibroblast cells (3T3) were used to observe the cytotoxic effect of MEDL and PEEDL against the normal cells. Data indicated that MEDL showed the most significant cytotoxicity effect against MDA-MB-231 cell at IC_{50} value of 22.4 µg/mL while PEEDL does not show cytotoxic effect against all of the cancer cells. MEDL also showed selective cytotoxic activity against the proliferation cancer cells (MDA-MB-231, HeLa and MCF-7) and did not harm the normal mouse fibroblast (3T3) cells. MEDL was able to inhibit the growth of MDA-MB-231 cell in a time dependent manner after incubation period of 24, 48 and 72 hours which showed that MEDL was able to prevent recurrent of cancer cells growth during the incubation period. Phase contrast microscopy examinations showed that MEDL was able to induce apoptosis in MDA-MB-231 cell which was characterized by the presence of cell shrinkage, cell rounding, cell detachment and membrane blebbing. AOPI

fluorescence microscopy examinations showed that there were presence of early apoptotic, late apoptotic and necrotic cells in MDA-MB-231 cells that were treated with MEDL after 24, 48 and 72 hours. Quantitative AOPI results showed that MEDL could induce apoptosis in MDA-MB-231 cell line in a time-dependent manner. Result showed that the percentage of early apoptotic cells increased significantly ($p < 0.05$) from 8.17% to 8.67% to 11.83% at 24, 48 and 72 hours, respectively. The percentage of late apoptotic cells was also seen to increase significantly ($p < 0.05$) from 10.67% to 11.17% to 17.50% at 24, 48 and 72 hours, respectively. As for the necrotic cells, the percentage was also seen to increase significantly ($p < 0.05$) from 5.50% to 16.00% to 24.00% at 24, 48 and 72 hours, respectively. Cell cycle analysis revealed that MEDL induced S phase cell cycle arrest in MDA-MB-231 cell effectively where the percentage of cells in the S phase was seen to increase significantly ($p<0.05$) from 32.58% at 24 hours to 55.82% at 48 hours and 69.62% at 72 hours. The proportion of cells in the S phase increased significantly ($p<0.05$) compared to the untreated cells and 5-FU treated cells. Early apoptosis induction in MDA-MB-231 cell was confirmed by Annexin V-FITC and Propidium Iodide (PI) staining. After 24 and 48 h of treatment with MEDL, the proportion of early apoptotic cells reduced from 6.60% to 5.87% at 24 and 48 h, respectively. Following this, the proportion of the late apoptotic cells in the MEDL treated group at 24 and 48 h increased significantly ($p < 0.05$) with values of 8.47% and 12.37%, respectively when compared to the untreated cells and 5-FU treated cells. These findings suggested that MEDL has the potential to be developed as a potent cytotoxic agent against MDA-MB-231 cancer cell line as well as complied with the regulation and halal requirements which is safe to be used since it does not harm the normal cells.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

KESAN RANGSANGAN EKSTRAK DAUN *Dicranopteris linearis* (Burm.f.) Underw. TERHADAP RENCATAN KITARAN SEL DAN APOPTOSIS SEL MDA-MB-231

Oleh

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Dicranopteris linearis adalah spesies paku pakis yang dikenali dalam kalangan orang Melayu sebagai 'Resam'. Secara saintikfiknya, tumbuhan ini telah dilaporkan mempunyai kesan antinosiseptif, antiradang, antipiretik, antikanser dan antioksidan yang boleh dikaitkan dengan potensi untuk merawat pelbagai jenis penyakit termasuk penyakit yang berkaitan dengan keradangan dan kanser. Walau bagaimanapun, potensi antikansernya masih belum disiasat secara meluas. Kajian ini dilakukan untuk menilai aktiviti sitotoksik ekstrak-ekstrak *D. linearis* terhadap beberapa sel kanser, untuk melihat perubahan morfologi pada sel MDA-MB-231, untuk menentukan aruhan perencatan kitaran sel terhadap sel MDA-MB-231 dan untuk menganalisis cara kematian sel MDA-MB-231. Ujian MTT digunakan untuk menentukan kesan sitotoksik ekstrak-ekstrak *D. linearis* iaitu ekstrak metanol (MEDL) dan ekstrak petroleum eter (PEEDL) pada kepekatan 100, 50, 25, 12.5, 6.25 and 3.125 µg/mL terhadap adenokarsinoma payudara (MCF-7 dan MDA-MB-231), adenokarsinoma serviks (HeLa) , karsinoma kolon (HT-29) dan karsinoma hati (HepG2), karsinoma paru-paru (A549) untuk tempoh eraman selama 72 jam. Keputusan MTT dibandingkan dengan sel-sel kanser yang tidak dirawat dengan MEDL dan PEEDL dan juga sel kanser yang dirawat dengan 5-fluorouracil (5-FU) sebagai kumpulan kawalan positif. Sel fibroblast tikus (3T3) digunakan untuk melihat kesan sitotoksik MEDL dan PEEDL terhadap sel normal. Data menunjukkan MEDL mempunyai kesan sitotoksik yang terbaik terhadap sel MDA-MB-231 dengan nilai IC_{50} 22.4 µg/mL manakala PEEDL sebaliknya gagal menunjukkan kesan sitotoksik terhadap semua sel dalam kajian ini. MEDL juga hanya menunjukkan aktiviti sitotoksik terhadap sel kanser yang aktif berproliferasi (MDA-MB-231, HeLa and MCF-7) tanpa menyerang sel-sel fibroblast tikus normal (3T3). MEDL juga dilihat dapat menghalang pertumbuhan sel MDA-MB-231 dengan bergantung kepada masa selepas tempoh eraman 24, 48 dan 72 jam dimana ia menunjukkan bahawa MEDL dapat mencegah pertumbuhan semula sel kanser semasa tempoh

eraman. Hasil analisa mikroskop fasa berbalik menunjukkan bahawa MEDL menggalakkan apoptosis berlaku dalam sel MDA-MB-231 yang dicirikan dengan kehadiran pengecutan sel, pembulatan sel, penanggalan sel dan pembengkakan membran. Analisa mikroskop fluoresen AOPI menunjukkan terdapat kehadiran sel apoptotik awal, sel apoptotik lewat dan sel nekrotik dalam sel-sel MDA-MB-231 yang dirawat dengan MEDL selepas 24, 48 dan 72 jam. Keputusan kuantitatif AOPI menunjukkan bahawa MEDL boleh menyebabkan apoptosis berlaku keatas sel sel MDA-MB-231 dalam cara yang bergantung pada masa. Keputusan menunjukkan bahawa peratusan sel apoptosis awal meningkat dengan berkesan *($p < 0.05$)* daripada 8.17% kepada 8.67% kepada 11.83% pada 24, 48 dan 72 jam. Peratusan sel apoptosis lewat juga dilihat meningkat dengan berkesan *($p < 0.05$)* dari 10.67% kepada 11.17% kepada 17.50% pada 24, 48 dan 72 jam. Bagi sel-sel nekrotik, peratusan juga meningkat dengan berkesan *($p < 0.05$)* dari 5.50% hingga 16.00% kepada 24.00% pada 24, 48 dan 72 jam. Analisis kitaran sel menunjukkan bahawa MEDL mampu merencatkan kitaran sel pada fasa S pada sel MDA-MB-231 dengan berkesan di mana peratusan sel dalam fasa S dilihat meningkat dengan berkesan *($p < 0.05$)* daripada 32.58% pada 24 jam kepada 55.82% pada 48 jam dan 69.62% pada 72 jam. Perkadaran sel dalam fasa S meningkat dengan ketara *($p < 0.05$)* berbanding dengan sel yang tidak dirawat dan sel-sel yang dirawat dengan 5-FU. Aruhan apoptosis awal terhadap sel MDA-MB-231 telah disahkan oleh ujian Annexin V-FITC dan pewarnaan propidium iodida (PI). Selepas 24 dan 48 jam rawatan dengan MEDL, peratusan sel apoptosis awal dilihat berkurangan dari 6.60% kepada 5.87% pada 24 dan 48 jam. Berikutan ini, peratusan sel apoptosis lewat yang telah dirawat dengan MEDL pada 24 dan 48 jam meningkat dengan berkesan *($p < 0.05$)* dengan nilai 8.47% dan 12.37%, berbanding sel yang tidak dirawat dan sel-sel yang dirawat 5-FU. Penemuan ini mencadangkan bahawa MEDL mempunyai potensi sebagai agen sitotoksik yang baik terhadap sel kanser MDA-MB-231 serta mematuhi peraturan dan keperluan halal yang selamat untuk digunakan kerana ia tidak membahayakan sel normal.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master Science. The members of the Supervisory Committee were as follows:

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This is to confirm that:

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

3T3	Normal mouse fibroblast cells
5-FU	5- Fluorouracil
A549	Lung carcinoma
AEDL	Aqueous extract of *D. linearis*
AOPI	Acridine Orange and Propidium Iiodide
Ca^{2+}	Calcium ions
CAKs	CDK-activating kinases
CDKs	Cyclin-dependent kinases
CO_2	Carbon dioxide
CEDL	Chloroform extract of *D. linearis*
DMBA	Dimethylbenz(a)anthracene
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2, 2-diphenyl-1-picrylhydrazyl
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
G_0 phase	Gap 0 phase
G_1 phase	Gap 1 phase
G_2 phase	Gap 2 phase
HeLa	Cervical adenocarcinoma
HL-60	Promyelocytic leukemia
HepG2	Hepatocellular carcinoma
HT-29	Colon carcinoma
IC_{50}	Median inhibitory concentration
K-562	chronic myelogenous leukemia
M phase	Mitosis phase
MCF-7	Breast adenocarcinoma
MDA-MB-231	Breast adenocarcinoma
MEDL	Methanolic extract of *D. linearis*
MTT	3-(4, 5- dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide

NF-κB	Nuclear factor-κB
ORAC	Oxygen radical absorbance capacity
PBS	Phosphate buffer solution
PEEDL	Petroleum ether extract of *D. linearis*
PS	Phosphatidylserine
RNA	Ribonucleic acid
RPMI 1640	Roswell Park Media Institute 1640
S phase	Synthesis phase
TPC	Total phenolic content
Trypsin EDTA	Trypsin ethylenediaminetetraacetic acid

CHAPTER 1

INTRODUCTION

Breast cancer is one of the most frequently diagnosed cancers, and the leading cause of cancer death in women with an estimation of 1.7 million cases and 521,900 deaths (Torre *et al.,* 2015). According to Malaysia's National Cancer Registry (2007) report, breast cancer is the most frequently diagnosed cancer among Malaysia population and also the most widely recognized cancer among female which accounts for 18.1% of all registered cancer cases (Omar and Tamin, 2011).

Age, family history of breast cancer, increased hormone exposure, early menarche, late menopause and nulliparity are the risk factors that involved in the increasing incidence of breast cancers (Baselga and Norton, 2002; Hortobagyi, 1998). The role of diet that can contribute to the risk of breast cancer has become one of the greatest interest for cancer research as it is seen as a potentially modified risk factor for cancer intervention (Michels *et al.,* 2007).

In the production of medicinal products, the concept of Halalan and Toyyiban must be taken into account as Muslims around the world are showing a growing interest concerning the halal status of pharmaceuticals (Sarriff and Razzaq, 2013). The pharmaceutical industry is ruled by gigantic pharmaceutical manufacturers specifically the Western Europe, North America and Japan. Interestingly, these companies are showing a growing awareness on the necessities of Muslims considering that the Muslim consumers around the world had developed extraordinarily (Nain *et al.,* 2013).

In halal products, apart from emphasizing the quality, safety and efficacy issues, the true concept of halal actually lies in the implementation of halalan toyyiban concept that associates with the source and manner of preparation according to Islamic teaching (Ismail and Ehsan, 2010). Therefore, a medicinal entity which is effective, but can cause serious adverse effects is still regarded as medicinal agent which cannot conform to the halalan toyyiban concept of pharmaceutical agent.

World Health Organization has recorded that more than 80% of the world's population depends on conventional medicine for their primary healthcare needs (Duraipandiyan *et al.,* 2006). Utilization of plants in cancer treatment has a long history and it is significant that more than 60% of anti-cancer agents that are currently used were originated from natural sources (Cragg and Newman, 2005). Some of the naturally occurring drugs that are used to fight against cancer includes vinca alkaloids, taxanes, podophyllotoxin, camptothecin and anthracyclines (Gordaliza, 2007; Nobili *et al.,* 2009). Nature is an appealing source of new remedial compounds as plenteous chemical diversity is found in millions of plant species as potential anti-cancer agent (Bhanot *et al.,* 2011).

At present bioactive compounds of natural sources, particularly from the traditionally used plant are gaining significance deliberations. *Dicranopteris linearis* (*D. linearis*) locally known to the Malays as 'resam' is a type of fern which is found in secondary forest. The leaves of *D. linearis* are used in the Malay's traditional medicine to reduce body temperature and to control fever (Chin, 1992; Derus, 1998). Several investigations have demonstrated that *D. linearis* plant extracts possess numerous health-promoting properties, such as antinocicepetive, anti-inflammatory, antipyretic activities (Zakaria *et al.,* 2008), and potential cytotoxic and antioxidant activity against various types of cancer (Zakaria *et al.,* 2011).

In studying the mechanisms of new drug reactions, neutraceuticals and pharmaceuticals, the usage of breast cancer cell lines are among a helpful tool. MDA-MB-231 cell line is a human breast cancer cell line known to be broadly used in cancer studies (Gibbs, 2000). The positive control drug used in this study is the fluoropyrimidines 5-fluorouracil (5-FU) is a a type of antimetabolite inhibitors of *de novo* purine and pyrimidine syntheses. It has shown a significant role in standard chemotherapy protocols for a range of solid tumors including breast and colorectal cancers (Cunningham and Coleman, 2001).

1.1 Problem Statement

It is undeniable that the current cancer treatments such as chemotherapy, radiotherapy and surgery are effective in treating patients who are suffering from cancer. However the presence of undesirable adverse effects such as nausea, anemia, hair loss and the rise of resistance cases in cancer patients have urged the demand for the development of new therapeutic agents. Continuous efforts are being made for the quest of novel source of bioactive compound which are more effective with less unintended consequences in order to combat breast carcinoma among cancer patients. *D. linearis* is one of the plants that have been studied to show significant cytotoxicity effect against breast carcinoma. Nevertheless, its anticancer potential has not been extensively investigated. Therefore an extensive investigation on *D. linearis* potentials as novel anticancer agent would contribute in the development of new cancer therapeutic agents. In addition, one of the focus in producing halal pharmaceutical product is to find the right source of ingredient that comply to the halalan toyyiban concept of effective and safe pharmaceutical ingredient, which still remain as a great challenge to the halal industry.

1.2 Justification of Study

D. linearis has been reported to have anti-inflammatory, antioxidant and possible antiproliferative activities. Antioxidant is believed to reduce the risk of cancer development by slowing down the lipid oxidation process (Alamed *et al.,* 2009*)*. There is also evidence that antioxidants and anti-inflammatory compounds could be used to modify the oxidation-reduction environment of cancer cells and their behavior (Schafer and Buettner, 2001). It was also suggested that antioxidants have

the ability to reduce the genetic instability of cancer cells and thus might be valuable in treatment (Reddy *et al.,* 2003). Therefore it is expected that *D. linearis* has the ability to exert significant anticancer activity against various cancer cell lines in particular breast adenocarcinoma and show excellent cancer cell growth retardation partly via induction of cell cycle arrest and apoptotic mode of cell death.

1.3 Objectives

1.3.1 General Objective

This study aimed to screen and investigate the *in vitro* antiproliferative properties of *Dicranopteris linearis* leaf extracts against a number of cancer cell lines.

1.3.2 Specific Objectives

1) to evaluate the cytotoxic activities of *D. linearis* extracts namely methanolic extract of *D. linearis* (MEDL) and petroleum ether extract of *D. linearis* (PEEDL) against several cancer cell lines namely breast adenocarcinoma (MCF-7) and (MDA-MB-231), cervical adenocarcinoma (HeLa), colon carcinoma (HT-29), hepatocellular carcinoma (HepG2) and lung carcinoma (A549). The extract showing the best cytotoxic activity on the selected cancer cell line were chosen for the next study (methanolic extract of *D. linearis* (MEDL)) on MDA-MB-231 cancer cell line.
2) to observe the morphological changes on MDA-MB-231 cells following treatment with MEDL via phase contrast and Acridine Orange/Propidium Iodide assay.
3) to determine the cell cycle arrest induction in MDA-MB-231 cells following treatment with MEDL using CycleTEST Plus DNA Reagent Kit.
4) to analyze the mode of cell death in MDA-MB-231 cells following treatment with MEDL via Annexin V-FITC/Propidium Iodide staining.

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