UNIVERSITI PUTRA MALAYSIA

ANTITUMOUR PROMOTING ACTIVITY AND MODE OF ACTION OF METHANOLIC EXTRACTS OF SELECTED MALAYSIAN SEAWEEDS AND OIL PALM FRONDS

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By

FARIDEH NAMVAR

Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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Dedicated

To

This thesis is dedicated to my lovely children Amin, Ali and Mohammad, my dear husband, and my parents that I owe them all of success in my life.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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Chairman: **Professor Suhaila Mohamed, PhD**

Institute: **Institute of Biosciences**

Dietary and non dietary biophenols may potentially be chemoprotective against various cancers. This research aims to investigate the cancer preventive and tumoricidal properties of selected Malaysian seaweeds and oil palm leaves as a source of phenolic compounds.

Initially, the anti-proliferation activities of, red (*Eucheuma cottonii*), green (*Caulerpa lentillifera*) and brown (*Sargassum polycystum*) seaweeds methanolic extract, against five important human cancer cell lines MCF-7, (human breast carcinoma cell line, estrogen positive) MDA-MB-231, (human breast carcinoma cell line, estrogen negative) HeLa, (human cervical adenocarcinoma cell line) HepG2, (human hepatocellular carcinoma cell line) HT-29, (human colon carcinoma) and normal Vero (African green monkey kidney cell line) were assessed. The MTT assays indicated that the 3 seaweeds extracts were cytotoxic against all these cancer cell lines in a dose-dependent manner,

with *Eucheuma cottonii* having the greatest inhibition activity. Cytotoxicity was not observed in normal Vero cell line. The MCF-7 cell line was most sensitive to *Eucheuma cottonii* methanol extract (ECME), with IC_{50} of 20±0.2 µg /mL, after 72 h incubation. The mode of action of ECME in MCF-7 cell was through apoptosis, characterised under SEM and TEM, by cell membrane blebbing, microvillus reduction or disappearance, shrinkage of cells, condensation of chromosomes and apoptotic bodies with complete membrane. The growth inhibited cells stained with AO/PI and Hoechst 33342 showed a time- and dose-depended apoptotic cell death suggesting that ECME caused irreversible cell damages in MCF-7 cells. The cell cycle analysis determined by flow cytometry analysis further confirmed that ECME induced apoptosis in MCF-7 cells without cell cycle arrest.

The *in vitro* investigation of oil palm leaves (*Elaeis guineensis*) methanol extract (OPLME) on MCF-7 cells showed proliferation at 17.5 µg /mL ($p < 0.05$) and an anti-proliferation effect at 150 µg/mL ($p < 0.05$) and 1200 µg/mL ($p < 0.01$) with an IC_{50} value of 678.5 µg/mL.

An *in vivo* study to compare the chemopreventive capabilities of ECME and OPLME on rat mammary gland tumour induced using rat cell line (CRL 2283), shows ECME has anti-estrogenic bioactivity on rat estrous cycle and serum hormone levels, causing an overall 37% increase in the length of the rat estrous cycle in a dose-dependent manner. The ECME also exerted a tempering effect on estrogen production in rats, which led to 18–33% reductions in circulating 17 β-estradiol concentrations. In comparison OPLME caused an overall 25% increase in the length of the rat estrous phase of the cycle in a

dose-dependent manner. The OPLME administration produced a statistically significant ($P < 0.001$), 2.54-fold increase in circulating 17 β-estradiol concentrations. The tumour incidence rate of each group was 87.5% (7/8) in control group, 37.5(3/8) in low dose ECME group, 12.5% (1/8) in high dose ECME group, 25% (2/8) in low dose OPLME, and 12.5% (1/8) in high dose OPLME group, respectively. The total tumour volume of each group was 10.7±1.2 cm^3 in control group, 0.95±0.7 cm^3 in high dose ECME group, 2.5±0.8 cm^3 in low dose ECME group, 0.8±0.7 cm^3 in high dose OPLME group, and 1.4±0.9 cm^3 in low dose OPLME group. Statistical analysis showed that the tumour incidence rate and total tumour volume for all treated groups were significantly lower ($p < 0.05$) than that of control group. The ECME decreased erythrocyte Malondialdehyde (MDA) level and increased catalase activity. Treatment with OPLME decreased erythrocyte MDA concentrations and increased erythrocyte GSH (reduced glutathione assay) and catalase activities. Electron microscopy and histopathology observation confirmed apoptosis in the mammary gland tumours of rats in both treatment groups.

Finally the research established the estrogenic properties of OPLME by showing significant increases in vaginal cornification, and uterine wet weight ($P < 0.001$), in a dose-dependent manner in ovariectomized rats. The OPLME also has a lowering effect on serum total cholesterol, and triglyceride concentration, in a dose-dependent manner. The estrogenic activity shown by OPLME can be attributed to the presence of flavonoids and phenolic compounds. This estrogenic activity of OPLME may be one possible mechanism for OPLME beneficial effects on serum lipid profile.

This study demonstrate that both OPLME and ECME showed *in vitro* and *in vivo* anti-breast tumour effects by inducing cancer cells apoptosis, improving whole animal antioxidative status and modulating the estrogen levels.

Abstrak Tesis Yang Dikemukakan Kepada Senat Universiti Putra Malaysia Sebagai Memenuhi Keprluan Untuk Ijazah Master Sains

AKTIVITI ANTI-KANSER DAN MEKANISME TINDAKAN OLEH RUMPAI LAUT DAN DAUN KELAPA SAWIT DARI MALAYSIA

Oleh

FARIDEH NAMVAR

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Biofenol diet dan bukan diet mungkin berpotensi untuk memberikan perlindungan terhadap pelbagai jenis kanser. Tujuan penyelidikan ini adalah untuk mengkaji ciri-ciri pengcegahan kanser serta ciri-ciri pemusnah sel kanser daripada rumpai laut Malaysia yang terpilih dan juga daun kelapa sawit sebagai sumber sebatian fenol.

Pada awalnya aktiviti anti percambahan oleh ekstrak metanol rumpai laut merah (*Eucheuma cottonii*), hijau (*Caulerpa lentillifera*) dan perang (*Sargassum polycystum*), menentang lima sel kanser manusia yang penting iaitu MCF-7 (sel karsinoma kanser payudara), MDA-MB-231 (sel karsinoma payudara manusia), HeLa (sel adenokarsinoma serviks), HepG2 (sel karsinoma hepatoselular manusia), HT-29 (sel karsinoma usus) dan sel Vero normal (sel buah pinggang monyet hijau afrika). Assay MTT menunjukkan bahawa 3 ekstrak rumpai laut adalah cytotoxic terhadap semua kanser sel tersebut dalam keadaan yang bergantung kepada dos, dengan Eucheuma

cottonii mempunyai aktiviti perencatan yang tertinggi. Ciri-ciri cytoxic tidak boleh ditemui dalam normal sel Vero. Sel MCF-7 adalah yang paling sensitif terhadap ekstrak methanol *Eucheuma cottonii* (ECME), dengan nilai 20±0.2µg/mL IC_{50}, selepas inkubasi 72 jam. Cara tindakan ECME dalam sel MCF-7 adalah melalui apoptosis, dikategorikan di bawah SEM dan TEM, dengan pembonjolan sel membran, pengurangan atau pelenyapan mikrovilli, pengecilan sel, kepadatan kromosom dan pemusnahan dengan membrane lengkap. Sel yang dicegah pertumbuhannya telah diwarnai dengan AO/PI dan Hoechst 33342 menunjukkan kematian sel apoptotic yang bergantung kepada masa dan dos, menyatakan bahawa ekstrak methanol *Eucheuma cottonii* (ECME) menyebabkan kerosakan sel dalam sel MCF-7 ireversibel. Analisis kitaran sel yang ditentukan oleh analisa aliran struktur dan fungsi sel telah mengesahkan lagi bahawa ECME menyebabkan apoptosis dalam sel MCF-7 tanpa penghentian kitaran sel.

Penyiasatan *in vitro* terhadap ekstrak methanol daun kelapa sawit (Elaeis guineensis) dalam sel MCF-7 menunjukkan proliferasi pada 17.5 µg/mL ($p<0.05$) dan kesan anti-proliferasi pada 150 µg/mL ($p<0.05$) dan 1200 µg/mL ($p<0.01$) dengan 6788.5 ug/mL untuk nilai IC_{50}. Kajian in-vivo yang membandingkan kemampuan pencegahan kanser oleh ECME dan OPLME terhadap kanser kelenjar mammary tikus yang berada di bawah induksi menggunakan sel tikus (CRL 2283), menunjukkan ECME mempunyai bioaktiviti anti-estrogenic terhadap kitaran estrus tikus dan tahap hormon serum, menyebabkan kenaikan 37% dalam tempoh kitaran estrus tikus bersama pergantungan dos. ECME juga mempunyai kesan besar terhadap pengeluaran estrogen dalam tikus yang meyebabkan kesusutan 18-33% dalam pengitaran konsentrasi 17 β-estradiol. Secara perbandingan, OPLME telah meyebabkan kenaikan 25% dalam tempoh fasa

estrus tikus yang bergantung kepada dos. Pembekalan OPLME menghasilkan kesan statistik ($P<0.001$) secara nyata, 2.54 kali kenaikan dalam pengedaran konsentrasi 17 β–estradiol. Kadar kejadian tumor dalam setiap kumpulan adalah 87.5 (7/8) untuk kumpulan kawalan, 37.5(3/8) untuk ECME dalam dos rendah, 12.5% (1/8) untuk kumpulan ECME dalam dos tinggi, 25% (2/8) OPLME dalam dos rendah, dan 12.5% (1/8) kumpulan OPLME dalam dos tinggi. Semua isipadu tumor untuk setiap kumpulan adalah 10.7 ± 1.2 cm^3 untuk kumpulan kawalan, 0.95 ± 0.7 cm^3 untuk kumpulan ECME dalam dos tinggi, 2.5 ± 0.8 cm^3 untuk kumpulan ECME dalam dos rendah, 0.8 ± 0.7 cm^3 untuk kumpulan OPLME dalam dos tinggi, dan 1.4 ± 0.9 cm^3 untuk kumpulan OPLME dalam dos rendah. Analisis statistik menunjukkan bahawa kadar kejadian tumor dan semua isipadu tumor untuk semua kumpulan dirawat adalah rendah ($p<0.05$) berbanding dengan kumpulan kawalan. ECME menurunkan tahap Malondialdehyde (MDA) sel darah merah dan menaikkan aktiviti catalse. Rawatan OPLME menurunkan konsentrasi MDA dan menaikkan GSH (assay reduced glutathione) sel darah merah dan aktiviti catalase. Mikroskop elektron dan pemerhatian histopatologi telah mengesahkan apoptosis untuk kelenjar kanser mammary tikus dalam kedua-dua kumpulan rawatan.

Akhirnya sekali, penyelidikan ini telah memperolehi ciri-ciri estrogenic OPLME dengan menunjukkan peningkatan yang nyata dalam penukaran sel kulit kepada keratin di dalam vagina dan berat basah kawasan rahim ($P<0.001$), dalam keadaan pergantungan dos untuk tikus yang telah dibuang rahimnya. OPLME juga mempunyai kesan penurunan terhadap jumlah kolestrol serum dan kepekatan trigliserida dalam pergantungan dos. Aktiviti estrogen yang ditunjukkan oleh OPLME boleh menyebabkan kehadiran

flavonoids dan sebatian fenol. Aktiviti estrogen OPLME mungkin adalah satu mekanisma untuk OPLME yang bermanfaat terhadap profil serum lipid

Kajian ini menyatakan bahawa kedua-dua OPLME dan ECME menunjukkan kesan anti-tumor payudara secara *in-vitro* dan *in-vivo* dengan menyebabkan apoptosis sel kanser, meningkatkan status keseluruhan antioksida haiwan dan mengimbangkan tahap estrogen.

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I certify that an Examination Committee met on 7 October 2009 to conduct the final examination of Farideh Namvar on her Doctor of Philosophy thesis entitled "ANTI TUMOUR PROMOTING ACTIVITY AND MODE OF ACTION OF METHANOLIC EXTRACT OF SELECTED MALAYSIAN SEAWEEDS AND OIL PALM FRONDS" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotation and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institute.

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FARIDEH NAMVAR

Date: 15 March 2009

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LIST OF ABBREVIATIONS

AO	**:**	**Acridine orange**
ATV	**:**	**Average tumour volume**
CAT	**:**	**Catalase**
Cdk	**:**	**Cyclin dependent kinase**
CO	**:**	**Carbon dioxide**
DMSO	**:**	**Dimethyl sulfoxid**
ECME	**:**	***Eucheuma cottonii* methanolic extract**
GSH	**:**	**Reduced glutathione assay**
h	**:**	**Hour**
LLC	**:**	**Lewis lung carcinoma**
MDA	**:**	**Malondialdehyde level**
MNC	**:**	**Maximal non-toxic concentration**
mg	**:**	**Milligram**
min	**:**	**Minutes**
mL	**:**	**Milliliter**
OPLME	**:**	**Oil palm leaf methanolic extract**
PI	**:**	**Propidium Iodide**
ROS	**:**	**Reactive oxygen species**
rpm	**:**	**Rotation per minutes**
SEM	**:**	**Scanning electron microscopy**
SOD	**:**	**Superoxide dismutase**
TEM	**:**	**Transmission electron microscopy**
TIR	**:**	**Tumor incidence rate**
UV	**:**	**Ultraviolet**
^{o}C	**:**	**Degree Celsius**
µ	**:**	**Micro**
%	**:**	**Percentage**

CHAPTER I

INTRODUCTION

Cancer is a class of diseases in which a group of cells display the traits of uncontrolled growth, invasion, and sometimes metastasis. These three malignant properties of cancers differentiate them from benign tumors, which are self-limited, do not invade or metastasize (Karp, 1999).

Cancer causes about 13% of all death. According to the American Cancer Society, 7.6 million people died from cancer in the world during 2007 (Coleman, 1995). In the U.S. and other developed countries, cancer is presently responsible for about 25% of all deaths. On a yearly basis, 0.5% of the population is diagnosed with cancer (American Cancer Society, 2008).

The problem of cancer in Malaysia is a growing one. It is now the fourth leading cause of death among medically certified deaths. Cancer of the lung is the most common killer among malignancies. It is estimated that the annual incidence of cancer is 30 000. The majority of patients are diagnosed at a late stage of the disease. According to the Malaysian National Cancer Registry by the Ministry of Health Malaysia, neoplasm which cover all types of cancer had the highest prevalence among the male and female

elderly patients admitted to Hospital Kuala Lumpur, the most common cancers in male were those of the lung and prostate, while cancers of the breast and colon were the two most common cancers in females (Ministry of Health Malaysia, 2000).

Nearly all cancers are caused by abnormalities in the genetic material of the transformed cells. Epithelial carcinogenesis is a multistep process in which an accumulation of genetic events within a single cell line leads to a progressively dysplastic cellular appearance, deregulated cell growth, and, finally, carcinoma (Lippman *et al*., 1994).

Chemoprevention is an active cancer preventive strategy to inhibit, delay or reverse human carcinogenesis, using naturally occurring or synthetic chemical agents (Surh, 2003). Dr Michael B. Sporn first introduced the term "chemoprevention", when he referred to the prevention of cancer development by natural forms of vitamin A and by its synthetic analogs (Sporn *et al*., 1976). Thereafter, a variety of naturally occurring dietary compounds have been shown to possess significant chemopreventive effects and many experimental attempts have been made to address their underlying mechanisms of action (Surh, 2003). Numerous cancer cell lines and animal cancer models have been used to evaluate the chemopreventive effects of phytochemicals as well as to elucidate their mechanisms of cancer prevention. These studies have resulted in the discovery of several new phytochemicals that possess cancer preventive effects, such as isothiocyanates from cruciferous vegetables, polyphenols from green and black tea, and flavonoids from soybeans. Several cellular mechanisms contribute to the overall cancer preventive effects of these dietary phytochemicals. These include oxidative or

electrophilic stresses that can trigger a wide variety of cellular events such as increasing expression of detoxifying enzymes and/or antioxidant enzymes, inhibiting cell cycle progression and cell proliferation, inducing differentiation and apoptosis, inhibiting expression and functional activation of oncogenes, increasing expression of tumour-suppressor genes, and inhibiting angiogenesis and metastasis by modulating cellular signaling pathways (Kupchan, 1976).

The "antioxidant hypothesis" in chemoprevention is strongly sustained in the literature. It asserts, "Being the antioxidants able to prevent or reduce oxidative damage, their increased uptake from the diet will reduce the risk of chronic disease" (Stanner *et al*., 2004). It is worthwhile to note that a large part of studies supporting the antioxidant against cancer is based on cell lines studies and on animal model where tumors were experimentally induced by high doses of carcinogens. However, growing experimental evidence suggest that antioxidants present in food act as chemopreventive agents independent of their ability to scavenge ROS. Many of them interfere with signal transduction regulation at different levels: modulating hormones/growth factors activities, inhibit oncogenes and activate tumour suppressor genes, induce terminal differentiation, activate apoptosis, restore immune response, inhibit angiogenesis, decrease inflammation (Russo, 2007). Although natural products have long been a fertile source of cures for cancer, there has been a desperate and continuous need for development of new anticancer drugs and chemotherapy strategies aimed at killing both primary and metastatic cancer cells.