UNIVERSITI PUTRA MALAYSIA

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DEDICATION

This thesis is dedicated to my loving family, who has been supporting me through thick and thin. Without them, none of this would have been possible.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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By

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Chairman: Associate Professor Rozita Rosli, PhD

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Cancer is a large group of diseases characterized by uncontrolled growth and spread of abnormal cells. Hundreds of research studies have demonstrated significant benefit of the use of natural products in the treatment of cancer and scientists believe examining new natural products will continue to turn up even more useful drugs to treat cancer. Marine organisms are a rich source for natural products and many compounds that are derived from these have generated interest for their cytotoxicities. *Gracilaria changii* is a type of red seaweed which comes from the family *Rhodophyta.* It is a relatively abundant seaweed in Malaysia can be found in the mangrove areas. In this study, the chemotherapeutic potential of *Gracilaria changii* in selected reproductive cancer cell lines was evaluated together with tamoxifen, a commercially used drug in cancer treatment. Exposure of breast, ovarian and cervical cancer cell lines, to a range of *Gracilaria changii* extracts demonstrated growth inhibition in some of these cancer cells in a dose-dependent manner. The *Gracilaria changii* extracts received from Kolej

Universiti Sains dan Teknologi Malaysia (KUSTEM) were methanol, buthanol and diethyl ether extracts. The methanol extract gave the most promising IC_{50} values, as determined by the 3-(4,5-dimethylthiazol–2-yl)–2,5-diphenyltetrazolium bromide (MTT) reduction assay and the results are as follows: MCF-7 (7.8 µg/ml), MDA-MB-231 (25 µg/ml) HeLa (70.26 µg/ml) and Caov-3 (90.46 µg/ml). Since the results for the breast cancer cell lines were significant compared to the ovarian and cervical cancer cell lines, they were chosen for further analysis. The normal breast cell line, MCF-10A was also tested and the IC_{50} value was found to be > 1000 µg/ml, indicating that the methanol extract was not cytotoxic to normal cells. AOPI staining was used to study the morphology of the cells treated with the extract. Apoptotic features that included membrane blebbing and nucleus condensation were evident in MCF-7 and MDA-MB-231 cancer cells. Subsequently, the TUNEL assay was conducted to determine and quantitate the apoptotic cells within a cell population. The results suggest that the methanol extract was better of inducing cell death by stimulating apoptosis than tamoxifen. This is based on the significantly higher percentage of apoptotic cells in the *G.changii* methanol extract treated cancer cells as compared to tamoxifen. For MCF-7 and MDA-MB-231 cell lines, p was <0.01 when compared with control (24 hours) and P of <0.001 when compared with control for 48 hours. In addition, gene expression analysis was performed using the microarray technology. This technology which allows the simultaneous analysis of a large number of nucleic acid hybridization experiments and was carried out to determine the gene expression profile. Preliminary work on micorarray was conducted using MCF-7 cell line only, due to time constraints and limited funding. Upon treatment with the methanol extract on MCF-7, several suppressed genes were

identified including haplotype n1b mitochondrion complete genome, melanoma-associated antigen p97 isoform 1 and damage-specific DNA binding protein 2 (ddb2). The results showed that the three genes regulated by the methanol extract encode proteins that belongs to DNA repair, protection against membrane-lipid peroxidation and maternal inheritance family, which may play an important role for the cancer treatment. It was further confirmed using Reverse Transcription Polymerase Chain Reaction (RT-PCR). Therefore, the methanol extract of *Gracilaria changii* is a potential candidate to be developed as a chemotherapeutic agent in the treatment of estrogen receptor-positive breast cancers.

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

EFFECTS OF *GRACILARIA CHANGII* EXTRACT ON APOPTOSIS AND GENE EXPRESSION OF MCF-7 AND MDA-MB-231 BREAST CANCER CELL LINES

Oleh

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Mei 2006

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Kanser merupakan satu kumpulan penyakit yang dikategorikan sebagai tumbesaran sel yang tidak dapat dikawal dan penyebaran sel-sel yang tidak normal. Sel-sel ini boleh membesar menjadi kelompok tisu yang dipanggil tisu malignan. Apabila kanser merebak, ia akan menyerang dan memusnahkan tisu normal dan juga akan merebak ke bahagian lain badan. Kanser yang merebak ke bahagian lain atau ke organ lain dipanggil kanser metastatik. Jika penyebaran kanser tidak dikawal, ia akan menyebabkan kematian. Kebanyakan kanser boleh dikawal atau dicegah jika dikesan awal dan dirawat dengan segera. Kebanyakan penyelidikan yang telah dijalankan menunjukkan kesan yang signifikan daripada penggunaan alam semulajadi dan saintis mempercayai bahawa ujian-ujian terhadap alam semulajadi yang baru akan membuahkan lebih banyak ubat antikanser. Organisma marin kaya dengan sumber alam semulajadi dan banyak sebatian daripadanya telah menarik minat terhadap kesan sitotoksik. *Gracilaria changii* merupakan rumpai laut merah daripada keluarga *Rhodophyta*. Ia merupakan rumpai laut

yang banyak terdapat di Malaysia dan boleh didapati di kawasan paya bakau. Di dalam kajian ini, potensi kemoterapeutik *Gracilaria changii* untuk kanser reproduktif terpilih dikaji bersama tamoxifen, drug antikanser komersil yang sekarang digunakan untuk rawatan kanser. Pendedahan sel-sel kanser payudara, ovari dan serviks kepada julat kepekatan ekstrak yang berbeza menghasilkan perencatan pertumbuhan bagi kedua jenis sel kanser dengan bergantung kepada nilai kepekatan. Ekstrak yang diterima daripada Kolej Universiti Sains dan Teknologi Malaysia (KUSTEM) merupakan ekstrak metanol, butanol dan dietil eter. Ekstrak methanol memberikan nilai IC_{50} yang paling baik, didapati daripada asai penurunan 3-(4,5-dimethylthiazol–2-yl)–2,5-diphenyltetrazolium bromide (MTT) adalah seperti berikut: MCF-7 (7.8 ug/ml), MDA-MB-231 (25 ug/ml) HeLa (70.26 ug/ml) and Caov-3 (90.46 ug/ml). Oleh kerana sel selanjar payudara memberikan keputusan yang baik berbanding dengan sel-sel selanjar ovari dan servik, maka ia dipilih untuk analisis seterusnya. Sel selanjar normal, MCF-10A juga diuji dan nilai IC_{50} lebih daripada 1000 ug/ml, menunjukkan ekstrak metanol tidak sitotoksik terhadap sel normal. Pewarnaan AOPI digunakan untuk melihat perubahan morfologi sel selepas rawatan dengan ekstrak tersebut. Ciri-ciri apoptosis seperti gelembung membran dan kondensasi nukleus telah didapati bagi sel kanser MCF-7 dan MDA-MB-231. Seterusnya, asai TUNEL dijalankan untuk menentukan apoptosis. Menariknya, ekstrak metanol didapati menyebabkan kematian sel dengan merangsang apoptosis lebih baik daripada tamoxifen berdasarkan peratusan sel-sel apoptotik yang menunjukkan perbezaan yang signifikan berbanding sel-sel kanser dengan rawatan ekstrak metanol. Sebagai tambahan, analisis pengekspresan gen dijalankan dengan menggunakan teknologi mikroarray. Teknologi mikroarray membenarkan analisis terhadap asid nukleik yang

banyak secara serentak bertujuan untuk menentukan profil pengekspresan gen. Sebagai permulaan, kajian mikroarray hanya dijalankan ke atas sel selanjar MCF-7 sahaja, disebabkan factor masa dan geran yang terhad.Daripada rawatan ekstrak methanol terhadap MCF-7, didapati tiga gen yang telah ditindas iaitu haplotype n1b mitochondrion complete genome, melanoma-associated antigen p97 isoform 1 dan damage-specific DNA binding protein 2. Keputusan kajian menunjukan gen tersebut terdiri daripada gen yang mengkodkan protein kumpulan pembaikkan DNA, perlindungan terhadap peroksidasi membrane-lipid dan warisan daripada ibubapa, yang mungkin memainkan peranan yang penting terhadap rawatan kanser. Seterusnya ia disahkan dengan menggunakan Reverse Transcription Polymerase Chain Reaction (RT-PCR). Oleh itu, ekstrak methanol *Gracilaria changii* didapati mempunyai potensi untuk dikembangkan sebagai agen kemoteraputik untuk rawatan kanser yang bergantung kepada hormone.

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LIST OF ABBREVIATIONS

µl	Microliter
µM	Micromolar
ATCC	American Type Culture Collection
cDNA	complementary deoxyribonucleic acid
cRNA	complementary ribonucleic acid
DMSO	Dimethylsulfoxide
dNTP	Deoxyribonucleotide triphosphate
dsDNA	Double stranded deoxyribonucleic acid
EDTA	Ethylenediaminetetra-acetic acid
mg	Milligram
ml	Milliliter
mm	Millimeter
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
mV	Millivolt
PBS	Phosphate buffer saline
pmol	picomole
RNA	Ribonucleic acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SDS	Sodium Deodecyl Sulphate

CHAPTER 1

INTRODUCTION

1.1 Cancer and natural product

Cancer is a collective term that covers hundreds of different diseases characterised by invasive and uncontrolled cell growth. It is a chronic disease that can consist of specific stages, from genetic predisposition through various forms of premalignant and malignant degeneration of cells to disease progression. Breast cancer is one of the most common cancers diagnosed in women worldwide and is a leading cause of cancer-related deaths (Greenlee *et al.*, 2000). In Malaysia, as of 2003, it accounted for 31 % of newly diagnosed female cases, and was the commonest cancer in all ethnic groups and all age groups in females from the age of 15 years (Lim and Halimah, 2004).

Mortality that results from the common forms of cancer is still unacceptably high. Natural or semisynthetic compounds may be used to block, reverse, or prevent the development of invasive cancers. Cellular carcinogenesis forms the biological basis for the identification of preventive products, the assessment of their activity, and ultimately the success or failure of a therapy (Reddy *et al.,* 2003).

Ideally, chemotherapeutic drugs should specifically target only neoplastic cells and should decrease tumor burden by inducing cytotoxic and/or cytostatic effects with minimal "collateral damage" to normal cells. (Ricky *et al.*, 2002). Many pharmaceutical

agents have been discovered by screening natural products from plants, animals, marine organisms and microorganisms. Despite major scientific and technological progress in combinatorial chemistry, drugs derived from natural products still make an enormous contribution to drug discovery today (Rocha *et al.*, 2001).

Epidemiological data indicated that ubiquitous consumption of seaweeds in Japan may be a possible protective factor against some types of tumor (Okai *et al.*, 1994). Therefore in this study, *Gracilaria changii,* from the family of *Rhodophyta* or red seaweed was chosen. It is indigenous agarophytic seaweed in Malaysia (Phang, 1994). *Gracilaria changii* when used as food provides substantial amounts of fiber, minerals, lipids and protein (Norziah *et al.*, 2000). At present, this seaweed is only consumed in certain coastal areas especially along the east coast of Peninsula Malaysia and in East Malaysia.

1.2 Significance of study

In recent years, improved diagnostic tools have made it possible to detect breast cancers at early, even pre-invasive stages leading to a significant decrease in breast cancer mortality rates over the past decades. Breast cancer has been the major killer in women all over the world, and the number is increasing year by year. The use of natural product in cancer treatment has shown good results, and extensive research is being carried out. In Japan, the cancer mortality rate is among the lowest in the world, and this has been associated with having seaweed in their dietary intake. Thus, this study is hoped to

highlight on our own seaweed as a potential anticancer agent. The molecular studies of *Gracilaria changii* on breast cancer may contribute insight on drug targets and formulation against novel apoptosis pathway.

1.3 Objectives

There has been no previous study reported on the effect of *Gracilaria* changii on cancer cells. Thus, the objectives of the study are:

1) to determine whether *Gracilaria changii* extract is effective in inhibiting the proliferation of selected breast cancer cell lines (MCF-7, MDA-MB-231), cervical cancer (HeLa) and ovarian cancer cell lines (Caov-3).
2) to select the cell lines most effective against the extracts and comparing this with a normal cell line.
3) to evaluate apoptosis inducing ability of the *Gracilaria changii* extract by morphology through the apoptotic features of the cells and to confirm through quantification using TUNEL assay
4) to determine the gene expression profile following treatment of the extract on the cancer cells using microarray technology and to validate the expression using RT-PCR.