UNIVERSITI PUTRA MALAYSIA

BIOLOGICAL ACTIVITIES OF ORYZANOL, STIGMASTEROL AND MICROMINUTININ ON HUMAN BREAST CANCER CELL-LINE, MCF7

ABDAH MD AKIM

FPSK(P) 2004 3

BIOLOGICAL ACTIVITIES OF ORYZANOL, STIGMASTEROL AND MICROMINUTININ ON HUMAN BREAST CANCER CELL-LINE, MCF7

ABDAH MD AKIM

DOCTOR OF PHILOSOPHY
UNIVERSITI PUTRA MALAYSIA

2004

BIOLOGICAL ACTIVITIES OF ORYZANOL, STIGMASTEROL AND MICROMINUTININ ON HUMAN BREAST CANCER CELL-LINE, MCF7

By

ABDAH MD AKIM

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

October 2004

UPM

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

BIOLOGICAL ACTIVITIES OF ORYZANOL, STIGMASTEROL AND MICROMINUTININ ON HUMAN BREAST CANCER CELL LINE, MCF7

By

ABDAH MD AKIM

October 2004

Chairman: **Associate Professor Asmah Rahmat, Ph. D.**

Faculty: **Medicine and Health Sciences**

Researchers are exploring better treatments in cancer. The identification of active plant chemicals and the study of their biological activities are extensively being pursued. The five objectives in this study were to determine the antioxidant activity of rice bran extracts, to isolate oryzanol from rice bran, to determine the cytotoxicity activity of oryzanol, stigmasterol and microminutinin, to investigate the morphological changes in MCF7 cells treated with oryzanol, stigmasterol and microminutinin and to study on the effect of these compounds on c-myc, c-fos and c-erbB2 gene expression. The antioxidant activities of various rice bran extracts were carried out to determine the best organic solvent extraction. In the Ferric Thiocyanate (FTC) and Thiobarbiturate (TBA) assays, chloroform extract (FTC; 92%, TBA; 82%) had the highest antioxidant activity followed by ethyl acetate (FTC;90%, TBA;79%) methanol (FTC;90%, TBA;79%) and hexane (FTC; 89%, TBA;77%) extracts. The antioxidant activities in the β-carotene degradation assay gave similar findings. Comparison between the antioxidant activities

of the hexane extract (non polar lipid) and chloroform: methanol (2:1) extract (total lipid) were then determined. The extraction time (0.5 and 1 hour) and temperature (33° and 60°C) were differed to gain the optimum method of extractions. Overall, the total lipid had higher antioxidant activity than the non polar lipid. The extraction time of 30 minutes and 33°C extraction temperature gave the highest antioxidant activity (97%). The total lipid was further investigated by varying the solvent to bran ratio 4:1 and 5:1 (v/w). Extraction time of thirty minutes, extraction temperature of 29°C and solvent to bran ratio (v/w) gave the highest antioxidant activity (15%). The oryzanol content in the rice bran was determined using high performance liquid chromatography (HPLC). In this study, extraction and saponification temperature at 29°C gave the highest yield of oryzanol (3964 ± 33 mg/kg). Under this condition, oryzanol was extracted using preparative HPLC. Oryzanol, stigmasterol and microminutinin were then tested for their free radical scavenging effect. Oryzanol gave the highest antioxidant activity followed by stigmasterol and microminutinin. Cytotoxicity assay of oryzanol, stigmasterol and microminutinin were then used on various cancer cell lines MCF7, MDA-MB-231, HepG2, Caco-2, Caov-3, HeLa, Chang and 3T3. The IC_{50} for oryzanol in MCF7 was the lowest (53.5±1.3 µg/ml). Stigmasterol inhibited colon cancer the best with IC_{50} at 132.5±3.3 µM. From all of the cancer cell lines, microminutinin exhibited anti-proliferative activity on MCF7 with IC_{50} at 203.0±4.0µM. Confocal microscopy using acridine orange and propodium iodide staining of treated MCF7 with oryzanol, stigmasterol and microminutinin showed nucleus fragmentation at 48 hours. Light microscopy of treated MCF7 using modified TUNEL assay showed dark stained apoptotic nuclei. For the Fluorometric TUNEL assay, intense yellow fluorescence of PI-FITC was observed at 24 hours. After 48 and 72 hours of treatment, apoptotic bodies

were visibly seen in all treatments. In the flow cytometry analysis, the RNAse/PI treatment in the treated MCF7 cells showed a marked decrease of G_1 phase and an increase in the apoptotic cells with increased concentration. Annexin V-FITC/PI staining showed an increase of early and late apoptotic cells in oryzanol-treated compared to increment of only early apoptotic cells in stigmasterol and microminutinin-treated MCF7 after 24 hours. After 48 hours, the percentages of late apoptotic cells in all treated cells were increased. Oryzanol and stigmasterol suppressed the oncogene c-fos, c-erbB2 and c-myc expression. Microminutinin only suppressed the oncogene c-fos and c-myc expression. In conclusion, the three compounds were shown to be anti-proliferative agents.

PERPUSTAKAAN SULTAN ABDUL SAMAD
UNIVERSITI PUTRA MALAYSIA

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

AKTIVITI BIOLOGI DARIPADA ORYZANOL, STIGMASTEROL DAN MICROMINUTININ KOUMARIN PADA TITISAN SEL KANSER PAYUDARA MANUSIA, MCF7

Oleh

ABDAH MD AKIM

Oktober 2004

Pengerusi: **Profesor Madya Asmah Rahmat, Ph.D.**

Fakulti: **Perubatan dan Sains Kesihatan**

Para penyelidik berusaha untuk mencari penyelesaian bagi mengubati kanser. Pengenalpastian kompaun aktif dan kajian tentang aktiviti biologi sedang giat dijalankan. Terdapat lima objektif iaitu kepastian aktiviti antioksidan di dalam dedak beras, pengasingan kompaun aktif dari dedak beras, kajian tentang anti-kanser kepada oryzanol, stigmasterol dan microminutinin, kajian tentang perubahan morfologi dalam sel yang mengalami apoptosis dan kajian tentang gen terekspres daripada sel yang teraruh. Dalam kaedah Ferik tiosianate (FTC) dan Tiobarbiturik (TBA) yang digunakan untuk menentukan peratus antioksidan, ekstrak klorofom (FTC, 92%; TBA, 82%) mempunyai aktiviti antioksidan yang tertinggi diikuti dengan ekstrak etil asetat (FTC, 90%; TBA, 79%), ekstrak methanol (FTC, 92%; TBA, 82%) dan ekstrak heksana (FTC, 89%; TBA, 77%). Peratusan aktiviti antioksidan di dalam kaedah penurunan beta-karoten

menunjukkan keputusan yang serupa. Aktiviti antioksidan dari kaedah penurunan beta-karotene menunjukkan ekstrak klorofom:metanol (2:1) mempunyai aktiviti antioksidan yang tinggi berbanding dengan ekstrak heksana. Masa pengekstrakan 30 minit dan suhu 33° C memberikan hasil yang tertinggi (8.6%) dan aktiviti antioksidan yang tertinggi (97%). Ekstrak klorofom:methanol (2:1) juga digunakan untuk melihat ratio larutan dedak 4:1 dan 5:1 (v/w). Pengekstrakan masa 30 minit, suhu 29°C dan ratio larutan ke dedak 5:1 (v/w) memberi penghasilan yang tinggi (12.15%) dan aktiviti antioksidan yang tinggi (15%). Kandungan oryzanol dalam dedak beras ditentukan dengan menggunakan HPLC. Kebiasaannya, saponifikasi dijalankan pada suhu yang tinggi. Di sini, suhu pengekstrakan dan saponifikasi pada 29°C memberi penghasilan oryzanol yang tertinggi (3964 ± 33 mg/kg). Di dalam keadaan ini, oryzanol dihasilkan menggunakan kolum HPLC preparatif. Aktiviti oksidan oryzanol, stigmasterol dan microminutinin diuji dalam kaedah DPPH. Oryzanol memberi aktiviti antioksidan yang tertinggi diikuti dengan stigmasterol dan microminutinin. Asai sitotoksik dijalankan menggunakan pelbagai titisan sel kanser MCF7, MDA-MB-231, HepG2, Caco-2, Caov-3, HeLa, Chang dan 3T3. Oryzanol memberi IC_{50} terendah kepada MCF7 (53.5±1.3 µg/ml). Stigmasterol menghalang pertumbuhan kanser kolon pada 132.5±3.3 µM. Microminutinin menghalang kanser MCF7 pada 203.0±4.0 µM. Konfokal mikroskopi digunakan untuk melihat perubahan morfologi MCF7 yang normal dan yang diberi ketiga-tiga kompaun dalam kaedah akridin oren dan propodium iodid. Kesemua perlakuan menunjukkan fragmentasi nukleus pada 48 jam. Mikroskopi cahaya digunakan untuk melihat fragmentasi nukleus yang telah diwarnakan gelap di dalam TUNEL asai yang dimodifikasikan. Mikroskopi pendaflour dan konfokal digunakan untuk melihat kesan fragmentasi nukleus yang dihasilkan oleh perlakuan ketiga-tiga

kompoun. Perlakuan dua puluh jam memperlihatkan pendaflour kuning yang terang dihasilkan oleh Propodium Iodide-FITC. Perlakuan 48 dan 72 jam memperlihatkan fragmentasi nukleus. Di dalam analisa flow sitometri, pengurangan peratus pada fasa G1 dan peningkatan peratusan sel apoptotik dilihat. Penglabelan annexin V-FITC/PI selepas 24 jam menunjukkan peningkatan sel apoptotik awal dan sel apoptotik akhir pada sel yang diberi oryzanol berbanding dengan peningkatan sel apoptotik awal sahaja pada sel yang diberi stigmasterol dan microminutinin. Selepas 48 jam, peratusan sel apoptotik akhir pada kesemua sel yang diberi ketiga-tiga kompaun meningkat. Perlakuan oryzanol dan stigmasterol ke atas sel MCF7 mengurangkan ekspresi c-myc, c-fos dan c-erbB2. Microminutinin mengurangkan ekspresi c-myc dan c-fos sahaja. Kesimpulannya, ketiga-tiga kompoun berpotensi menjadi agen anti-proliferasi.

ACKNOWLEDGEMENTS

Behind every work stretches a long line of the people who made it possible. In the immediate past these people are in sharp focus, but as the line gets farther back the image of those who contributed gets dimmer and is finally totally obscured by the fog of time itself. Others go unsung, since many who have contributed to an idea have themselves passed into total obscurity. Thus, here first and foremost I thank Associate Professor Dr Asmah Rahmat for her guidance and support. I wish to express my deep appreciation to Associate Professor Dr Patimah Ismail and Associate Professor Dr Taufiq Yap Yun Hin for their understanding and advice throughout the study. I am greatly indebted to Associate Professor Dr Fauziah Othman for her invaluable advice. I am also thankful to my family for their moral support. To my friends, thank you for being beside me.

UPM

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AFP	Alfa-Feto Protein
APC	Anaphase-Promoting Complex
CDK	Cyclin Dependent Kinase
DNA	Deoxyribose Nucleic Acid
FAP	Familial Adenomatous Polyposis
FTC	Ferric Thiocyanate
HCC	Hepatocellular Carcinoma
HPLC	High Performance Liquid Chromatography
ROS	Reactive Oxygen Species
SPF	S-Phase Promoting Factor
SOD	Superoxide Dismutase
TBA	Thiobarbituric Acid
mRNA	messenger Ribose Nucleic Acid
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
VHL	Von Hipplel-Lindau syndrome

UPM

CHAPTER I

INTRODUCTION

Cancer is one of the three main causes of death among the economically active populations. The two other main causes of mortality worldwide are accidents and cardiovascular diseases. Annually, there are more than 6 million deaths from a type of cancer worldwide (Tovar-Guzman *et al.*, 2001). The number of new cancer cases has been increasing over the past nine decades. It was reported that cancer (45%) is the major cause of death in Government Hospitals, which is 2.8 times higher than that of the heart diseases (16%) (Rosemawati and Sallehudin, 2001). A total of 26,089 cancers were diagnosed among all residents in Peninsular Malaysia in the year 2002, comprising 11,815 males and 14,274 females. An estimated 10,656 cases were however not registered. In terms of risk, 1 in 5.5 Malaysians can be expected to get cancer in his/her lifetime. Taking into account the unregistered cases, the risk would be 1 in 4 Malaysians. The crude rate for all cancers in the year 2002 was 118.9 per 100,000 males and 148.4 per 100,000 females (Lim *et al.*, 2002).

Among all types of cancer, breast cancer is the most common malignancy affecting women and the second highest cause of cancer death (Sakorafas *et al.*, 2002). Every woman is at risk for getting breast cancer. Close to 200,000 cases of breast cancer were diagnosed in the United States in 2001. In 2003, an estimated 211,000 women were diagnosed with breast cancer. Breast cancer is the second leading cause of cancer death in American women after lung cancer. Breast cancer affects more than

1,000 men in United States each year. The lifetime risk of any particular woman getting breast cancer is about 1 in 8 although the lifetime risk of dying from breast cancer is much lower at 1 in 28 (Jatoi and Miller, 2003). The National Cancer Registry of Malaysia reported 4337 cases of female breast cancer making it the most commonly diagnosed cancer in Malaysian women. Breast cancer is the commonest cancer in all ethnic groups and all age group in females from the age of 20 years (Lim *et al.*, 2002).

Research has led to better treatments in cancer. Researchers are learning more about what causes cancer and are exploring new ways to prevent, detect, diagnose, and treat this disease (Jayaprakasam *et al.,* 2003). Even though numerous early works, including classical Greek and Latin texts and mediaeval Latin herbals, record the value of many plant species for treating all kind of diseases, few of these have been investigated in the context of modern biology (Wang and Xu, 1995). Furthermore, modern medicine has mostly abandoned plants as a source of new medicines in favour of chemical synthesis. The problem is that many drugs, particularly for cancer, are not as effective or free from unwanted side effects as they might be. However, recently modern pharmacognosy has been used in a judicious association with the ancient data. The identification of active plant chemicals is an essential component of modern pharmacognosy. The biological activity and clinical value of the whole plant, as in medicinal herbalism, is also being pursued (Pasquale, 1984).

Whole grains contain many of the same compounds and therefore may share some of the beneficial properties of fruits and vegetables (Jacobs *et al.*, 1995). In one study,

grain consumption has been inversely associated with colorectal cancer (Armstrong and Doll, 1975). The grains include wheat and rice. One of the objectives of this study will look at the potential anti-cancer effect of rice bran. This is given attention because rice bran was once considered as one of the most wasted food resources and was only used as diet for the laying hens. Since rice is heavily consumed in this country, this country produces a lot of the milling by-product that is the rice bran.

One of the few bioactive compounds being studied is plant sterols. Plant sterols (phytosterols) such as sitosterol and stigmasterol are ubiquitous in occurrence in higher plants (Tapeiro *et al.*, 2003). They are structurally similar to cholesterol, differing only by a methyl or ethyl group in their side chains. Thus, they are able to inhibit the uptake of cholesterol from the small intestine (Neil and Huxley, 2002) and rendering them to be anti-hypercholesterolemic (Normen *et al.*, 2001). On the other had, it was reported on their ability to prevent colorectal cancer (Normen *et al.*, 2001). Another bioactive compound that is currently being looked into is oryzanol. It is a ferulate ester of triterpene alcohols and plant sterols. This compound is also able to reduce hypercholesterolemia (Rogers *et al.*, 1993). Coumarins, a family of phenylpropanoids, are among the naturally occurring phenolic compounds that are being investigated. Only one earlier study had reported on its effect in lung cancer (Lopez-Gonzalez *et al.*, 2004).

New advances in understanding cancer provide the basis for screening bioactive plant chemicals using relevant bioassays. Procedures which are simple, rapid and