UNIVERSITI PUTRA MALAYSIA

ANTI-CANCER ACTIVITY AND MECHANISM OF ACTION OF RICE BRAN PHYTIC ACID ON COLON CANCER IN VITRO AND IN VIVO

NURUL HUSNA SHAFIE

IB 2013 15

ANTI-CANCER ACTIVITY AND MECHANISM OF ACTION OF RICE BRAN PHYTIC ACID ON COLON CANCER *IN VITRO* AND *IN VIVO*

By

NURUL HUSNA SHAFIE

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

May 2013

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

ANTI-CANCER ACTIVITY AND MECHANISM OF ACTION OF RICE BRAN PHYTIC ACID ON COLON CANCER *IN VITRO* AND *IN VIVO*

By

NURUL HUSNA SHAFIE

May 2013

Chairman : Norhaizan Mohd Esa, Ph.D

Institute : Biosciences

Colorectal cancer or colon cancer is a major neoplastic disease affecting men and women worldwide. Since Burkitt's pioneering research that pointed to inverse relationship between colon cancer risk and consumption of fiber-rich foods, many epidemiological and laboratory animal studies have tested this hypothesis. The protective effects of dietary fiber on colon cancer development depend on the nature and source of fiber. Rice bran is one of the richest sources of dietary fiber and contains phytonutrients, including phytic acid, known to possess various medicinal properties. Phytic acid, or inositol hexaphosphate (IP_6), is a polyphosphorylated carbohydrate, that has been suggested to play a significant role in the inhibition of colorectal cancer. Thus, our attention was drawn to the possibility to utilize the local source of phytic acid in identifying non-toxic anti-cancer agents that can potentially lead to the development of better treatments for colorectal cancer.

In particular, the present study was aimed at investigating the anti-cancer effect of IP_6 extracted from rice bran, in colon cancer model *in vitro* and *in vivo*. It began with the investigation of the inhibitory effect and associated mechanisms of rice bran IP_6 on human colorectal cancer cell line, HT-29. IP_6 induced marked growth inhibition in a dose and time dependent manner as evaluated by the MTT proliferation assay (IC_{50} = 12 μg/mL). Indeed, IP_6 also did not cause any cytotoxicity towards normal 3T3 cells. Cell cycle progression studies were performed by flow cytometric analysis following propidium iodide (PI) staining of the cells. IP_6 treatment (9.5, 12.0 and 14.5 μg/mL IP_6) for 24, 48 and 72 hours, resulted in significant accumulation of G_0/G_1 phase cells (63 – 65 %) compared to control (50.53 %) (p<0.05). Together, these results suggested that IP_6 causes the inhibition of cell growth through G_0/G_1 phase arrest in the cell cycle progression of HT-29 cells.

Additionally, investigation of the ability of IP_6 to induce colon tumor cell apoptosis was carried out. It was proven that IP_6 significantly induced early apoptotic cell death (30 %) in a dose- and time dependent manner compared to control cells showing <1 % of cell death as confirmed by Annexin V assay (p<0.05). Quantitative real-time polymerase chain reaction (qRT-PCR) analysis was performed next followed by Western blotting to further determine apoptosis at the molecular level. Interestingly, IP_6 showed an extensive significant reduction of anti-apoptotic Bcl-xl and a coherent increment of pro-apoptotic Bax at the mRNA and protein level. These results showed that IP_6 caused a marked increase in apoptosis, which was accompanied by significantly increased mRNA and protein levels of caspase 3 and caspase 8 (p<0.05). These molecular alterations provide an insight into the apoptotic death of human colon cancer, HT-29 cells, elicited by IP_6.

The second part of this study in which the hypothesis whether IP_6 extracted from rice bran had any effect on colon carcinogenesis was further investigated in an animal model of experimental colon cancer. Male Sprague-Dawley, weanling rats were divided into 5 groups with 12 rats in each group. Rats received two intraperitoneal (*i.p.*) injections of azoxymethane (AOM) in saline (15 mg/kg body weight) over a 2-week period for colon cancer induction. The IP_6 treatments were given in three concentrations: 0.2 % (w/v), 0.5 % (w/v) and 1.0 % (w/v) via drinking water and the treatment were divided into two termination timelines. For the first termination, 6 rats from each group were killed after 8 weeks of IP_6 treatment. The colons of these rats were analyzed for detection and quantification of aberrant crypt foci (ACF), an early biomarker of colon cancer. Analysis of ACF incidence demonstrated that administration of IP_6 extracted from rice bran significantly reduced the total number of ACF ($p<0.05$). Furthermore, IP_6 also significantly reduced the number of dysplastic and non-dysplastic ACF in a dose-dependent manner.

For the second termination, the other 6 rats in each group were killed after 16 weeks of IP_6 treatment. Colons of these rats were assessed for tumor incidence. It was shown that administration of phytic acid in the drinking water, significantly suppressed the total number of tumor incidences compared to the control ($p<0.05$). In another case, deregulation of the Wnt/β-catenin signaling pathway has been implicated in colorectal tumorigenesis resulting in accumulation of β-catenin, a major mechanism in the AOM-induced colon carcinogenesis model. Indeed, expression of enzymes associated with inflammation, such as inducible cyclooxygenase-2 (COX-2), has been shown to play a role in colon tumor progression. Therefore, the potential of IP_6 in targeting key components of the

Wnt/β-catenin signaling pathway and COX-2 as a rational for cancer drug discovery was demonstrated. Colon tumors were further analyzed for β-catenin and COX-2 expression at mRNA and protein level by qPCR and Western blot respectively. It was shown that administration of IP_6 significantly down-regulated β-catenin and COX-2 expression in colon tumors as compared with control (no IP_6 treatment) ($p<0.05$). Therefore, it can be suggested that β-catenin and COX-2 could be potential targets for colon cancer chemoprevention.

Collectively, results presented in this study demonstrated that IP_6 extracted from rice bran inhibited the proliferation of colon cancer *in vitro* selectively by arresting the cell cycle and thus leading to programmed cell death, which was later confirmed to be through the regulation of pro- and anti-apoptotic proteins and caspase dependent pathways. Moreover, the study *in vivo* with the AOM induced rat colon carcinogenesis model clearly showed that IP_6 had inhibited the development of colon cancer through the suppression of ACF, leading to reduction in tumor incidence, which was via the down-regulation of β-catenin and COX-2 expression.

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

AKTIVITI ANTI-KANSER DAN MEKANISME TINDAKAN ASID FITIK DEDAK BERAS KE ATAS KANSER KOLON *IN VITRO* DAN *IN VIVO*

Oleh

NURUL HUSNA SHAFIE

Mei 2013

Pengerusi : Norhaizan Mohd Esa, Ph.D

Institut : Biosains

Kanser kolon atau usus besar ialah antara penyakit neoplasia yang utama di kalangan lelaki dan wanita di seluruh dunia. Sejak bermulanya penyelidikan kanser Burkitt's yang membuktikan hubungan terbalik di antara risiko kanser kolon dan penggunaan makanan kaya serat, banyak kajian epidemiologi dan makmal haiwan telah menguji hipotesis ini. Perbandingan antara serat diet dengan kejadian dan kadar kematian kanser kolon menyokong hipotesis di mana serat diet, terutamanya serat daripada sumber bijirin dapat menghalang penyakit kanser kolon. Sebagai contoh, dedak beras juga merupakan salah satu daripada bahan yang kaya dengan serat dan mengandungi pelbagai fitonutrien dan fitokimia termasuk asid fitik yang berpotensi sebagai bahan ubatan. Asid fitik atau juga dikenali sebagai inositol heksafosfat ialah sejenis karbohidrat polifosfat, telah dibuktikan mempunyai peranan penting dalam mencegah dan merawat kanser kolon. Oleh itu, ianya telah menarik perhatian kami

untuk menggunakan asid fitik dari sumber tempatan yang tidak toksik dan dikembangkan potensinya sebagai rawatan penyakit kanser kolon.

Secara khususnya, kajian ini adalah bertujuan untuk mengkaji keberkesanan asid fitik yang diekstrak daripada dedak beras sebagai anti-kanser menggunakan model kanser kolon *in vitro* dan *in vivo*. Kajian ini dimulakan dengan penyelidikan kesan perencatan pertumbuhan dan mekanisme tindakan yang terlibat oleh asid fitik (IP_6) pada sel kanser kolon manusia (HT-29). IP_6 telah dibuktikan dapat menghalang pertumbuhan sel kanser kolon (HT-29) bergantung pada dos dan masa yang dinilai menggunakan asai proliferasi MTT (IC_{50} = 12 μg/mL). Malahan dapat dibuktikan bahawa asid fitik tidak mempunyai kesan toksik terhadap sel normal, 3T3. Selain itu, kajian terhadap proses kitaran sel telah dijalankan menggunakan pewarna 'propidium' iodida diikuti dengan analisis sitometri aliran. Selepas perawatan dan pendedahan asid fitik ke atas sel kanser kolon (HT-29) dengan dos-dos tertentu (9.5, 12.0 and 14.5 μg/mL IP_6) selama 24, 48 and 72 jam, terbukti bahawa asid fitik menyebabkan pengumpulan sel pada fasa G_0/G1 sebanyak 63 – 65 % berbanding dengan kawalan atau sel tanpa rawatan dengan asid fitik hanya sebanyak 50.53 % (p<0.05). Oleh itu, dapat dibuktikan bahawa asid fitik (IP_6) merencatkan pertumbuhan sel kanser kolon (HT-29) melalui penahanan kitaran sel, iaitu pada fasa G_0/G_1.

Selain itu, penyelidikan ke atas asid fitik dalam mengaruh proses apoptosis terhadap sel kanser kolon telah dijalankan. Ini telah dibuktikan di mana IP_6 menyebabkan peningkatan sel apoptosis awal sebanyak 30 % berbanding dengan kawalan hanya < 1 % sel yang mati dan ini bergantung pada dos dan masa seperti yang ditunjukkan

oleh asai Annexin V (p<0.05). Analisis qRT-PCR seterusnya dijalankan dan diikuti dengan 'Western blot' untuk kajian yang lebih mendalam dalam membuktikan proses apoptosis oleh $IP_{6,}$ iaitu pada peringkat molekul. Menariknya, IP_6 menunjukkan kesan 'down'-modulasi atau pengurangan yang nyata ke atas anti-apoptosis Bcl-xl dan pada masa yang sama meningkatkan atau 'up'-regulasi ke atas pro-apoptosis Bax pada peringkat mRNA dan protein. Semua data ini menunjukkan keberkesanan IP_6 dalam meningkatkan sel apoptosis, dan turut disokong dengan penemuan peningkatan aktiviti 'caspase' 3 and 'caspase' 8 setelah sel kanser kolon (HT-29) dirawat dengan asid fitik (IP_6). Perubahan peringkat molekul ini menyediakan penemuan yang mendalam terhadap kesan asid fitik (IP_6) dalam mengaruh apoptosis ke atas sel kanser kolon (HT-29).

Bahagian kedua penyelidikan ini ialah kajian ke atas kesan asid fitik (IP_6) yang diekstrak daripada dedak beras terhadap proses 'karsinogenesis' atau tumor kolon yang diuji menggunakan model haiwan yang diaruh dengan kanser kolon. Pada peringkat awal, tikus jantan Sprague-Dawley dibahagikan kepada 5 kumpulan dengan 12 ekor bagi setiap kumpulan. Tikus-tikus tersebut menerima dua kali suntikan azoksimetana (AOM) dalam 'saline' (15 mg/kg body weight) pada dua minggu berturut-turut bagi tujuan pengaruhan kanser kolon. Rawatan dengan asid fitik (IP_6) adalah berdasarkan tiga dos yang berbeza: 0.2 % (w/v); 0.5 % (w/v) dan 1.0 % (w/v) melalui air minuman dan dibahagikan kepada dua kali masa penamatan. Untuk penamatan pertama iaitu selepas 8 minggu rawatan dengan asid fitik, 6 ekor tikus dibunuh dan kolon atau usus tikus tersebut diambil dan dianalisa untuk tujuan pengesanan dan pengiraan 'aberrant crypt foci' (ACF), iaitu penanda awal kanser kolon. Analisis terhadap kejadian pembentukan ACF ini membuktikan tikus yang

dirawat dengan asid fitik yang diekstrak daripada dedak beras ini telah mengurangkan jumlah kejadian ACF. Tambahan pula, IP_6 juga turut mengurangkan jumlah pembentukan ACF 'dysplasia' dan bukan 'dysplasia' bergantung kepada dos (p<0.05).

Bagi penamatan kedua pula, 6 ekor tikus yang berbaki dibunuh selepas 16 minggu dirawat dengan asid fitik. Kolon atau usus tikus tersebut diambil dan dikaji untuk analisis kejadian pembentukan tumor. Dapat dibuktikan bahawa terdapat pengurangan terhadap kejadian pembentukan kanser setelah rawatan asid fitik ke atas tikus yang diaruh dengan kolon kanser berbanding dengan tikus yang tidak dirawat (p<0.05). Perubahan kawalan terhadap tranduksi isyarat Wnt/β-catenin telah diimplifikasikan dalam proses karsinogenesis kolon menyebabkan pengumpulan β-catenin adalah mekanisme terbesar di dalam haiwan yang diaruh dengan AOM. Selain itu juga, ekspresi enzim yang melibatkan inflamasi contohnya COX-2 dibuktikan memainkan peranan penting dalam proses karsinogenesis. Oleh itu, potensi asid fitik terhadap kunci komponen tranduksi isyarat Wnt/β-catenin dan COX-2 sebagai terapi kanser dijalankan. Kolon tikus yang diambil pada masa penamatan kedua dikaji untuk ekspresi β-catenin and COX-2 pada peringkat mRNA dan protein oleh qRT-PCR dan 'Western blot'. Kajian ini membuktikan rawatan tikus dengan asid fitik telah mengurangkan atau 'down'-regulasi ekspresi β-catenin and COX-2 di dalam kanser kolon berbanding dengan kawalan. Oleh itu, telah dibuktikan bahawa β-catenin dan COX-2 mungkin berpotensi sebagai sasaran dalam pencegahan kanser kolon. Secara kolektif, keputusan yang dibentang dalam kajian ini membuktikan asid fitik yang diekstrak daripada dedak beras telah menghalang proliferasi kanser kolon secara *in vitro,* melalui perencatan pada kitaran sel dan

seterusnya membawa kepada kematian sel yang diprogramkan, yang mana kemudiannya disahkan melalui regulasi antara molekul pro- dan anti- apoptosis dan laluan ‘caspase’. Tambahan pula, kajian *in vivo* pada model tikus yang diaruh dengan kanser kolon menggunakan AOM menunjukkan IP_6 telah menghalang pembentukan kanser kolon melalui penyekatan terhadap ACF dan seterusnya mengurangkan pembentukan tumor, iaitu melalui ‘down’-regulasi β-catenin dan COX-2.

ACKNOWLEDGEMENTS

This has been a learning journey for me with new experiences and obstacles that come with undertaking a PhD degree. I am forever thankful to my supervisor, Assoc. Prof. Dr. Norhaizan Mohd Esa for all the opportunities that she has given me to excel in my field of research. Dr Norhaizan has been a wonderful source of encouragement and guidance. Her frankness plus friendly and enthusiastic approach has made the journey that much more pleasant, so I sincerely thank her.

To Prof. Dr. Hairuszah Ithnin, thank you for being there, whenever I had questions or was in need. Your expert guidance has been most helpful and appreciated. I also want to thank to Dr Abdah Md Akim for all her guidance and assistance, particularly in the molecular studies.

Special thanks to Miss Norazalina Saad for your time and assistance in many aspects. I would like to thank the entire staffs of the Laboratory of Cancer Research UPM-MAKNA including Mrs Noraini Mohd Ain, Mrs Tommini Salleh, Mrs Norlela Ahmad and Mrs Noor Haizi Ruslan for their help as well as MOSTI and MOHE for scholarship and financial assistance.

To my fellow postgraduates students especially my housemates, Nursyuhada, Siti Syazani, Salmiah and Munira Saira special thanks to you all for your continuous support and friendship.

I also would like to take this opportunity to acknowledge my best friends, Nurhidayah and Nur Suzana and not to forget Siti Suriani for being there during my critical preparation for thesis submission. Thank you girls!

Lastly, I want to give my most heartfelt thanks to my mother, Mrs Che Fadzilah Saad and the rest of my family for all the endless encouragement, love and support.

I certify that a Thesis Examination Committee has met on (22nd May 2013) to conduct the final examination of Nurul Husna Shafie on her thesis entitled "Anti-cancer activity and mechanism of action of rice bran phytic acid on colon cancer *in vitro* and *in vivo*" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The committee recommends that the student be awarded the Doctor of Philosophy.

Members of the Thesis Examination Committee were as follows:

Asmah Rahmat, PhD
Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

Patimah Ismail, PhD
Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Internal Examiner)

Noorjahan Banu Binti Mohammed Alitheen, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Klaus W.J. Wahle, PhD
Professor
School of Medicine and Dentistry
Aberdeen University
United Kingdom
(External Examiner)

NORITAH OMAR, PhD
Assoc. Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 2 AUGUST 2013

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

Norhaizan Mohd Esa, PhD
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

Hairuszah Ithnin, PhD
Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

Abdah Md Akim, PhD
Senior Lecturer
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

BUJANG KIM HUAT, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

TABLE OF CONTENTS