UNIVERSITI PUTRA MALAYSIA

POTENTIAL OF PHYTIC ACID EXTRACTED FROM RICE BRAN AS ANTIOXIDATIVE COMPOUND AND ANTI-PROLIFERATION AGENT IN COLON CANCER CELL LINE

NORASHAREENA MOHAMED SHAKRIN

FPSK(m) 2013 23
POTENTIAL OF PHYTIC ACID EXTRACTED FROM RICE BRAN AS ANTIOXIDATIVE COMPOUND AND ANTI-PROLIFERATION AGENT IN COLON CANCER CELL LINE

By
NORASHAREENA MOHAMED SHAKRIN

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

April 2013
COPYRIGHT

All materials contained within the thesis, including without limitation text, logos, icons, photographs and all other artworks is copyright material of Universiti Putra Malaysia unless otherwise stated. Use may be made of any material contained within the thesis for non-commercial purposes from the copyright holder. Commercial use of material may only be made with the express, prior, written permission of Universiti Putra Malaysia.

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

POTENTIAL OF PHYTIC ACID EXTRACTED FROM RICE BRAN AS ANTIOXIDATIVE COMPOUND AND ANTI-PROLIFERATION AGENT IN COLON CANCER CELL LINE

By

NORASHAREENA MOHAMED SHAKRIN

April 2013

Chairman : Associate Professor Norhaizan Mohd Esa, PhD

Faculty : Medicine and Health Sciences

Phytic acid (IP6 or PA) which occurs at 9.5-14.5% in weight in rice bran has been reported to possess various significant health benefits including a potential as antioxidant and anticancer. It is a powerful inhibitor of iron mediated generation of hydroxyl radical (OH) that proposes may lower the incidence of colonic cancer. This research was done to study the potential of PA extracted from rice bran as an antioxidant agent and as antiproliferative agent on colon cancer cell line, HT-29. For optimization of PA extraction from defatted rice bran, three types of acidic solution were used (hydrochloric acid, sulphuric acid and trichloroacetic acid) with different concentration, pH and time of extraction. Neutralization of the sample extract was then performed to formulate the sample to be applied for in vitro followed by the purification by Anion-Exchange chromatography. The isolation and quantitation of PA was done by using reverse-phase High Performance Liquid Chromatography (HPLC). Five methods were used to analyze the antioxidant activity of the rice bran...
PA and to compare its antioxidant activity with corn PA and butylated hydroxytoluene (BHT). For the assessment of cytotoxicity effect of PA on HT-29 colon cancer cell, MTS (3-4,5-dimethylthiazol-2-yl-5-3-carboxymethoxy phenyl-2-4-sulfophenyl-2H tetrazolium, inner salt assay) was used. The conjugated of FITC (fluorescein isothiocyanate) and Annexin V was used to quantitate apoptotic cells on a single-basis by flow cytometry and the qualitative apoptosis assay by Tunel assay (microscopy). Results showed the highest amount of PA extract yielded (11.14mg/g) by using 5% of sulphuric acid in pH 0.6 for 30 minutes of extraction. For antioxidant assays, by using β-carotene bleaching method, the results for rice bran PA, corn PA and BHT are 93.075%±3.099, 92.550%±9.635 and 109.610%±4.238, respectively. Meanwhile, for FTC and TBA method, the results for rice bran PA, corn PA and BHT are (74.76%±0.05 and 40.05%±0.03), (74.89%±0.03 and 34.42%±0.09) and (86.02±0.05 and 64.30%±0.04) respectively. The order of ferric reducing effect power (FRAP value) was: corn PA (2.738 mM±0.013) > rice bran PA (2.107 mM±0.006) > BHT (1.567 mM±0.039). DPPH assay indicates that PA has low radical scavenging effect, which was in the range of 10.1 to 41.0% merely. The antioxidant activities of both PA measured by all methods are not significantly different between each other. For cytotoxicity assays, rice bran PA and corn PA produced a 50% net of inhibition growth (IC$_{50}$) at the dose of 25.3±5.23 µg/mL and 35.2±3.11 µg/mL respectively. The IC$_{50}$ value of rice bran PA is significantly different with corn PA. Result from the quantitative apoptosis assay showed at the IC$_{50}$ concentration, the percentage of treated cells undergone early apoptosis and late apoptosis for rice bran PA is 18.54%±1.55 and 38.41%±1.78, while the corn PA is 1.7%±0.10 and 4.35%±0.26 respectively. The percentage of total apoptotic cells at IC$_{50}$ concentration for the rice bran PA is higher (56.9%) compared to corn PA.
(6.1%). The results exposed that both samples induced the exposure of phosphatidylserine on outer cell membrane detected by using Annexin V-FITC. Conversely, the qualitative apoptosis assay by Tunel assay are demonstrated that none of the both phytic acid induced cells emits green fluorescence indicated that none of the treated cells underwent apoptosis. Though, since the results from both quantitative and qualitative apoptosis assays were not comparable to each other, further assessment is needed. In conclusion, our findings showed that PA extracted from rice bran posses the anti proliferative effect on colon cancer cell line (HT-29). One possible mechanism is by antioxidant activity that showed high in PA extract as demonstrated in our findings. The PA showed its strong characteristic of antioxidant activity by minimizing the coupled oxidation of linoleic acid (free radical) and beta carotene in β-Carotene bleaching assay, capability as ferric reducing power in FRAP assay and as a hydrogen peroxide scavenger at the initial stage of lipid oxidation in FTC assay but not as a free radical scavenger in DPPH assay.
Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

POTENSI ASID FITIK (INOSITOL HEKSAFOSFAT) YANG DIEKSTRAK DARIPADA DEDAK BERAS SEBAGAI SEBATIAN ANTIOKSIDAN DAN ANTIPROLIFERASI KE ATAS SEL KOLON KANSER

Oleh,
NORASHAREENA MOHAMED SHAKRIN

April 2013

Pengerusi : Profesor Madya Norhaizan Mohd Esa, PhD
Fakulti : Perubatan dan Sains Kesihatan

Asid fitik (PA atau IP6) yang terdapat dalam anggaran berat 9.5-14.5% di dalam dedak beras dilaporkan mempunyai pelbagai manfaat kesihatan termasuklah berpotensi sebagai agen antioksidan dan antikanser. Ia adalah perencat perantara iron dalam pembentukan radikal hidroksi (OH) yang berkesan untuk merendahkan kadar insiden kancer kolon. Kajian ini telah dijalankan bagi mengkaji potensi PA yang diekstrak daripada dedak beras sebagai agen antioksidan dan antiproliferasi pada sel kancer kolon (HT-29). Bagi pengoptimum proses pengekstrakan PA dari dedak beras, tiga jenis larutan asid telah digunakan (asid hidroklorik, asid sulfurik dan asid trikloroasetik) dengan kepekatan, pH dan masa pengekstrakan yang berbeza. Proses peneutralan ekstrak sampel dijalankan bagi memformulasikannya untuk digunakan bagi ujian in vitro diikuti proses penulenan melalui kaedah Kromatografi Pernukaran-Anion. Pemisahan PA dijalankan menggunakan kaedah Kromatografi Cecair Berprestasi Tinggi (HPLC). Lima kaedah dijalankan bagi penentuan aktiviti antioksidan PA dedak beras dan bagi membandingkan dengan PA komersial dan
Bagi menentukan kesan sitotoksik PA pada sel kolon kanser, HT-29, kaedah MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium) telah digunakan. Konjugat FITC dan Annexin V digunakan untuk pengiraan sel apoptotik secara kuantitatif menggunakan flowcytometry dan asai Tunel sebagai kaedah kualitatif. Keputusan menunjukkan amaun PA yang tertinggi (11.14mg/g) diperolehi dengan 5% asid sulfurik pada pH 0.6 dan jangkamasa pengekstrakan selama 30 minit. Bagi analisis antioksidan oleh kaedah pelunturan β-carotene, aktiviti antioksidan bagi PA dedak beras, PA jagung (komersial) dan BHT masing-masing adalah 93.075%±3.099, 92.550%±9.635 dan 109.610%±4.238. Manakala bagi kaedah FTC dan TBA, nilai aktiviti antioksidan masing-masing adalah (74.76%±0.05 dan 40.05%±0.03), (74.89%±0.03 dan 34.42%±0.09) dan (86.02±0.05 dan 64.30%±0.04) bagi PA dedak beras, PA komersial dan BHT. Turutan bagi kesan kuasa penurunan ferik adalah: PA komersial (2.738mM±0.013) > PA dedak beras (2.107mM±0.006) > BHT (1.567mM±0.039). Kaedah DPPH menunjukkan PA mempunyai kesan pemerangkapan radikal yang rendah iaitu hanya dalam anggaran 10.1 hingga 41.0%. Aktiviti antioksidan bagi kedua-dua PA yang dianalisis oleh kesemua kaedah menunjukkan perbezaan yang tidak signifikan antara satu sama lain. Bagi analisis sitotoksik nilai IC₅₀ bagi PA dedak beras dan PA komersial (jagung) ialah 25.3±5.23 μg/mL dan 35.2±3.11 μg/mL. Nilai IC₅₀ bagi kedua-dua PA ini adalah berbeza secara signifikan. Analisis kuantitatif menunjukkan pada kepekatan IC₅₀ peratus sel apoptotik bagi peringkat awal dan akhir apoptosis bagi PA dedak beras ialah 18.54%±1.55 and 38.41%±1.78 manakala bagi PA komersial adalah 1.7%±0.01 dan 4.35%±0.26. Peratus jumlah sel apoptotik pada kepekatan IC₅₀ PA dedak beras (56.5%) adalah lebih tinggi berbanding PA komersial (6.10%). Keputusan menunjukkan yang kedua-dua sampel PA
merangsang pendedahan fosfatidilserine pada sel luaran membran yang dikesan oleh Annexin V-FITC. Walau bagaimanapun, kaedah apoptosis kualitatif oleh asai Tunel menunjukkan sel yang dirawat dengan sampel PA tidak mempunyai ciri-ciri sel apoptotik iaitu menunjukkan warna hijau fluorescense. Oleh sebab itu, kajian seterusnya harus dijalankan bagi mengenalpasti kesan PA kepada morfologi sel kanser. Secara kesimpulannya, kajian ini menunjukkan sampel PA yang diekstrak dari dedak beras menunjukkan kesan antiproliferasi pada sel kolon kanser, HT-29. Satu dari mekanismanya ialah keupayaan aktiviti antioksidan yang tinggi di dalam ekstrak PA seperti yang ditunjukkan oleh kajian ini. PA menunjukkan ciri-ciri antioksidan yang kuat dimana ia dapat meminimakan pengoksidaan berganda bagi asid linoleik (radikal bebas) dan beta karotene di dalam asai pelunturan beta karotene, mempunyai kuasa penurunan ferik dalam asai FRAP dan sebagai pemerangkap hidrogen peroksida di peringkat awal pengoksidaan lipid seperti ditunjukkan oleh asai FTC tetapi bukan sebagai pemerangkap radikal bebas di dalam asai DPPH.
ACKNOWLEDGEMENTS

Bismillahirrahmanirrahim,

Alhamdulillah. Thanks to Allah SWT, for this incredible journey given and His willing in giving me the opportunity to complete this thesis. This thesis is the end of my long journey in obtaining my master degree in Nutritional Biochemistry. It is a pleasure to thank the many people who made this thesis possible.

Foremost, I am deeply indebted to my supervisor Associate Professor Dr. Norhaizan Mohd Esa of my master study and research for the continuous support, guidance, patience, motivation, and enthusiasm. Her understanding and personal encouragement helped me in all the time of research for and writing of this thesis.

My sincere thanks to my co-supervisors, Dr. Abdah Md Akim and Dr. Loh Su Peng for their stimulating and valuable guidance during my project progress. I am also thankful to Prof Dr. Maznah Ismail who gave me the opportunity to work with them in her laboratory for my research project. All these have provided a good basis for the present thesis.

Deepest thanks and appreciation dedicated to my parents, family and special mate of mine, Mr. Nazmi for supporting me spiritually throughout my life. Without their cooperation, encouragement and understanding from the beginning till the end, it would have been impossible for me to finish this thesis.

My warm thanks are due to all of my lovely colleagues especially Ms Norazalina, the team of Faculty of Medicine & Health Sciences and everyone, those have been contributed by supporting my work and help myself during the project progress till it is fully completed.
I certify that a Thesis Examination Committee has met on 4 April 2013 to conduct the final examination of Norashareena binti Mohamed Shakrin on her thesis entitled "Potential of Phytic Acid Extracted from Rice Bran as Antioxidative Compound and Anti-Proliferation Agent in Colon Cancer Cell Line" 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 Master of Science.

Members of the Thesis Examination Committee were as follows:

**Huzwah binti Khaza’ai, PhD**  
Senior Lecturer  
Faculty of Medicine and Health Sciences  
Universiti Putra Malaysia  
(Chairman)

**Asmah binti Rahmat, PhD**  
Professor  
Faculty of Medicine and Health Sciences  
Universiti Putra Malaysia  
(Internal Examiner)

**Latifah binti Saiful Yazan, PhD**  
Associate Professor  
Faculty of Medicine and Health Sciences  
Universiti Putra Malaysia  
(Internal Examiner)

**Suzana binti Makpol, PhD**  
Associate Professor  
Faculty of Medicine  
Universiti Kebangsaan Malaysia  
Malaysia  
(External Examiner)

\[Signature\]

**NORITAH OMAR, PhD**  
Associate Professor and Deputy Dean  
School of Graduate Studies  
Universiti Putra Malaysia

Date: 26 June 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 Master Science. 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)

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

**Loh Su Peng, PhD**  
Associate Professor  
Faculty of Medicine and Health Sciences  
Universiti Putra Malaysia  
(Member)

........................................................................

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

Date :
DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

..............................................

NORASHAREENA BINTI MOHAMED SHAKRIN

Date: 4 April 2013
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>SECTION</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>ABSTRAK</td>
<td>v</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENT</td>
<td>viii</td>
</tr>
<tr>
<td>APPROVAL</td>
<td>ix</td>
</tr>
<tr>
<td>DECLARATION</td>
<td>xi</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xiii</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xv</td>
</tr>
</tbody>
</table>

## CHAPTER

### I INTRODUCTION

1

### II LITERATURE REVIEW

2.1 Cancer
   2.1.1 Colon Cancer
   2.1.2 Cell death
   2.1.2.1 Apoptosis
   2.1.2.2 Necrosis
   2.1.2.3 Apoptosis assays
2.2 Free radical and Cancer
2.3 Antioxidant and Cancer
2.4 Rice (*Oryza sativa L.*) and rice bran
2.5 Phytic acid (Inositol Hexaphosphate)
   2.5.1 Inhibitory effect of phytic acid on mineral bioavailability
   2.5.2 Potential positive roles of phytic acid
      2.5.2.1 Antioxidant properties
      2.5.2.2 Anti-cancer properties

### III MATERIALS AND METHODS

3.1 Materials
   3.1.1 Preparation of sample
   3.1.2 Antioxidant studies
   3.1.3 Cell Culture studies
   3.1.4 Instrument and equipments
3.2 Preparation of phytic acid
   3.2.1 Rice bran preparation
The content of the page is a list of sections and subsections from a scientific paper. Here is the plain text representation:

3.2.2 Microwave heat stabilization  
3.2.3 Oil extraction (defating process)  
3.2.4 Optimization of extraction process of phytic acid  
3.2.5 Spectrophotometrical Determination  
3.2.6 Neutralization of phytic acid extract  

3.3 Purification and isolation of phytic acid  
3.3.1 Anion-Exchange Purification  
3.3.2 High Performance Liquid Chromatography (HPLC) Analysis  

3.4 Antioxidant assays  
3.4.1 Ferric thiocyanate (FTC) method  
3.4.2 Thiobarbituric acid (TBA) method  
3.4.3 B-carotene bleaching method  
3.4.4 Diphenylpcyrylhidrazine (DPPH) method  
3.4.5 Ferric reducing antioxidant power (FRAP) assay  

3.5 Cell culture studies  
3.5.1 Cell maintenance  
3.5.1.1 Cell culturing  
3.5.1.2 Cells Cryopreservation  
3.5.1.3 Subculturing the Cells  
3.5.2 Cytotoxicity assay  
3.5.2.1 Cell plating  
3.5.2.2 Cell treatment  
3.5.2.3 Cell proliferation assay (MTS)  
3.5.2.4 Determination of IC50  
3.5.3 Apoptosis assay  
3.5.3.1 Flowcytometry analysis using AnnexinV-FITC/PI  
3.5.3.2 Tunel assay with Fluorometric method  

3.6 Statistical Analysis  

IV RESULTS AND DISCUSSION  

4.1 Optimization of phytic acid extraction process from rice bran  
4.1.1 Optimization of extraction process by using different conditions  
4.1.2 Neutralization of phytic acid extracts  
4.2 Purification and isolation of phytic acid sample  
4.2.1 Anion-Exchange Purification  
4.2.2 Separation of phytic acid using High Performance Liquid Chromatography (HPLC)  
4.3 Antioxidant studies of phytic acid  
4.3.1 Determination of antioxidant by Ferric Thiocyanate (FTC) and Thiobarbituric acid (TBA) method  
4.3.2 Determination of antioxidant activity by Beta-carotene
bleaching method

4.3.3 Determination of antioxidant by Diphenyl-1-picryl hydrazyl (DPPH) method  97
4.3.4 Determination of antioxidant by Ferric Reducing Antioxidant Power (FRAP) assay  101

4.4 Cytotoxicity assay by MTS cell proliferation assay  104

4.5 Apoptosis analysis
   4.5.1 Flowcytometry analysis using AnnexinV-FITC/PI  110
   4.5.2 Microscopy analysis by Fluorometric Tunel System  118

V GENERAL DISCUSSION  123

VI CONCLUSION AND RECOMMENDATION FOR FUTURE RESEARCH  130

REFERENCES/BIBLIOGRAPHY  133
APPENDICES  147
BIODATA OF STUDENT  156
LIST OF PUBLICATIONS  157