UNIVERSITI PUTRA MALAYSIA

TOXICOLOGICAL EVALUATION OF GERMINATED ROUGH RICE CRUDE EXTRACT AND ITS CHEMOPREVENTIVE EFFECTS IN INHIBITING AZOXYMETHANE-INDUCED ABBERANT CRYPT FOCI FORMATION IN Sprague dawley RATS

ELNAZ SAKI

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By

ELNAZ SAKI

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

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DEDICATION

This dissertation is dedicated to my dearest Mom and Dad, Batoul Selahvarzi and Mohammad Kazem Saki for their endless love, support and never ending encouragement.

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

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June 2015

Chairman: Associate Prof PhD. Zalinah Ahmad
Faculty : Institute of Bioscience

Rice is a nutritious staple food with health-promoting activity. Germination of rough rice (GRR) causes significant changes in several chemical compositions and bioactive compounds that might prevent or postpone the inception of cancer. This research was carried out to study the chemopreventive properties of GRR crude extract in *Sprague dawley* rats induced with azoxymethane. The antioxidant properties of GRR crude extract were determined by TPC, ABTS, DDPH and FRAP assays. The level of antioxidant activity of GRR crude extract as determined by TPC and FRAP assay was 105.75 and 69.16 (mg GAE/g GRR crude extract), respectively. The level of antioxidant activity of GRR crude extract as determined by ABTS and DDPH assay was 105.75 and 69.16 mg (Trolox Equivalent/g GRR crude extract), respectively. The correlation between antioxidant assays (ABTS, DDPH and FRAP) and total phenolic content was roughly high (R^2 = 0.9984) and showed the antioxidant property of GRR crude extract. The cytotoxic effect of GRR crude extract on HT29 cells after 72 hours was determined by MTT assay. IC_{50} value of GRR crude extract was 43 µg/mL. For the acute toxicity study of GRR crude extract, the OECD Guidelines 423 was followed. Forty male (6 weeks of age) *Sprague dawley* rats were divided in 4 groups (n=5) which were (G1) 2000 mg/kg body weight (GRR crude extract (g)/body weight of rat (kg)), (G2) 1500 mg/kg body weight, (G3) 500 mg/kg body weight and (G4) distilled water alone. GRR crude extract was administered orally one time per week for 14 days. No evidence of toxicity attributable to the treatment with GRR crude extract was observed based on the body and organ weight, hematological parameters and histological evaluation. The sub-acute toxicity study of GRR crude extract followed OECD Guidelines 407. Fifteen male *Sprague dawley* rats (6 weeks of age) were divided in to 4 groups (n=5) which were (G1) 2000 mg/kg body weight (GRR crude extract (g)/body weight of rat (kg)), (G2) 1000 mg/kg body weight, (G3) 500 mg/kg body weight and (G4) distilled water alone. GRR crude extract was administered orally one time daily for 8 weeks. No evidence of toxicity attributable to the treatment with GRR crude extract was observed based on the body and organ weight, hematological parameters and histological evaluation. For the chemopreventive properties of GRR crude extract, fifty male *Sprague dawley* rats (6 weeks of age) were randomly divided into 5 groups (n=10) which were (G1) positive control (with AOM, unfed with GRR crude extract), (G2) with AOM, fed with 2000 mg/kg body weight (GRR crude extract (g)/body weight of rat (kg)), (G3) with AOM, fed with 1000 mg/kg body weight, (G4) with AOM, fed with 500 mg/kg body weight and (G5) negative control (without AOM, unfed with GRR crude extract). In order to induce colon cancer, the rats received two intraperitoneal injection of azoxymethane (AOM) in saline (15 mg/kg body weight) for two subsequent weeks. Then, GRR crude extract was administrated orally once

daily for eight weeks. Following the treatment, animals were sacrificed. Colons and all the organs (liver, kidney, lung, heart and spleen) were removed and weighed. Colonic aberrant crypt foci (ACF) were evaluated histopathologically. Treatment with 2000 mg/kg GRR crude extract gave the greatest reduction in the formation of ACF ($p<0.05$). From the histological classification of ACF, treatment with 2000 mg/kg GRR crude extract also had the highest percentage of non-dysplastic ACF. Expression of β-catenin was determined by Western blot analysis. The highest dose of GRR crude extract (2000 mg/kg (GRR crude extract (g)/ body weight of rat (kg)) showed the lowest level of β-catenin expression. In summary, GRR crude extract was not toxic to the animals and exhibited chemopreventive properties in rats induced with azoxymethane. GRR crude extract can be a promising dietary supplement component that might prevent or postpone the inception of cancer.

Abstrak thesis dikemukakan kepada Senat Universiti Putra Malaysia bagi memenuhi syarat untuk mendapatkan ijazah Master Sains

PENENTUAN KETOKSIKAN EKSTRAK KASAR BERAS KASAR CAMBAH DAN KESAN PENGHALANG KIMIA DALAM MERENCAT PEMBENTUKAN FOKUS KRIPT TIDAK NORMAL DALAM TIKUS *Sprague Dawley*

Oleh

ELNAZ SAKI

Jun 2015

Pengerusi: Prof Madya PhD. Zalinah Ahmad
Fakulti : Institut Biosains

Beras adalah makanan ruji yang berkhasiat dangan aktiviti yang boleh meningkatkan kesihatan. Pencambahan beras kasar (GRR) menyebabkan perubahan ketara dalam beberapa komposisi kimia dan sebatian bioaktif yang mungkin menghalang atau menangguhkan permulaan kanser. Kajian ini dijalankan untuk mengkaji ciri-ciri pencegahan kanser ekstrak GRR kasar dalam tikus *Sprague dawley* yang diaruh dengan azoxymetana. Ciri-ciri antioksida ekstrak mentah GRR telah ditentukan dengan ujian TPC, ABTS, DPPH dan FRAP. Tahap aktiviti antioksidan ekstrak kasar GRR seperti yang ditentukan oleh ujian TPC dan FRAP adalah 105.75 dan 69.16 (mg GAE/g GRR ekstrak mentah), masing-masing. Tahap aktiviti antioksida ekstrak mentah GRR seperti yang ditentukan oleh ujian ABTS dan DPPH adalah 105.75 dan 69.16 mg (Trolox Equivalent/g GRR ekstrak mentah), masing-masing. Hubungan diantara ujian antioksida (ABTS, DPPH dan FRAP) dan jumlah kandungan fenolik adalah tinggi (R2=0.998) dan ia menunjukkan ciri-ciri antioksida bagi ekstrak kasar GRR.Kesan sitotoksik ekstrak kasar GRR ke atas sel HT29 selepas 72 jam telah ditentukan oleh ujian MTT. Nilai IC_{50} ekstrak kasar GRR adalah 43 μg/mL. Untuk kajian ketoksikan akut ekstrak mentah GRR, Garis Panduan OECD 423 diikuti. Empat puluh ekor tikus *Sprague dawley* jantan (berumur 6 minggu) dibahagikan kepada 4 kumpulan (n=5) iaitu (G1) 2000 mg/kg berat badan (ekstrak mentah GRR (g) berat badan/badan tikus (kg)), (G2) 1500 mg/kg berat badan, (G3) 500 mg/kg berat badan dan (G4) air suling sahaja. Ekstrak kasar GRR telah diberikan secara oral. Tiada bukti ketoksikan dikaitkan dengan rawatan dengan ekstrak kasar GRR diperhatikan berdasarkan berat badan dan organ, parameter hematologi dan penilaian histologi. Kajian ketoksikan sub-akut ekstrak kasar GRR mengikut Garis Panduan OECD 407. Lima belas ekor tikus *Sprague dawley* jantan (berumur 6 minggu) telah dibahagikan kepada 4 kumpulan (n=5) iaitu (G1) 2000 mg/kg berat badan (ekstrak kasar GRR (g) berat badan/badan tikus (kg)), (G2) 1000 mg/kg berat badan, (G3) 500 mg/kg berat badan dan (G4) air suling sahaja. Tiada bukti ketoksikan dikaitkan dengan rawatan dengan ekstrak mentah GRR diperhatikan berdasarkan berat badan dan organ, parameter hematologi dan penilaian histologi. Bagi ciri-ciri pencegahan kanser ekstrak kasar GRR, lima puluh ekor tikus *Sprague dawley* jantan (berumur 6 minggu) dibahagikan secara rawak kepada 5 kumpulan (n=10) iaitu (G1) kawalan positif (dengan AOM, tanpa rawatan dengan ekstrak kasar GRR), (G2) dengan AOM, diberi makan dengan 2000 mg/kg berat badan ekstrak mentah GRR (g) berat badan/badan tikus (kg)), (G3) dengan AOM, diberi makan dengan 1000 mg/kg berat badan, (G4) dengan AOM, diberi makan dengan 500 mg/ kg berat badan dan (G5) kawalan negatif (tanpa AOM, tidak diberi makan dengan ekstrak mentah GRR). Bagi mengaruh kanser kolon, tikus tersebut menerima dua suntikan intraperitoneum azoxymetana (AOM) dalam salina (15 mg/kg berat badan) selama dua minggu berturut-turut. Ekstrak mentah GRR diberi secara oral sekali sehan hari selama lapan

minggu. Selepas rawatan, haiwan dikorbankan. Usus dan semua organ (hati, buah pinggang, paru-paru, jantung dan limpa) telah dikeluarkan dan ditimbang. Colonic aberrant crypt foci (ACF) telah dikaji secara histopatologi. Rawatan dengan 2000 mg/kg ekstrak kasar GRR memberikan pengurangan paling besar dalam pembentukan ACF. Berdasarkan klasifikasi histologi ACF, rawatan dengan 2000 mg/kg ekstrak mentah GRR juga mempunyai peratusan tertinggi ACF bukan displastik. Ekspresi β-catenin telah ditentukan oleh analisis Western blot. Dos tertinggi ekstrak mentah GRR (2000 mg/kg (GRR ekstrak mentah (g) berat badan/badan tikus (kg)) menunjukkan tahap ekspresi β-catenin yang paling rendah. Kesimpulannya, GRR ekstrak mentah tidak toksik kepada haiwan tersebut dan mempamerkan ciri-ciri pencegahan kanser pada tikus yang diaruh dengan azoxymetana. Ekstrak kasar GRR boleh menjadi komponen tambahan diet yang berpotensi menghalang atau menangguhkan permulaan kanser.

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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 of Science. The members of the supervisory committee were as follows:

Zalinah Ahmad, PhD
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairperson)

Latifah Saiful Yazan, PhD
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

Loh Su Ping, PhD
Associate Professor
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:

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This is to confirm that:

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Name of Chairman of Supervisory Committee: Zalinah Ahmad, PhD	Name of Member of Supervisory Committee: Latifah Saiful Yazan, PhD

Signature: _____
Name of Member of Supervisory Committee: Loh Su Ping, PhD

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ACF	Aberrant crypt foci
AOM	Azoxymethane
APC	Adenomatous polyposis coli
BERNAS	PadiBeras Nasional Berhad
CRC	Colorectal cancer
COX-2	The cyclooxygenase 2
FRAP	Ferric reducing ability of plasma
GAE	Gallic acid equivalent
GABA	Gamma-aminobutyric acid
GRR	Germinated rough rice
MAM	Methylazoxymethanol
MMR	Mismatch repair
Tcf	T cell factor
TGF-β	The transforming growth factor beta
TPC	Total phenolic content
TE	Trolox equivalents

CHAPTER 1

INTRODUCTION

1.1. History

Cancer today is a considerable public health concern that affects both developed and developing countries. The American Cancer Society estimated that in year 2008 alone, there were about 1.5 million new cancer cases diagnosed worldwide. Furthermore, 10 million people were diagnosed with all types of cancer, and it was estimated that about 60,000 died from cancer in 2014 (Cancer Facts and Figures, 2014).

Colorectal cancer (CRC) is a cancer that originates in the colon or the rectum. It can also be known separately as colon or rectal cancer, based on the origin. The rate of CRC is increasing worldwide (Pignone et al., 2002). It is the second highest cancer in frequency and one of the most common causes of death, affecting both men and women, worldwide. According to the American Cancer Society, there were 102,480 new cases of CRC and 40,340 new cases of rectal cancer in the US alone in 2014 (Cancer Facts and Figures, 2014).

In Malaysia, CRC is the most common cancer in males and the third most common in females after breast and lung cancer (National Cancer Registry, 2012) with majority of patients are above 50 years of age (National Cancer Patient Registry, 2012). It also contributes to the highest number of hospital admissions due to neoplasm-related problems (Yusoff et al., 2012).

CRC treatment consists of either single sense modality or a mixture of surgery, radiation therapy, chemotherapy or immunotherapy. Cancer patients receiving these treatments often experience unpleasant side effects such as body pain, hair and appetite loss, diarrhea, fatigue (a persistent sense of tiredness or exhaustion), constipation (the infrequent or difficult passage of stool), blood disorders and nervous system effects that compromise the quality of their life (Nobili et al., 2009; Mann, 2002).

The most conspicuous among the etiological factors of CRC are physical inactivity and obesity, both of which are strongly and consistently related to a higher risk of CRC (Willer, 2003). Furthermore, research has shown that environmental factor especially that of dietary related is evidently involved in the etiology of CRC. Many studies have found that there is a close relationship between CRC and dietary habit. Therefore, healthy dietary strategies were suggested to bring about a reduction in the risk of this cancer (Kim & Milner, 2007). In other research, it was found that consumption of whole meal based food may be helpful in lowering the risk of CRC (Schatzkin et al., 2007; Larsson et al., 2005; Slattery et al., 2004). Nevertheless, the consumption of refined cereals has been claimed to be still increase the risk of developing CRC (Anderson et al., 2010; Chatenoud et al., 1999) however, Larsson et al. (2005).

In the regions of Asia, it has been observed that there is an association between dietary pattern and CRC (Hixson et al., 1994). The occurrence of CRC in the region, where rice (*Oryza sativa*) is the staple diet, was significantly lower than in Western countries (Verschoyle et al., 2007; Hudson et al., 2000). Thus, it is believed that consumption of rice help to prevent CRC. In recent years, there has been an increase of attention in germinated seeds diet such as rice (Saman et al., 2008), barley (Rimsten et al., 2003) and wheat (Yang et al., 2010). During germination, chemical composition and active compounds level significantly will increase, resulting in the increase of simple sugars, peptides and amino acids levels in the germinated rice. Other than modifying the level of nutrients, the biochemical activities that take place during the germination process also release bioactive components, some of which contain more antioxidants such as ascorbic acid, tocopherols, tocotrienols and phenolic compounds, therefore resulting in an increase of antioxidant activity as well (Fernandez-Orozco et al., 2008; Frias et al., 2005).

Both *in vivo* and *in vitro* experiments provide convincing evidence for the positive prevention or treatment effect of rice-germ (Kawabata et al., 1999), brown rice, rice bran, polished rice (Li et al., 2011), phytic acid extracted from rice bran (Norazalina et al., 2010) and germinated brown rice (GBR) (Latifah et al., 2010) on colon carcinogenesis.

GBR and germinated rough rice (GRR) have been viewed as enormously interesting germinated cereal products that had grown in popularity and received much attention particularly in Asia. Significant changes in the level of γ-aminobutyric acid, glycine, lysine and leucine levels, which were shown to have anticancer effect, were observed in GRR and germinated rice extract powder (Moongngaerm & Saetung ., 2010).

Aberrant crypt foci (ACF) have been identified as biomarkers of colonic carcinogenesis and putative precursors of CRC (Bird & Good, 2000). Several studies have been carried out using animal models as well as human pathologic specimens to investigate the role of ACF in colonic carcinogenesis. ACF as biomarkers in azoxymethane-treated rodents have been established (Derdák et al., 2006; Ochiai et al., 2005; Corpet & Pierre, 2003; Hixson et al., 1994).

β-catenin is a cadherin-binding protein, which has been acknowledged as transcriptional activator when it binds with the T cell factor (Tcf) family of DNA binding protein. Activation of the β-catenin-Tcf pathway results in the accumulation of β-catenin in the cytosol and nucleus. Mutation in the *β-catenin* gene is associated with majority of human and rat colon cancer (Takahashi et al., 2000; Yamada et al., 2000).

1.2. Problem Statement

The staple components of human diet have received less attention as sources of cancer chemopreventive substances. This notion is exemplified by rice, *Oryza sativa*, the staple food of over half the world's population. Rice possesses special dietary importance in Asia, where the incidence of breast and colon cancer is markedly below that in the Western world (Hudson et al., 2000).

In addition, the low cost of rice production and the accessibility of rice bran make it an appealing candidate for global dietary chemoprevention. Therefore, the establishment of dietary rice bran as a practical food-derived chemopreventive agent has the potential to have a significant impact on cancer prevention for the global population (Henderson et al., 2012.a).

As has been initially mentioned, incidence of CRC, in particular, has increased significantly worldwide. Chemotherapy as a CRC treatment leads to serious problems in patients, causing neuropathy, low blood counts, loss of appetite, nausea, vomiting, hair loss, diarrhea and anemia. Therefore, chemoprevention modality using natural resources needs to be developed mainly due to the ineffectiveness of the current therapeutic modalities especially those related to the side effects.

Whole grain food has been proposed as an imperative step to reduce the risk of CRC, due to the abundance of indigestible fiber. Rice bran, which is the outer membrane of GRR, has been proven to be a rich source of health-beneficial compounds to prevent cancer, hyperlipidaemia, fatty liver, hypercalciuria, kidney stones and heart disease (Jariwalla, 2001). However, no studies have examined the effect of GRR crude extract on CRC, although most studies reported that individual components of rice may have a protective effect.

1.3. Objective

The general objective of this research was to determine the chemopreventive properties of germinated rough rice (GRR) crude extract in *Sprague dawley* rats induced with azoxymethane (AOM).

The specific objectives of this study were:

- To determine the antioxidant properties of GRR ethanolic crude extract (ECE)
- To evaluate the chemotherapeutic effects of GRR-ECE on the colon cancer cell line
- To determine the acute and sub-acute toxicity of GRR-ECE in Sprague Dawely rats
- To evaluate the chemopreventive effect of GRR-ECE in inhibiting the formation of ACF and the expression of β-catenin in the colon of *Sprague dawley* rats

1.4 Null hypothesis

- GRR crude extract will exhibit antioxidant and cytotoxic properties
- GRR crude extract will not toxic to the animals
- GRR crude extract will inhibit the formation of ACF and the expression of β-catenin in the colon of *Sprague dawley* rats

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