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EFFECTS OF COCOA POLYPHENOL RICH DIET IN PREVENTION OF COLITIS-ASSOCIATED COLON CANCER

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By

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the Degree of Master of Science

EFFECTS OF COCOA POLYPHENOL RICH DIET IN PREVENTION OF COLITIS-ASSOCIATED COLON CANCER

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Chairman : Associate Professor Norhaizan Mohd Esa, PhD
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Colorectal cancer (CRC) is the second highest mortality and the third most diagnosed in both men and women. Colitis-associated cancer is a subtype of CRC that is associated with inflammatory bowel disease. Cocoa has a rich source of polyphenols and inhibits the cancer cell proliferation and decrease the risk of different type of cancers, cardiovascular disease and diabetes. This study was aimed to determine the anti-cancer effects of cocoa rich diets on dextran sulfate sodium (DSS) and azoxymethane (AOM)-induced colitis associated cancer in BALB/c mice. Natural Forastero cocoa powder was used for this study and diets were prepared from an AIN-93G formulation. The 5% and 10% cocoa diets are produced by adding 50 g/kg and 100 g/kg cocoa to AIN-93G at the expense of starch, cellulose and casein. The total polyphenol content of the cocoa powder was determined. Cocoa rich diet was modified to supplement 1 g and 2 g of polyphenols per kg of diet respectively. Total number of 50 female BALB/c mice (*Mus musculus*) weighing 25-30 g were divided into 5 different groups and each group consist of 10 mice. Data are presented as mean (n = 10 mice per group). The mice in groups 2, 3 and 4 were initiated by a single intraperitoneal (*i.p.*) injection of AOM (10 mg/kg body weight). Starting 1 week after the injection, 2% DSS in drinking water was administrated to mice of group 2, 3 and 4 for 7 days and 14 days and followed by normal drinking water for the recovery period. Totally 3 cycles of 2% DSS were treated. Group 1 (control) and Group 2 received AIN-93G diet, group 3 and 4 were treated with cocoa diet of 5% and 10%, respectively. Group 5 treated with 10% of cocoa diet alone to assess the toxicity of cocoa. On day 62 of the experiment, all mice were sacrificed and the entire colon and rectum were processed for histopathology examination and further evaluation.

Pro-inflammatory mediators and cytokines were measured by enzyme-linked immunohistochemical assay; real-time–PCR and western blot analysis. The tissue samples were examined for ultrastructural changes in experimental mice by Transmission Electron Microscopy. Change in colon length in mouse model of colitis-associated cancer was significantly improved in animals receiving cocoa-

enriched diet. Spleen weight was significantly decreased in animals treated with cocoa diet ($P<0.05$). Colon tumor number was increased upon DSS/AOM administration and fed with cocoa-enriched diet showed reduced number of tumors/mice. Cocoa diet modulates histological alterations caused by AOM/DSS. Control and cocoa diet alone treated group of mice shown normal architecture of microvilli. AOM/DSS treated mice showing the invasive gland in the submucosa layer of a large size adenoma. Treatment 5% and 10% of cocoa-enriched diet showed small polyps in the muscular layer.

In AOM/DSS group of animal, increased expressions of iNOS and COX-2 was observed by immunohistochemistry. However, treatment with 5% and 10% cocoa diet showed decreased expression of iNOS and COX-2, whereas control and cocoa alone groups showed fewer positive expressions. Deregulation of the JAK/STAT3 signaling pathway has also been implicated in colorectal tumorigenesis resulting in accumulation of cytokines and growth factors, Janus kinases.. Therefore, the potential of polyphenols in cocoa powder in targeting key components of the STAT3 signaling pathway along with iNOS and COX2 as a rational for cancer drug discovery was demonstrated. Colon tumors were further analyzed the mRNA levels of pro-inflammatory cytokines such as IL-6, TNF-α, IL-1β, IL-17 by RT-PCR and BcI-xL, Bax, Caspase 3 and Caspase 8 at protein levels by Western Blot analysis. It was shown that administration of cocoa significantly down-regulated inflammatory factors in colon cancer animal as compared with control (no cocoa treatment) ($p<0.05$).

In summary, the ability of cocoa to prevent the development of the colon carcinogenesis was demonstrated by lower tumor incidence, number and size of DSS/AOM-treated mice. In this present study, shown that after cocoa-enriched diet, the colitis presented a statistical improvement and tumors burden decreased significantly, this was accompanied by lower activity of p-STAT3^{Y705}, decreased expressions of COX-2 and iNOS, lower expression of cytokines in the colons of CAC mice. We suggest that the chemopreventive effect of cocoa enriched diet on colitis-associated carcinogenesis could be mediated mainly through the IL-6/STAT3 pathway. Taken together, the present data provide evidence that cocoa polyphenols would offer a natural approach to improve individual health status including the prevention of colonic inflammation with no toxicity.

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

KESAN DIET KOKO KAYA POLIFENOL DALAM MENGHALANG KANSER KOLON BERKAIT KOLITIS

Oleh

ZEINAB SAADATDOUST

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Kanser kolorektal (CRC) adalah mortaliti yang kedua tertinggi dan ketiga paling banyak didiagnosis di kalangan lelaki dan juga wanita. Kanser berkait kolitis adalah subjenis CRC yang berkaitan dengan penyakit radang usus. Ia sukar dirawati dan mempunyai mortaliti yang tinggi. Di tahun kebelakangan ini, penggunaan bahan semulajadi untuk rawatan pelbagai penyakit keradangan di kalangan pesakit yang mengalami kesan sampingan akibat rawatan dadah, semakin meningkat. Polifenol tumbuhan yang merupakan komponen utama di dalam diet manusia mempunyai pelbagai kesan kebaikan. Polifenol telah difikirkan mempunyai ciri-ciri anti-oksidatif dan anti-keradangan tersendiri. Koko adalah sumber yang kaya dengan polifenol dan menghalang proliferasi sel kanser dan mengurangkan risiko pelbagai jenis kanser, penyakit kardiovaskular dan diabetes. Kajian ini bertujuan untuk menentukan kesan anti-kanser diet koko kaya polifenol terhadap mencit BALB/c yang diaruh kanser kolon berkait kolitis oleh dekstran sulfat sodium (DSS) dan azoksimetana (AOM).

Serbuk koko 'Natural Forastero' digunakan untuk kajian ini dan diet disediakan dengan formulasi AIN-93G. Diet koko 5% dan 10% dihasilkan dengan menambahkan 50 g/kg dan 100 g/kg koko ke dalam diet AIN-93G dengan mengambilkira kanji, selulosa dan kasein. Kandungan jumlah polifenol serbuk koko ditentukan dengan kaedah spektrofotometrik *Folin – Ciocalteu* menggunakan epicatechin sebagai piawaian. Diet koko kaya polifenol diubahsuai untuk memberikan 1 g dan 2 g polifenol per kg diet, masing-masing sebagai suplemen. Sejumlah 50 ekor mencit BALB/c (*Mus musculaus*) betina dengan berat 25-30 g dibahagikan kepada 5 kumpulan berlainan dan setiapnya mengandungi 10 ekor mencit. Data dibentangkan sebagai min (n = 10 ekor mencit setiap kumpulan). Mencit di kumpulan 2, 3 dan 4 diberi satu suntikan awal *intraperitoneal* (*i.p.*) AOM (10 mg/kg berat badan). Bermula satu minggu selepas suntikan, 2% DSS dalam air minuman diberi kepada mencit di kumpulan 2, 3 dan 4 selama 7 hari dan 14 hari, diikuti dengan air minuman biasa untuk tempoh pemulihan. Sejumlah 3 kitaran 2% DSS diberi. Kumpulan 1 (kawalan) dan kumpulan 2 menerima diet AIN-93G, kumpulan 3 dan 4 dirawat dengan diet koko 5% dan 10%, masing-masing.

Kumpulan 5 diberi diet koko 10% sahaja, untuk menilai ketoksikan koko. Pada hari ke 62 ujikaji, mencit dibunuh dan keseluruhan kolon, rektum diproses untuk penilaian histopatologi dan penilaian lain seterusnya.

Pengantara pro-keradangan dan sitokin diukur dengan asai *enzyme-linked immunohistochemical*, *real-time–PCR* dan analisis *Western Blot*. Sampel tisu dari ujikaji mencit ditentukan perubahan ultrastruktur menggunakan *Transmission Electron Microscopy*. Perubahan panjang kolon di dalam model mencit kanser berkait kolitis diperbaiki dengan signifikan pada kumpulan yang menerima diet kaya koko. Berat limpa berkurangan dengan signifikan pada mencit yang dirawat dengan diet koko ($p<0.05$). Bilangan tumor kolon meningkat dengan pemberian DSS/AOM dan dengan memberi diet koko kaya polifenol menunjukkan penurunan bilangan tumor/mencit. Diet koko, modulasi perubahan histologi yang disebabkan oleh AOM/DSS. Mencit dalam kumpulan kawalan (normal) dan yang diberi diet koko sahaja menunjukkan struktur microvili yang normal. Mencit yang diberi AOM/DSS menunjukkan kelenjar yang invasif di dalam lapisan submukosa adenoma bersaiz besar. Rawatan dengan diet kaya koko, 5% dan 10% menunjukkan polip yang kecil di dalam lapisan berotot.

Analisis immunohistokimia *cyclooxygenase-2* (COX-2) dan *inducible nitric oxide synthase* (iNOS) dilakukan ke atas haiwan kumpulan kawalan dan ujikaji. Bagi haiwan dalam kumpulan yang diberi AOM/DSS, didapati ekspresi iNOS dan COX-2 meningkat. Walaubagaimanapun, rawatan dengan diet koko 5% dan 10% menunjukkan ekspresi iNOS dan COX-2 adalah lebih rendah. Manakala kumpulan kawalan (normal) dan menerima koko sahaja menunjukkan ekspresi yang sedikit. Penyah-regulasi tapakjalan isyarat JAK/STAT3 juga mempunyai implikasi didalam tumorigenesis kolorektal yang mengakibatkan pengumpulan sitokin dan faktor tumbesaran, Janus kinases. Tambahan pula, ekspresi enzim berkait dengan keradangan seperti iNOS dan COX-2, ditunjukkan memainkan peranan yang penting di dalam perkembangan tumor kolon. Oleh itu, potensi polifenol di dalam serbuk koko dalam mensasarkan komponen utama tapak jalan isyarat STAT3 bersama iNOS dan COX2 sebagai penemuan ubat kanser yang rasional, telah ditunjukkan. Tumor kolon dianalisis lebih lanjut diperingkat mRNA sitokin pro-keradangan seperti IL-6, TNF-α, IL-1β, IL-17 melalui *RT-PCR* and protein Bcl-xL, Bax, Caspase 3 dan Caspase 8 dianalisis dengan *Western Blot*. Keputusan menunjukkan bahawa pemberian koko *down-regulate* faktor keradangan di dalam haiwan yang mempunyai kanser kolon dengan signifikan ($p<0.05$) berbanding dengan kumpulan kawalan yang tidak menerima rawatan koko. Ini mencadangkan bahawa kesan chemopreventif diet kaya koko terhadap karsinogensis berkait kolitis mungkin diperantarai oleh tapak jalan IL-6/STAT3.

Kesimpulannya, kebolehan koko untuk mencegah perkembangan karsinogenesis kolon pada mencit yang diberi dengan DSS/AOM ditunjukkan melalui pelemahan keradangan kolorektal dan pengurangan kejadian, bilangan dan saiz tumor. Kajian yang dijalankan ini juga menunjukkan bahawa selepas diet kaya koko diberikan, keadaan kolitis adalah lebih baik dengan sifnifikan dan ini disertai dengan aktivit p-STAT3^{Y705} yang lebih rendah, pengurangan ekspresi COX-2 dan iNOS, merendahkan ekspresi sitokin pro-radangan pada kolon mencit CAC. Kami mencadangkan kesan chemopreventif diet kaya koko keatas karsinogenesis berkait kolitis, mungkin diperantarai oleh tapak jalan IL-6/STAT3. Keseluruhannya, data

yang diperolehi ini membuktikan bahawa polifernol koko menawarkan pendekatan semulajadi untuk memperbaiki status kesihatan individu termasuklah menghalang keradangan kolon tanpa memberikan kesan toksik dan juga mempunyai potensi sebagai diet yang menghalang keradangan kolon dan perkembangan kanser berkaitan.

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Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) were adhered to.

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LIST OF ABBREVIATIONS

Abbreviation	Full Term
%	Percentage
ºC	Degree Celsius
ΔΔCt	Delta-delta threshold cycle
ΔΨm	Mitochondrial membrane potential
µg	Microgram
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
AOM	Azoxymethane
Bcl-2	B-cell lymphomal/leukemia
BSA	Bovine serum albumin
CAC	Colitis associated colon cancer
COX-2	Cyclooxygenase-2
CRC	Colorectal cancer
Chol	Cholesterol
Creat	Creatinine
DNA	Deoxyribionucleic Acid
DSS	Dextran sulfate sodium
dH2O	Distilled Water
FBS	Foetal Bovine Serum
H&E	Hematoxylin and Eosin
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
IL-17	Interleukin 17
iNOS	Inducible isoform of nitric oxid synthase
kDa	Kilo dalton
kb	Kilo base pair
mg	Miligram
mg/dl	Miligram/decilitre
mg/kg	Miligram/kilogram
mRNA	Messenger Ribonucleic Acid
NaCl	Sodium chloride
NAOH	Sodium Hydroxide
NF-κB	Nuclear Factor κB
NOAEL	No-observable adverse effect levels
PBS	Phosphate Bovine Serum,
PCR	Polymerase chain Reaction

SDS-PAGE	Sudium–dodecyl Sulfate Polyacrylamide Gel
PG	Prostaglandin
PGE2	Prostaglandin endoperoxide synthase2
p-STAT3	Phosphorylated STAT3
PVDF	Polyvinylidene difluoride
qPCR	Quantitative Real –time polymerase chain reaction
qRT-PCR	Quantitative real-time reverse transcriptase polymerase Chain Reaction
RNA	Ribonucleic Acid
SD	Standard Deviation
TBA	Thiobarbituric Acid
TBST	Tris Buffer saline and twin 20
TEM	Transmission Electron Microscopy
TEMED	Tetramethylethylenediamine
TNF	Tumor Necrosis factor
TNF-α	Tumor Necrosis factor -alpha
WHO	World Health Organization

CHAPTER ONE

INTRODUCTION

1.1 Research Background

Colon cancer is the third most common cancer and the second leading cause of cancer death among both men and women in the United States (Zeller et al., 2008). It is responsible for around 700,000 deaths per year, worldwide (Lim & Halimah, 2003). Colon cancer, also known as colorectal cancer (CRC), is currently one of the most common cancers in Malaysia. According to Ministry of health, colorectal cancer is the second disease causing death after heart disease in Malaysia, and contributes 9.23% of total death cases (Balraj & Ruhana, 2007). Epidemiological and experimental studies have shown that patients with inflammatory bowel disease (IBD) are at a greater risk of developing CRC than the general population, and colitis-associated cancer (CAC) is the major cause of death in inflammatory IBD patients (Eaden et al., 2001).

CAC is the type of colon cancer which is preceded by clinically detectable IBD, such as Crohn's disease (Caprioli et al., 2008) or ulcerative colitis (UC) (Feagins et al., 2009; Rubin et al., 2012; Saleh & Trinchieri, 2010). UC increases cumulative risk of CAC by up to 18–20 %, while CD by up to 8 % after 30 years of active disease (Canavan et al., 2006; Eaden et al., 2001; Rubin et al., 2012). One of the important underlying etiologies of carcinogenesis in the colon is inflammation (Fran Balkwill & Mantovani, 2001). The microenvironment of chronic intestinal inflammation facilitates cell proliferation, migration and angiogenesis, thereby promotes tumor development, growth and progression (Boland et al., 2005). In particular, the incidence of colitis-associated cancer (CAC) approaches to ~40% in patients with colitis (Munkholm, 2003).

Polyphenols are phytochemicals derived from phenylalanine and contain an aromatic ring with a reactive hydroxyl group (Signorelli & Ghidoni, 2005). Polyphenols have gained much interest recently due to its antioxidant capacity and possible benefits to human health such as anti-carcinogenic, anti-atherogenic, anti-ulcer, anti-thrombotic, anti-inflammatory, immune modulating, anti-microbial, vasodilatory and analgesic effects (Hii et al., 2009). Furthermore, polyphenols, which constitute the active substances found in many medicinal plants, modulate the activity of a wide range of enzymes and cell receptors (Keen, 2001). Indeed, polyphenols have been reported to interfere with cancer initiation, promotion, and progression, acting as a strong chemopreventive agent (Araújo et al., 2011).

Cocoa (*Theobroma cacao L.*) consist rich source of polyphenols and cocoa beans has 6-8% total polyphenol (Ali et al., 2014). A reported by Vinson et al., (1999) had found phenolic compounds in cocoa are higher than 23 types of vegetables and several types of fruits. In this study the effect of combination of polyphenols on

colitis associated cancer was investigated since mixture of polyphenols may target overlapping and complementary phases of the carcinogenic process (de Kok et al., 2008) thus increasing the efficacy and potency of the chemopreventive effect.

1.2 Problem Statement

Colorectal cancer is a major cause of morbidity and mortality throughout the world. It accounts for over 9% of all cancer incidences. It is the third most common cancer worldwide and the fourth most common cause of death. It affects men and women almost equally, with just over 1 million new cases recorded in 2002 (Botteri et al., 2008). Countries with the highest incidence rates include Australia, New Zealand, Canada, the United States, and parts of Europe. The countries with the lowest risk include China, India, and parts of Africa and South America (Society, 2009). Worldwide, colorectal cancer represents 9.4% of all cancer incident in men and 10.1% in women. In Malaysia, colorectal cancer is the second most common cancer in males and females. A total of 2,246 cases were diagnosed in 2007 and reported to NCR, represent 12.3 % of all cases reported (Balraj & Ruhana, 2007).

Current treatment of colorectal cancer generally employs surgical resection combined with radiation of chemotherapy with synergistic cytotoxic drugs. Anti-inflammatory and also chemotherapeutic drugs have important effect for inflammation and cancer but they have a number of side-effects that can limit their efficacy (Wang et al., 2011). Moreover, the anti-inflammatory drugs in 2005 was cost around $ 31.1 billion and this number is increasable to reach $ 47.8 in 2010 (Kearney et al., 2006). Hence, finding a suitable treatment for oxidative stress- and inflammation- related diseases with minimal or no side effects is still warranted.

Polyphenol has attracted many attentions because of its significant role in human health. Accumulating evidence showing that these compounds possess a high number of protective biologic properties such as antioxidant, anti-carcinogenic, anti-inflammatory (Scalbert et al., 2005), anti-allergic, anti-diarrheal, antiulcer, antibiotic (Howells et al., 2007) antilipidemic, vasorelaxing and antithrombotic properties (Scalbert et al., 2005). Because of these effects, polyphenols may confer protection against pathologies with very high incidence and mortality in occidental countries: cardiovascular and neurodegenerative diseases and cancer (Jemal et al., 2008). In relation to cancer, numerous case-control (Johnson, 2007) and animal and cell culture studies have corroborated a protector role of polyphenols and of foods and drinks that contain them (especially fruits and vegetables) in distinct cancer types (e.g., breast, lung, colon, stomach, esophagus, larynx, and oral cavity) (Jemal et al., 2008). Hence, examining the potent effect of Malaysian cocoa extract will enlarge using of cocoa as anti-oxidative or as anti-inflammatory agent.

1.3 Significance of Study

In the continuing effort to reduce the public health burden of cancer there is a constant search for more effective cancer treatment and increased interest in the

concept of prevention, as a promising approach to the control of cancer. A major target in current research is to identify cancer reduction strategies based on dietary modification including looking at natural sources that may have anticancer properties. Besides that, selective destruction of tumor cells without damaging normal cells is an important goal for cancer treatment (Keen, 2001).

Since the current treatment such as radiotherapy, chemotherapy and drugs possess unwanted side effects, the move to use potential bioactive compounds as the alternative should be made. Besides, in order to maintain full health as well as a broad range of nutraceutical compounds that has been demonstrated to have remarkable therapeutic properties, the opportunities to develop an alternative compound from the local source is the main reason why this study should be done and carried out successfully (Keen, 2001).

Cocoa is a rich source of bioactive compounds with potential chemopreventive ability but there are not enough studies that support its effectiveness in animal models of colon carcinogenesis. Rodríguez-Ramiro et al., (2011) have examined effect of cocoa rich diet on early levels of bowl tumorigenesis for the first time *in vivo*, so my research will provide the first in *vivo* evidence that cocoa-rich diet may inhibit the advance stage of colon carcinogenesis.

As compared with other flavonoid- containing foods, cocoa products exhibit a high concentration of procyanidins that are poorly absorbed in the intestine and consequently its beneficial effects would be more focused on the gastrointestinal tract where they may have an important local function neutralizing oxidants. Despite these evidences, the efficacy of cocoa against CRC initiation and development *in vivo* remains largely unexamined. Moreover, the effect of polyphenol from cocoa to inhibit the proliferation of colon cancer cell line (*in vitro*) has also been shown by Carnesecchi et al., (2002). However, we do not know whether it has similar effect in the body due to low bioavailability of polyphenol in our digestion system. So, this study was conducted in *vivo* to confirm this finding and also to know the possible mechanism how polyphenol acts as an anti-cancer agent.

In addition, Malaysia is one of the main cocoa-based product producer in the world and the biggest in Asia. However, Malaysian cocoa-based markets preferred cocoa beans/powder from African origins because cocoa are lacking in flavor. Although lack of flavor quality, a group of research based at University Putra Malaysia (UPM) has reported that Malaysian cocoa beans and its derived products could contribute toward decreasing of chronic diseases risk factors (Amin, Koh, et al., 2004; Othman et al., 2007). Through this study, it could be provide information that cocoa has a good potential in decreasing the risk of colon cancer. So, if the result from this study is able to show that Malaysian cocoa beans are able to prevent and treatment of colon cancer the beans then can be used as an alternative ways to reduce the incidence of colon cancer and hence boost the value of Malaysian cocoa beans. Therefore, in the present study, the chemopreventive activity of cocoa will be evaluated in the mouse model of azoxymethane (AOM)/ dextran sulfate sodium (DSS)-

induced colitis-associated cancer.

1.4 Objectives

1.4.1 General Objective

To investigate the protective effect of cocoa polyphenol rich diet from Malaysian cocoa powder on colitis associated colon cancer *in vivo.*

1.4.2 Specific Objectives

1. To determine the total amount of polyphenol and to evaluate chronic toxicity of Malaysian cocoa powder at dietary levels of 10%.

2. To analyze the effect of cocoa rich diet on incidence of tumor neoplasm, Histological alterations, Inflammatory mediators (iNOS and COX-2), pro-inflammatory cytokines (*TNF-α, IL-1β, IL-6, IL-17*), and $STAT3^5$ signaling pathway during AOM/DSS-induced colon cancer.

3. To quantify the expression of apoptotic proteins (Bax, Bcl-xL, caspase-3 and -8*)* induced by cocoa in colitis associated cancer.

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