UNIVERSITI PUTRA MALAYSIA

CYTOTOXICITY EFFECT OF COCOA (Theobroma cacao L.) POLYPHENOL EXTRACT ON MCF-7 CELLS, AND MODE OF CELL DEATH

HAZIRAH BINTI ABDUL RADZAK

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By

HAZIRAH BINTI ABDUL RADZAK

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

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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.

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May 2014

Chairman : Abdah binti Md Akim, PhD

Faculty : Medicine and Health Sciences

The Incidence of breast cancer in Malaysia is alarming and it increased every year. It is one of the common causes of deaths among cancer patients in women. Despite of several drugs have been formulated for breast cancer treatment, these drugs can cause undesired side effects to the breast cancer patients. Therefore, scientists are searching for potential cancer chemopreventive and chemotherapeutic agents through dietary approaches. Cocoa (*Theobroma cacao* L.) is rich in specific antioxidants such as catechin, epicatechin and procyanidins. Previous research exhibited that cocoa possessed antioxidant and cytotoxic properties. The objective of the present study was to investigate the cytotoxicity effect of cocoa polyphenol extract (CPE) towards MCF-7 cells and its effect on mode of cell death. The phenolic constituents of CPE were evaluated by phytochemical screening, HPLC profiling and total phenolic content (TPC) assay. The antioxidant activity of CPE was determined using DPPH radical scavenging and ferric reducing antioxidant power (FRAP) assay. Cell viability was measured using MTT assay. The morphological alteration was observed using inverted light and fluorescence microscope (acridine orange/ propidium iodide dual staining). The mode of cell death was investigated using annexin V FITC-PI and DNA fragmentation assay. The apoptotic marker of cell death was carried out using p53 and caspase-9 ELISA kits. The phytochemical screening and HPLC profiling exhibited CPE contained phenolic compound particularly saponins, flavonoids and condensed tannins. TPC, DPPH IC_{50} and FRAP value of CPE were 13558.99±420.10 mg GAE/100g dry weight of sample, 14.73±1.47 µg/ml and 2130.33±2.33 µM FE/1 mg dry weight of sample respectively. CPE exhibited highest cytotoxicity towards MCF-7 cells with the lowest IC_{50} value (3 mg/ml) and exhibited significant difference ($p<0.05$) compared to other cancer cell lines. The difference of IC_{50} value was significant ($p<0.05$) between 24 h (4.50±0.50 mg/ml), 48 h (2.85±0.20 mg/ml) and 72 h (1.60±0.10 mg/ml). The morphological alteration of MCF-7 cells upon 48 h CPE treatment showed apoptosis and necrosis characteristics including cell membrane blebbing, cell

shrinkage, nuclear condensation, apoptotic bodies and cell membrane rupture. The cell cycle analysis revealed that CPE was able to cause mild cell cycle arrest at G0/G1 phase and also induced sub-G1 peak, indicating apoptosis. Annexin V-PI assay proved that CPE induced early and late apoptosis in treated MCF-7 cells. The DNA fragmentation assay confirmed that DNA fragmentation had occurred during apoptosis in treated MCF-7 cells. The expression level of p53 and caspase-9 were increased upon CPE treatment in MCF-7 cells indicating that apoptosis was executed via mitochondria pathway. In conclusion, these findings suggested that CPE demonstrated cytotoxicity effect towards MCF-7 cells through inhibition of cell proliferation by arresting G0/G1 phase and apoptosis execution via p53 and caspase-9 activation. Based on the current findings, further research is required to develop CPE as chemopreventive agents for breast cancer.

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

KESAN SITOTOKSISITI EKSTRAK (*Theobroma cacao* L.) POLIFENOL KOKO KE ATAS SEL MCF-7, DAN MOD KEMATIAN SEL

Oleh

HAZIRAH BINTI ABDUL RADZAK

Mei 2014

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Insiden kanser payudara di Malaysia semakin membimbangkan dan meningkat setiap tahun. Ia merupakan satu daripada penyebab umum kematian di kalangan pesakit kanser wanita. Walaupun pelbagai ubat telah diformulasi untuk rawatan kanser payudara, ubat-ubat ini akan menyebabkan kesan sampingan yang tidak diingini kepada para pesakit kanser payudara. Oleh itu, para saintis sedang mencari agen yang berpotensi sebagai pencegah kanser dan perawatan kanser melalui pendekatan pemakanan. Koko (*Theobroma cacao* L.) kaya dengan kandungan antioksidan yang spesifik seperti katekin, epikatekin dan prosianidin. Penyelidikan sebelum ini menunjukkan koko mempunyai ciri-ciri antioksidan dan sitotoksik. Objektif kajian ini untuk menyiasat kesan sitotoksisiti ekstrak polifenol koko (CPE) terhadap sel MCF-7 dan kesannya ke atas mod kematian sel. Kandungan fenolik CPE telah diuji dengan saringan fitokimia, pemprofilan HPLC dan asai jumlah kandungan fenolik (TPC). Aktiviti antioksidan yang terdapat pada CPE ditentukan menggunakan asai penyahbebas radikal DPPH dan kuasa penurunan ferik (FRAP). Viabiliti sel diukur menggunakan asai MTT. Perubahan morfologi diperhatikan menggunakan mikroskop inversi cahaya dan fluoressen (dwi perwarnaan akridina oren/propidium iodida). Mod kematian sel disiasat menggunakan asai annexin V FITC-PI dan fragmentasi DNA. Mekanisme kematian sel dijalankan menggunakan kit ELISA p53 dan caspase-9. Saringan fitokimia dan pemprofilan HPLC menunjukkan CPE mengandungi bahan fenolik terutamanya saponins, flavonoid dan tannin tersejat. TPC, nilai IC_{50} untuk asai DPPH dan FRAP masing-masing ialah 13558.99±420.10 mg GAE/100g berat kering sampel, 14.73±1.47 µg/ml dan 2130.33±2.33 µM FE/1 mg berat kering sampel. CPE menunjukkan sitotoksik yang tertinggi terhadap sel MCF-7 dengan nilai IC_{50} yang terendah (3 mg/ml) dan menunjukkan perbezaan yang signifikan ($p<0.05$) berbanding dengan kanser sel yang lain. Perbezaan nilai IC_{50} adalah signifikan ($p<0.05$) diantara 24 jam (4.50±0.50 mg/ml), 48 jam (2.85±0.20 mg/ml) dan 72 jam (1.60±0.10 mg/ml). Perubahan morfologi sel MCF-7 sebaik sahaja rawatan CPE menunjukkan ciri-ciri

apoptosis dan nekrosis termasuklah pembengkakkan membran sel, pengecutan sel, kondensasi nukleus dan sel membran ruptur. Analisis kitar sel mendedahkan CPE boleh menyebabkan sedikit penahanan kitar sel pada fasa G0/G1 dan juga merangsang fasa sub-G1 yang menandakan apoptosis. Asai annexin V-PI membuktikan bahawa CPE telah merangsang apoptosis peringkat awal dan apoptosis peringkat lewat pada sel MCF-7 yang dirawat. Asai fragmentasi DNA mengesahkan fragmentasi DNA telah berlaku semasa apoptosis pada sel MCF-7 yang dirawat. Peringkat ekspresi p53 dan kaspase-9 meningkat sebaik sahaja rawatan CPE pada sel MCF-7 menandakan bahawa apoptosis dilaksanakan melalui aliran mitokondria. Secara kesimpulannya, penemuan-penemuan diatas ini membuktikan CPE menunjukkan kesan sitotoksisiti terhadap sel MCF-7 melalui perencatan pembahagian sel dengan penahanan pada fasa G0/G1 dan pelaksanaan apoptosis melalui pengaktifan p53 dan kaspase-9. Berdasarkan pada penemuan-penemuan ini, penyelidikan lanjutan diperlukan untuk menjadikan CPE sebagai agen pencegah kanser untuk kanser payudara.

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I certify that a Thesis Examination Committee has met on 5 May 2014 to conduct the final examination of Hazirah binti Abdul Radzak on her thesis entitled "Cytotoxicity Effect of Cocoa (*Theobroma cacao* L.) Polyphenol Extract on MCF-7 Cells, and Mode of Cell Death" 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.

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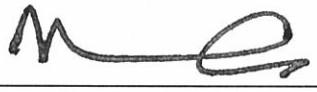

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfiillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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Committee : Dr Huzwah Binti Khaza'ai

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AIP1	Actin-interacting protein 1
Akt	Protein kinase
ANOVA	Analysis of variance
AO	Acridine orange
Apaf-1	Apoptotic protease activation factor-1
ARF	Alternative reading frame
ASPP1/2	Apoptosis-stimulating protein of p53 1 and 2
ATCC	American Type Culture Collection
ATM	Ataxiatelangiectasia mutated
ATP	Adenosine triphosphate
Bak	Bcl-2 antagonist killer
Bax	Bcl-2-associated X protein
Bcl-2	B-cell-lymphoma 2
BRCA 1	Breast cancer type 1 susceptibility protein
BRCA 2	Breast cancer type 2 susceptibility protein
BSA	Bovine serum albumin
CADP	Collagen-ADP
cAMP	cyclic adenosine monophosphate
CARD	Caspase recruitment domain
Caspase	Cysteine-aspartic protease
CAT	Catalase
CDC	Cell division cycle
Cdc2	Cell division cycle 2
Cdc25	Cell division cycle 25

CDK	Cyclin-dependent kinase
CDKI	Cyclin-dependent kinase inhibitor
CEPI	Collagen epinephrine
CF	Cocoa rich fiber
chk2	Checkpoint kinase 2
cIAP	cellular Inhibitor of Apoptosis
CO_2	Carbon dioxide
CPE	Cocoa Polyphenol Extract
CPF	Cocoa procyanidin fraction
Cu^+	Copper(I) ion
DAPI	4',6-diamidino-2-phenylindole
DBP	Diastolic blood pressure
DD	Death domain
DED	Death effector domain
DISC	Death-inducing signaling complex
DMEM	Dulbecco's Minimum Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
dATP	Deoxyadenosine triphosphate
DU145	Androgen nonresponsive prostate cancer cell line
EDTA	Ethylenediamine Tetraacetic Acid
ELISA	Enzyme-Linked Immunoabsorbent Assay
ER	Estrogen receptor

ERK/Cx43	Extracellular signal- regulated kinase/connexin 43
FADD	Fas-Associated protein with Death Domain
FBS	Fetal Bovine Serum
FE	Ferric equivalents
Fas/CD95	Cell death signaling receptor
Fe^{2+}	Ferric (II) ion
Fe^{2+}-TPTZ	Ferrous-tripyridyltriazine
Fe^{3+}-TPTZ	Ferric tripyridyltriazine
FFAs	Free fatty acids
FITC	Fluorescein isothiocyanate
FMD	Flow mediated dilation
FRAP	Ferric reducing antioxidant power
FRIM	Forest Research Institute of Malaysia
GAE	Gallic acid equivalent
GJIC	Gap junctional intracellular communication
GPx	Glutathione peroxidase
GPIIb/IIa-act	Glycoprotein Iib/IIIa receptor
GRx	Glutathione reductase
GSH	Glutathione
g	Gram
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HDL	High-density lipoprotein
HeLa	Helacyton gartleri
HepG2	Hepatocellular carcinoma

HIPK2	Homedomain-interacting protein kinase 2
HNE	4-hydroxynenal
HPLC	High Performance Liquid Chromatography
HRP	Horseradish peroxide
HRT	Hormone Replacement Therapy
HT-29	Human colon adenocarcinoma grade II cell line
h	Hours
IC_{50}	Inhibition concentration 50% of cell viability
IgG	Immunoglobulin G
IU	International Unit
i.d	Internal diameter
JMY	Junction mediating and regulatory protein
JNK	c-Jun N-terminal protein kinase
kb	Kilobase
kDa	Kilodaltons
LDL	Low-density lipoprotein
MCF-7	Michigan Cancer Foundation -7
MCP-1	Monocyte chemoattractant protein-1
MDA	Malondialdehyde
MDA-MB-231	Mammary gland adenocarcinoma
MDA-MB-436	Mammary gland adenocarcinoma
MDA-MB-468	Mammary gland adenocarcinoma
MDM2	Mouse doubling minute 2 homolog
MKK4-JNK	Mitogen activated protein kinase kinase 4-c-Jun N-terminal protein kinase

MOMP	Mitochondrial outer membrane permeabilization
MPTPs	Mitochondrion permeability transition pores
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide
mg	Milligram
min	Minutes
ml	Mililiter
mM	Milimolar
mm	Milimeter
NCR	National Cancer Registry
NF-κB	Nuclear factor-κB
NO	Nitric oxide
NOX-1	NADPH oxidase-1
ng	Nanogram
nm	Nanometer
OH·	Hydroxyl radical
PA	α-amylase
PAH	Polycyclic aromatic hydrocarbon
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffer saline
PC12	Pheocromocytoma
PI	Propidium iodide
PIG3	p53-inducible gene 3

PKA	Protein Kinase A
PL	Pancreatic lipase
PLA_2	Phospholipase A_2
PolII	Polymerase II
pRb	Retinoblastoma protein
PS	Phosphatidylserine
Puma	p53-upregulated modulator of apoptosis
p38-MAPK-	p38 mitogen-activated-protein-kinase-
p53	Tumor suppressor gene
p63	Tumor protein 63
p73	Tumor protein 73
p107	Retinoblastoma-like protein 1
p130	Retinoblastoma-like protein 2
p53AIP1	p53-regulated apoptosis-inducing protein 1
P53DINP1	p53-dependent damage-inducible nuclear protein 1
RIP	Receptor-interacting protein
RLE	Rat liver epithelial
RNA	Ribonucleic acid
ROS	Reactive species oxygen
rpm	Revolutions per minute
RWEP-1	Normal prostate cancer cell line
SBP	Systolic blood pressure
SEM	Standard error mean
Ser46	Serine 46
SHR	Spotaneously hypertensive rats

SKRB-3	Retinoid receptor- positive breast cancer cell line
SOD	Superoxide dismutase
SPSS	Statistical package for social science
s	Seconds
TAB	TAK-1 binding protein
TAK	Transforming growth factor-β activated kinase
TBE	Tris/Borate/Ethylenediaminetetraacetic acid
t-BOOH	*tert*-butylhydroperoxide
TGs	Triacylglycerols
TNF	Tumor necrosis factor
TNF- α	Tumor necrosis factor-α
TNFR	TNF receptor
TPC	Total phenolic content
TPTZ	2,4,6-tri(2-pyridyl)-1,3,5-triazine
TRADD	TNFR-associated death domain
TRAF	TNFR-associated factor
TUNEL	TdT-mediated dUTP nick-end-labeling
t	Time
U	Units
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
UV-VIS	Ultraviolet-visible
V	Volt

vWF	von Willebrand Factor
WHO	World Health Organization
WISP-1	WNT1-inducible-signaling protein 1
WRL-68	Hepatic human cell line
WST	Water soluble Tetrazolium salts
XIAP	X-linked inhibitor of apoptosis
XTT	Sodium 2,3,-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium inner salt
22Rv1	Androgen responsive prostate cancer cell line
cm^2	Square centimetre
µg	Microgram
µl	Microliter
µm	Micrometer
µM	Micromolar
±	Approximately or about
ºC	Celcius
%	Percentage
v/v	Volume per volume
w/v	Weight per volume

CHAPTER 1

INTRODUCTION

1.1 Background of study

Cancer is an abnormal mass of tissue as a result of uncontrollable proliferation which continued in excessive manner once the stimuli that induce the alteration terminated (Stricker and Kumar, 2007). Development of cancer frequently occurs in any region of organ or tissues such as skin, breast, lung, colon, nerve tissue and bones. According to the GLOBOCAN in the year of 2008, it was estimated approximately 7.6 million of people died due to cancer and about 12.7 million of people were diagnosed with cancer. From this statistic, about 56% of the incidence and 64% of the mortality arise from the economically developed countries (Jemal et al., 2011).

In Malaysia, cancer is one of the major public health problems. A sum of 18 219 new cancer cases was discovered in 2007 as recorded in the National Cancer Registry (NCR). The cancer incidence rate for males and females were 8123 (44.6%) and 10 096 (55.4%) peoples respectively. Five most frequent cancer cases among Malaysian males in 2007 were lung, colorectal, nasopharynx, prostate and lymphoma, while the five most common cancer cases in females were breast, colorectal, cervix, ovary and lung (Zainal Ariffin and Nor Saleha, 2011).

Breast cancer is the most common cancer diagnosed in women and the principal cause of death among women in most parts of the world. According to statistic, breast cancer contributed approximately 410 000 death cases per year (Coughlin and Ekwueme, 2009). In Malaysia, breast cancer is prevalent in women amongst all races as early age of 20. It is most common cancer disease in Malays, followed by Chinese and Indian. Based on the NCR 2007, breast cancer incidence was approximately 32.1% of overall cancer occurring among women population in Malaysia (Zainal Ariffin and Nor Saleha, 2011).

Polyphenol is vital part of the human diet and mainly present in berries, grapes/wine, chocolate/cocoa, coffee, soybeans and other fruits and vegetables. Several studies reported that polyphenol possessed antimutagenic properties and powerful free radical scavengers (Stoner and Mukhtar, 1995; Rice-Evans et al., 1995). These properties exhibited that polyphenol is one of the dietary compounds which obviously emerged as potential cytotoxicity agents against cancer.

Currently, cocoa is one of the famous dietary polyphenol that contain rich source of polyphenol which possessed beneficial effect for human health. Cocoa tree or scientifically known as *Theobroma cacao* L., belongs to family of *Sterculiaceae* which originated from the area of central, southern and southeastern Mexico (Rusconi and Conti, 2010). Catechin, epicatechin, flavanol glycosides, anthocyanins

procyanidins are among polyphenol identified in cocoa beans and cocoa products (Rimbach et al., 2009). Furthermore, cocoa demonstrated higher antioxidant capacity and contains more phenolic phytochemicals than teas and red wine (Arteel and Sies, 1999). Research on pharmacological potential of cocoa has been done over the past few years. Numerous investigators reported that cocoa phenolic contained bioactive phenolic compounds that possessed antioxidant, anticarcinogenic, and antiradical properties (Sanbongi et al., 1998; Wollgast and Anklam, 2000; Ren et al., 2003). However, the cytotoxicity study against cancer cell lines that had been done in previous research is insufficient to answer the present research objective due to different origin of cocoa polyphenol extract (CPE) (United States of America and France) and different cancer cell lines used such as Caco-2, 22Rv1 and DU145, MDA MB-436, MDA MB-468 and SKRB-3. Furthermore, the data on mode of cell death including AOPI, annexin V FITC-PI, DNA fragmentation, p53 and caspase-9 assay were scanty. Thus, the aforementioned parameters were conducted to elucidate the mode of cell death and prove the pharmacological potential of cocoa for cytotoxicity study *in vitro*.

1.2 Problem statement

The prevalence of breast cancer and mortality rate among women suffered with this disease is increasing every year. In fact, there are a number of factors which contribute to the breast cancer risk such as age, genetic hereditary, radiation, lifestyle, environmental and hormonal (Washbrook, 2006). Moreover, breast cancer was confirmed to be a challenging disease to cure and only several effective drugs are available. To date, surgery, hormone therapy, chemotherapy and radiotherapy are several conventional strategies for breast cancer treatment (El Saghir et al., 2011). However, these treatments were insufficient in order to prevent breast cancer patients from intermittence and metastasis of the tumor.

In addition, slowing action of chemotherapeutic drugs in breast cancer treatment will cause body developing resistance towards the drugs and trigger tumor recurrence (Yaacob et al., 2010). On top of that, the drugs cause critical side-effects to the patients such as cardiac and other toxicities (Beer and Bubalo, 2001; Leonard et al., 2009; Wonders and Reigle, 2009). Due to adverse effects of drug, a great deal of research has been conducted to explore natural products from plants and to search for potential cytotoxic activity towards breast cancer. Instead of medicinal herbs, dietary approaches have attracted tremendous attention among nutritionist, scientist and consumers due to its role to prevent, slow and delay the development of breast cancer without causing excessive damage to normal cells in human body. Therefore, this study is designed to elucidate the cytotoxicity effect of CPE originated from cocoa bean clone KKM4, KKM22, PBC123 and PBC159 towards breast cancer cell line (MCF-7).

1.3 Research objectives

1.3.1 General objective:

To investigate the cytotoxicity effect of CPE towards MCF-7 cells and elucidates mode of cell death.

1.3.2 Specific objectives:

1. To determine CPE constituents by qualitative phytochemical screening and HPLC profiling analysis.
2. To determine total phenolic and antioxidant activity of CPE using TPC, DPPH and FRAP assay.
3. To determine the IC_{50} value of CPE on various cancers cell lines using MTT assay.
4. To determine the IC_{50} value of CPE against MCF-7 at different time points (24, 48 and 72 h) using MTT assay.
5. To determine the mode of cell death of CPE towards MCF-7 using inverted light microscope, acridine orange/propidium iodide, cell cycle, annexin V FITC and DNA fragmentation assay.
6. To determine the apoptotic pathway of CPE towards MCF-7 using p53 and caspase-9 ELISA kits.

1.4 Research hypothesis

CPE possess high total phenolic content, antioxidant activity and exhibit cytotoxicity towards MCF-7 cells through morphological alteration of cells, cell cycle phase arrest, phosphatidylserine (PS) externalization, DNA fragmentation and inducing apoptosis via increasing level of p53 and caspase-9 expression.

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