UNIVERSITI PUTRA MALAYSIA

ANTIPROLIFERATIVE EFFECTS OF ZERUMBONE-HYDROXYPROPYL-β-CYCLODEXTRIN INCLUSION COMPLEX ON HepG2 LIVER CANCER CELLS In Vitro

NABILAH BINTI MUHAMMAD NADZRI

IB 2013 35

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By

NABILAH BINTI MUHAMMAD NADZRI

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

May 2013

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In the Name of Allah, the Most Merciful and Most Compassionate Dedication

We all have dreams. But in order to make dreams come into reality, it takes an awful lot of determination, dedication, effort and continuous support from our loved ones.

Specially dedicated to,

Allah SWT & Prophet Muhammad (SAW)
My beloved parents
My brothers & sisters
My fiancée

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

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Chair: Ahmad Bustamam Abdul, PhD

Faculty: Institute of Bioscience

Zingiber zerumbet Smith locally known as 'lempoyang' or wild ginger belongs to the Zingiberaceae family. Previous investigations on *Z. zerumbet* proved that it contained a bioactive compound, zerumbone (ZER), a crystalline sesquiterpene possessing suppressive effect in cancers. The purpose of encapsulating ZER with hydroxylpropyl-β-cyclodextrin (HPβCD) is to modify ZER's solubility and pharmacokinetic properties making it less harmful to the human body. The objective of this study is to investigate antiproliferative activities of ZER-HPβCD inclusion complex towards HepG2 liver cancer cells. The MTT assay showed that the inclusion complex is cytotoxic towards HepG2 cells with an IC_{50} of 11.43 µg/ml. Morphological evaluation showed structural changes associated with apoptosis including membrane blebbing, cell shrinkage and chromatin condensation. HepG2 cells treated with the inclusion complex further resulted in cell cycle arrest at G2/M phase with increments of apoptotic cells. Further investigations showed the release of

cytochrome c and loss of mitochondrial membrane potential, proving mitochondrial dysfunction upon the ZER-HPβCD treatment as well as modulating pro-apoptotic and anti-apototic Bcl-2 family members with no significant change of p53. The activated Bax will be translocated to the mitochondria, which activates the transformation of mitochondrial function and release of cytochrome c. Upregulation of caspase 3/7, caspase 9 and caspase 8 were also detected with the depletion of BID cleaved by caspase 8 proving that both extrinsic and instrinsic pathway were involved upon ZER-HPβCD induction. Collectively, these results demonstrate that the highly soluble inclusion complex of ZER with HPβCD induce apoptosis programmed cell death in HepG2 cells and can be extrapolated to postulate that caspase 8's activation is indirectly involved as an interconnection between the extrinsic and intrinsic pathway. Further investigations to this complex are needed to substantiate its use as an anticancer against hepatocellular carcinoma in humans.

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

KESAN ANTIPROLIFERASI KOMPLEKS ZERUMBON-HIDROKSILPROPIL-β-SIKLODEKSTRIN TERHADAP SEL KANSER HATI HepG2 *In Vitro*

Oleh

NABILAH MUHAMMAD NADZRI

Mei 2013

Pengerusi: Ahmad Bustamam Abdul, PhD

Fakulti: Institut Biosains

Zingiber zerumbet Smith yang dikenali sebagai 'lempoyang' atau halia liar yang termasuk dalam famili Zingiberaceae. Kajian sebelum ini telah membuktikan bahawa *Z. zerumbet* mengandungi kompaun bioaktif, zerumbon (ZER), sesquiterpen kristal yang mampu menindas pertumbuhan pelbagai jenis kanser. Pengkapsulan ZER dengan hidroksilpropil-β-siklodekstrin adalah bertujuan untuk memodifikasi kadar kelarutan ZER dan ciri-ciri farmakokinetiknya sekaligus manjadikannya kurang bahaya kepada tubuh manusia. Objektif kajian ini adalah bertujuan untuk menyelidik aktiviti antiproliferasi kompleks inklusi ZER-HPβCD terhadap sel kanser hati, HepG2. Keputusan asai MTT menunjukkan kompleks inklusi adalah sitotoksik terhadap sel HepG2 dengan bacaan IC_{50} 11.43 µg/ml. Penilaian morfologi menunjukkan pertukaran struktur yang berkaitan dengan apoptosis termasuk pengelembungan membran, pengecutan sel dan pemekatan kromatin. Sel HepG2 yang dirawat oleh kompleks inklusi ZER-HPβCD seterusnya mencetuskan

penghentian kitaran sel pada fasa G2/M serta peningkatan sel apoptotik. Kajian lanjutan menunjukkan pembebasan sitokrom c dan kehilangan potensi membran mitokondria membuktikan rawatan kompleks inklusi ZER-HPβCD menyebabkan kehilangan fungsi mitokondria sekali gus memodulasi ahli keluarga pro-apoptotik dan anti-apoptotik tanpa perubahan p53 yang signifikan. Bax yang teraktif akan ditranslokasikan ke mitokondria dan mengaktifkan transformasi fungsi mitokondria sekali gus pembebasan sitokrom c. Peningkatan kaspase 3/7, kaspase 8 dan kaspase 9 yang signifikan serta pemotongan BID oleh kaspase 8 juga dikesan menunjukkan kedua-dua tapak jalan ekstrinsik dan intrinsik terlibat dengan induksi ZER-HPβCD. Secara keseluruhan, kajian menunjukkan bahawa kompleks inklusi ZER-HPβCD berkebolehan untuk mengaruhkan kematian sel secara terprogram apoptosis dalam sel HepG2 dan boleh dicadangkan bahawa pengaktifan kaspase 8 secara tidak langsung terlibat dalam menghubungkan di antara tapak jalan ekstrinsik dan intrinsik. Penyelidikan yang lebih mendalam terhadap kompleks inklusi ini adalah diperlukan untuk digunakan sebagai antikanser terhadap hepatoselular karsinoma manusia.

ACKNOWLEDGEMENTS

First and foremost, my praise to Almighty Allah for giving me the strength to complete this study and peace upon final Prophet and Messenger Muhammad, SAW.

I would like to express my gratitude to the chairman of my supervisor committee, Dr Ahmad Bustamam Abdul who was abundantly helpful and offered invaluable assistance, support and guidance. I would like to extend my deepest gratitude and appreciation to the members of the supervisory committee, Professor Dr. Mohd. Aspollah Sukari and Associate Professor Dr. Siddig Ibrahim Abdelwahab, without their knowledge and assistance this study would not have been successful.

I gratefully acknowledge Universiti Putra Malaysia, Universiti Malaya and Ministry of Higher Education Malaysia for giving me this opportunity and their financial support during the course of my Master programme.

I would like to thank all my lab mates in UPM-MAKNA lab and in Department of Pharmacy, UM, and all the staff of Bioscience for their help and wishes for the successful completion of this project. Special thanks to my fiancée, Muhammad Faiz and my lovely friends Theebaa Anasamy, Nurul Hafiza, Nurul Nadia, Nur Farhana, and Rohayati for their friendship and companion. Finally, yet importantly, I would like to express my heartfelt thanks to my siblings and beloved parents, Muhammad Nadzri and Wan Nor Aishah for their blessings and continuous support in completing my study.

I certify that a Thesis Examination Committee has met on 14 May 2013 to conduct the final examination of Nabilah Binti Muhammad Nadzri on her thesis entitled "**Antiproliferative Effects of Zerumbone-Hydroxypropyl-β-Cyclodextrin Inclusion Complex on HepG2 Liver Cancer Cells *In Vitro***" 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 degree of Master of Science.

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DECLARATION

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

NABILAH BINTI MUHAMMAD NADZRI

Date: 14 May 2013

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LIST OF ABBREVIATIONS

%	Percentage
µg	Microgram
µl	Microliter
10^5	100 000
AAF	Acetylaminofluorene
AFB1	Aflatoxin B1
AIF	Apoptosis-inducing factor
ANOVA	Analysis of variance
AO	Acridine orange
AOM	Azoxymethane
Apaf-1	Apoptotic protease-activating factor-1
APS	Ammonium persulfate
ATP	Adenosine-5'-triphosphate
Bad	Bcl-2-associated death promoter
Bak	Bcl-2 homologous antagonist killer
Bax	Bcl-2 associated X protein
Bcl-2	B cell lymphoma 2
Bcl-$_X$L	B cell lymphoma extra large
BID	BH3 interacting domain death agonist
BIR	Baculovirus inhibitor of apoptosis protein repeat
BSA	Bovine serum albumin
CA	Chromosome aberrations
Caov-3	Ovarian cancer cells

Caspases	Cysteine-dependent aspartate-directed proteases
CD	Cyclodextrins
Cdk	Cyclin-dependent kinase
CEMss	T-acute lymphoblastic leukemia cells
CIN	Cervical intraepithelial neoplasia
CMC	Sodium carboxymethyl cellulose
CO_2	Carbon dioxide
dATP	2'-Deoxyadenosine 5'-triphosphate
DEN	Diethylnitrosamine
DES	Diethylstilboestrol
dH_2O	Distilled water
DISC	Death-inducing signaling complex
DISC	Death-inducing signalling complex
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSS	Dextran sodium sulfate
EMB	Embryonal histologic
FADD	Fas-associated death domain
FasL	Fas ligand
FDA	Food and Drug Administration
FH	Fetal histologic
FITC	Fluorescein isothiocyanate
G0	Gap 0 at cell cycle
G1	Gap 1 at cell cycle
G2/M	Gap 2/ Mitosis at cell cycle

h	Hour
HB	Hepablastoma
HBV	Hepatitis B virus
HBx	Hepatitis B virus X
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HeLa	Cervical cancer cells
HepG2	Liver cancer cells
HO	Heme oxygenase
HP-β-CD	Hydroxypropyl-β-cyclodextrin
Hsp70	Heat shock protein 70
HT-29-D4	Colon cancer cells
IAP	Inhibitor of apoptosis protein
IC_{50}	Inhibitory concentration
KCl	Potassium chloride
M	Metastasis for TNM staging
MCF-7	Estrogen receptor positive breast cancer cells
MDA-MB-231	Estrogen receptor negative breast cancer cells
ml	Mililitre
MMP	Mitochondrial membrane potential
MOMP	mitochondrial outer membrane permeabilization
MPT	Mitochondrial permeability transition
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N	Node for TNM staging
NaCl	Sodium chloride

NCE	New chemical entity
NCI	National Cancer Institute
NF-κB	Nuclear Factor-KappaB
P53	Tumor protein 53
PBL	Human peripheral blood lymphocytes
PBS	Phosphate buffer saline
pH	Potential of hydrogen
PI	Propidium Iodide
PMSF	Phenylmethylsulfonyl fluoride
PS	Phospatidylserine
RNA	Ribonucleic acid
Rpm	Rotation per minute
SCU	Small cell undifferentiated histologic
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SMAC	Second mitochondria-derived activator of caspase
T	Size for TNM staging
tBID	Truncated BH3 interacting domain death agonist
TEMED	Tetramethylethylenediamine
TNF	Tumor necrosis factor
TNF-R1	Tumor necrosis factor receptor 1
TRAIL	Tumor necrosis factor related apoptosis-inducing ligand
TRAIL-R2	TNF related apoptosis-inducing ligand receptor 2
TUNEL	TdT-mediated dUTP nick end labelling
UPM	Universiti Putra Malaysia

WRL-68	Normal liver cells
XIAP	X-linked inhibitor of apoptosis protein
ZER	Zerumbone
ΔΨm	Mitochondrial transmembrane potential

CHAPTER I

INTRODUCTION

1.1 Introduction

Liver cancer can be classified into primary and secondary types. The primary liver cancers are Hepatocellular Carcinoma (HCC) and Hepatoblastoma whilst secondary liver cancer is a metastatic liver cancer is originated from other organs such as the colon, stomach, pancreas, breast, and lung. Liver cancer is the sixth most frequent cancer in the world. Nearly 626,000 or 5.7% new cases are reported each year. It is also the third highest cancer-related death after lung and stomach cancer. In Malaysia, it is the tenth highest cancer-related death among man with 378 cases reported for every 100,000 population (Lim and Halimah, 2004).

The use of plants as the preferential treatment for cancer has been known for centuries. To date about 3000 plant species have been identified to possess anticancer properties. This historical information was utilized by modern scientists in search for better compounds as anticancers agent (Cragg *et al.*, 2005). One of the most important plant contributions for the world health is the anticancer agents derived from natural plants (Cragg *et al.,* 1997). This is proven by the production of more than 60 types of clinical chemotherapy drugs derived from plants. A number of promising new agents are in clinical development based on selective activity against cancer-related molecular targets, including flavopiridol and combretastin-A4-

phosphate, while some agents which failed in earlier clinical studies have stimulating renewed interest (Cragg *et al.*, 2005).

Zingiber zerumbet is a member of the family *Zingiberaceae*. It is an erect herb up to two meters tall arising from a bulky yellowish pungent underground rhizome. This plant can be found in the moist forest, beach thickets and mangrove margin from sea-lever to over 500m. In peninsular Malaysia, it is found around villages and in secondary growth. It is indigenous in south-east Asia, widely distributed and naturalize all over the south pacific (Kho, 2005). Other names for this plant are lempoyang, 'lempoyang gajah', 'halia putih', shampoo ginger and pinecones ginger (Ahmad *et al.,* 1994). Some of the traditional usages of *Z. Zerumbet* include the treatment of fever, indigestion, diarrhea, severe sprains, inflammation, constipation, toothache and some of the Malays use the rhizomes to cure edema and worm infestation in children (Zakaria *et al.,* 2010).

Zerumbone (ZER), is a crystalline monocyclic sesquiterpene derived from the rhizomes wild ginger, *Z. zerumbet*. This bioactive component has its unique structure with cross-conjugated ketone in an 11-membered ring, as well as having interesting biological activities. It has been reported that the compound ZER constitute about 37% of *Z. zerumbet* (Sakinah *et al.,* 2007).

Hydroxypropyl-β-cyclodextrin (HPβCD) is a cyclodextrin derivative that is widely studied in the field of drug encapsulation because of its inclusion ability as well as its high water solubility. HPβCD is well tolerated by human body both by intravenous and oral administration (Gould and Scott, 2005). Eid *et al.* (2011a) investigated the

inclusion complex between ZER and HPβCD and its characterization. The ZER penetrates completely into the cavity of HPβCD and the solubility of ZER was enhanced >30 fold after complexation. The current results showed that HPβCD is a suitable encapsular capable of forming thermodynamically stable complex with ZER for delivery of the compound as an anticancer. Hence, taking into consideration the importance of biopharmaceutical characterization, the antiproliferative effects of the new ZER-HPβCD inclusion complex was carried out on HepG2 cells in this study.

1.2 Hypothesis

Treated HepG2 liver cancer cells with zerumbone-hydroxypropyl-β-cyclodextrin (ZER-HPβCD) inclusion complex will induce apoptosis, which will involve modulation of pro-apoptotic and anti-apoptotic family member proteins.

1.3 Objective

To investigate the *in vitro* anti-proliferative properties and the apoptosis pathway determination of the inclusion complex of zerumbone with hydroxypropyl-β-cyclodextrin (ZER-HPβCD) on HepG2 liver cancer cells.

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