ANTITUMOR EFFECTS OF ZERUMBONE ON RAT HEPATOCARCINOMA AND HEPG2 CELLS

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MANAL MOHAMED ELHASSAN TAHA

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

April 2011

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

Specially dedicated to,

Allah SWT, Prophet Mohamed (SAW)

The soul of my late brother
My beloved parents
My husband
My sisters
Our families
My daughters
My supervisor

For their invaluable support, love, patience and intellectual stimulation.....

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

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Chairman: Ahmad Bustamam Abdul, PhD

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Zerumbone (ZER), a monosesquiterpene found in the subtropical ginger (*Zingiber zerumbet* Smith), possesses antiproliferative properties to several cancer cell lines, including cervical, skin and colon cancers. In this study, the antitumor effects of ZER were assessed in rats induced to develop liver cancer with a single intraperitoneal injection of diethylnitrosamine (DEN, 200 mg/kg body wt.) followed two weeks later by daily dietary 2-acetylaminofluorene (AAF, 0.02%) for another two weeks. Eighty rats were divided into 16 equal groups (n=5), comprising of normal (control) rats at three sacrifice times (3 groups), rats with induced hepatocarcinogenesis at three sacrifice times [positive control (DEN/AAF)] (3 groups), rats with induced hepatocarcinogenesis given three ZER treatment doses (15, 30 or 60 mg/kg body wt.) each at three sacrifice times (9 groups) and normal rats treated with ZER (60 mg/kg body wt.) and sacrificed at end of experiment (1 group). Treated rats received intraperitoneal ZER injections twice weekly for 4, 8 or 11 weeks after AAF treatment. The effects of ZER on rat hepatocarcinogenesis

were studied through body weight profile, apoptosis proteins (Bax and Bcl-2), α-fetoprotein (AFP), glutathione (GSH), glutathione S-transferase (GST), glutathione peroxidase (GPx), aspartate aminotransferase (AST), alanine transaminase (ALT), alkaline phosphatase (AP) and γ-glutamyltranspeptidase (γGT) concentrations, TUNEL and lipid peroxidation assays, histopathological examinations, immunohistochemical staining of cell proliferation marker (PCNA), transmission electron microscopy and AFP and apoptosis genes expressions using realtime quantitative polymerase chain reaction. The current study also used the liver cancer cell line (HepG2) as the *in vitro* model. The effects of ZER on liver cancer cells were determined by the MTT, caspase-3 and reactive oxygen species assays, inverted light microscopy, Hoechst DNA stain, and estimation of lactate dehydrogenase (LDH), AST, ALT and γGT concentrations in HepG2 cell culture media.

The *in vivo* study showed that, the hepatocytes of positive control (DEN/AAF) rats were smaller with larger hyperchromatic nuclei than normal, showing cytoplasmic granulation and intracytoplasmic violaceous material, which were characteristic of hepatocarcinogenesis. Histopathological evaluations showed that ZER protects the rat liver from the carcinogenic effects of DEN and AAF. Liver weights and body weights were not statistically ($P>0.05$) different among groups. Rats in DEN/AAF group showed the relative liver weights. Serum ALT, AST, AP and AFP concentrations were significantly lower ($P<0.05$) in ZER-treated than untreated rats with liver cancer. The liver malondialdehyde (MDA) concentrations significantly ($P<0.05$) increased in the untreated DEN/AAF rats indicating hepatic lipid peroxidation. There was also significant

($p<0.05$) reduction in the hepatic tissue GSH concentrations in these rats. The liver sections of untreated DEN/AAF rats also showed abundant PCNA, while in ZER-treated rats the expression of this antigen was significantly ($P<0.05$) lowered.

Formalin-fixed paraffin-embedded liver sections of untreated DEN/AAF rats also showed abundant AFP immunoexpression, while in ZER-treated rats the expression of this protein was significantly ($P<0.05$) lowered. The realtime qPCR analysis also showed significantly ($P<0.05$) lower expression of AFP mRNA in DEN/AAF rats treated with ZER than those untreated. From the TUNEL assay, the numbers of apoptotic cells were significantly ($P<0.05$) higher in DEN/AAF rats treated with ZER than those untreated. Antitumor activities of ZER were further confirmed by transmission electron microscopy, which showed distinctive morphological changes corresponding to typical apoptosis. Zerumbone treatment had also increased Bax and decreased Bcl-2 proteins expression with a concurrent increase in Bax/Bcl-2 ratio with respect to DEN/AAF rats, which again suggested increased apoptosis. This was supported by similar changes in levels of Bax and Bcl-2 mRNAs. The *in vivo* study suggests that ZER reduces oxidative stress, inhibits proliferation, induces mitochondria-regulated apoptosis, thus minimising DEN/AAF-induced carcinogenesis in rat livers. The MTT assay suggested that ZER (IC_{50}=3.9 ± 0.64 µg/mL) was cytotoxic to the liver cancer cell while harmless to the normal liver cells. This shows that the mode of death in ZER-treated liver cancer cells was apoptosis, as indicated by the up-regulation of caspase-3 in these cells. The ZER-triggered apoptosis in liver cancer cells was preceded by redox change that generated of reactive oxygen species. Therefore, ZER has great potential in the treatment of hepatocellular carcinomas.

Keywords: Zerumbone; liver cancer; rat hepatocarcinogenesis; alphafetoprotein; antiproliferation; apoptosis; reactive oxygen species; mitochondrial regulated cell death.

Abstrak tesis yang dikemukakan keapda Senat Universiti Putra Malaysia sebagai memenunhi keperluan untuk ijazah Doktor Falsafah

KESAN ANTITUMOR ZERUMBON TERHADAP HEPATOKARSINOMA TIKUS DAN SEL HEPG2

Oleh

MANAL MOHAMED ELHASSAN TAHA

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Zerumbon (ZER), suatu monoseskuiterpena yang terdapat dalam halia subtropika (*Zinger zerumbet* Smith), mempunyai sifat antiproliferatif terhadap beberapa titisan sel kanser, termasuk kanser serviks, kulit dan kolon. Dalam kajian ini, kesan antitumor ZER telah dinilai dalam tikus yang diaruh untuk mengembangkan kanser hati melalui satu suntikan intraperitoneum dietilnitrosamina (DEN, 200 mg/kg) dan dikuti dua minggu kemudian dengan 2-asetilaminofluorena (AAF, 0.02%) dalam diet selama dua minggu lagi. Lapan puluh ekor tikus dibahagikan kepada 16 kumpulan sama rata (n=5), terdiri daripada tikus normal (kawalan) pada tiga masa korban (3 kumpulan), tikus diaruh untuk hepatokarsinogenesis sahaja pada tiga masa korban [kawalan positif (DEN/AAF)] (3 kumpulan), tikus diaruh untuk hepatokarsinogenesis diperlakukan dengan dos ZER (15, 30 atau 60 mg/kg berat badan) setiap satu pada tiga masa korban (9 kumpulan), and tikus normal di perlakukan dengan ZER (60 mg/kg berat badan) dan dikorbankan pada akhir ujikaji (1 kumpulan). Tikus terperlaku menerima suntikan ZER intraperitoneum dua kali

seminggu selama 4, 8 atau 11 minggu selepas perlakuan dengan AAF. Kesan ZER terhadap hepatokarsinogenesis tikus dikaji melalui profil berat badan, protein apoptosis (Bax dan Bcl-2), α-fetoprotein (AFP), kepekatan glutation (GSH), glutation S-transferase (GST), glutation peroksidase (GPx), aspartat aminotransferase (AST), alanina aminotransferase (ALT), alkalin fosfatase (AP) dan γ-glutamiltransferase (γGT) dan asai TUNEL dan pemperoksidaan lipid, pemeriksaan histopatologi, pewarna imunohistokimia untuk penanda pemproliferatan sel (PCNA), mikroskopi electron pancaran dan penyataan gen AFP dan apoptosis menguna tindak balas rantaian polymerase kualitatif masa nyata. Kajian ini juga mengguna titisan sel kanser hati (HepG2) sebagai model *in vitro*. Kesan ZER terhadap sel kanser hati ditentukan melalui asai MTT, caspase-3 dan spesies oksigen reaktif, mikroskopi cahaya terbalik, pewarnaan Hoechst, penentuan kepekatan laktat dehidrogenase (LDH), AST, ALT, γGT dalam medium kultur sel HepG2.

Kajian *in vivo* menunjukkan yang hepatosit pada tikus kawalan positif (DEN/AAF) lebih kecil dengan nucleusnya hiperkromatik dan lebih besar daripada normal, menunjukkan pengranulan sitoplasma dan bahan intrasitoplasma keungguan yang cirian untuk hepatokarsinogenesis. Penilaian histopatologi menunjukkan ZER melindungi hati tikus daripada kesan karsinogen DEN dan AAF. Kepekatan ALT, AST, AP dan AFP serum lebih rendah tererti ($p<0.05$) dalam tikus terpelaku ZER daripada yang mengalami kanser hati dan tidak diperlakukan. Kepekatan malondialdehid (MDA) meningkat secara tererti ($p<0.05$) dalam tikus DEN/AAF tidak diperlaku menunjukkan berlakunya pemperoksidaan lipid hepar. Kepekatan GSH tisu hepar juga menurun tererti ($p<0.05$) dalam tikus ini. Irisan hati tikus DEN/AAF juga menunjukkan banyak PCNA, sambil

dalam tikus terperlaku ZER, penyataan antigen ini menurun secara tererti ($p<0.05$). Peningkatan aras AFP serum dicerapkan dalam kanser hati tikus teraruh DEN/AAF. Zerumbon secara tererti ($p<0.05$) menurunkan aras AFP kepada julat normal. Irisan hati tikus DEN/AAF tertetap formalin terbenam paraffin juga menunjukkan banyak pengimunonyataan AFP, sambil dalam tikus terperlaku ZER, penyataan protein ini menurun secara tererti ($p<0.05$). Analisis qPCR masa nyata juga menunjukkan penurunan tererti ($p<0.05$) dalam penyataan mRNA AFP dalam tikus DEN/AAF terperlaku ZER berbanding tikus yang tidak diperlaku. Daripada asai TUNEL, bilangan sel apoptosis lebih tinggi tererti ($p<0.05$) dalam tikus DEN/AAF terperlaku ZER daripada yang tidak diperlakukan. Aktiviti antitumor ZER seterusnya disahkan melalui mikroskopi elektron pancaran yang menunjukkan perubahan morfologi nyata, padan dengan apoptosis tipikal. Penemuan ini disokong oleh perubahan serupa dalam aras mRNA Bax dan Bcl-2. Kajian *in vivo* ini menyarankan yang ZER mengurangkan tekanan oksidatif, merencat pemproliferatan, mengaruh apoptosis terkawalatur mitokondrion and dengan ini meminimumkan karsinogenesis teraruh DEN/AAF dalam hati tikus. Asai MTT menyarankan ZER sitotoksik kepada sel kanser hati sambil tidak mencederakan sel hati normal. Ini menunjukkan yang mod kematian sel kanser hati terperlaku ZER ialah apoptosis, seperti yang ditunjukkan melalui naik pengawalaturan kaspase-3 dalam sel tersebut. Apoptosis yang dicetus oleh ZER dalam sel kanser hati didahului dengan perubahan redoks yang menjanakan spesies oksigen reaktif. Kesimpulannya, ZER mempunyai potensi tinggi dalam rawatan karsinoma hepatosel.

Kata-kata kunci: Zerumbon; kanser hati; hepatokarsinogenesis tikus; α-fetoprotein; antiproliferasi; apoptosis; spesies oksigen reaktif; kematian sel mitokondria ditetapkan

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LIST OF ABBREVIATIONS

µl	Microlitre
0.05	Level of Significance (Type 1 error)
10^6	1000,000
200X	Two Hundred Times
AAF	2-Acetylaminoflourine
Abs	Absorbance
ACUC	Animal Care and Use Committee
AFP	Alpha-fetoprotein
AP	Alkaline phosphatase
ALT	Alanine Aminotransferase
ANOVA	Analysis of Variance
AST	Aspartate aminotransferase
B.W.	Body weight
Bax	Bcl-2–associated X protein
Bcl-2	B-cell lymphoma 2
Bp	Basepair
cDNA	Complementary DNA
Cm	Centimeter
CO_2	Carbon dioxide
DAB	3, 3' –diaminobenzidine
DEN	Diethylnitrosamine
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
EDTA	Disodium Ethylene Diaminetetracetate
EtOH	Ethanol
FFPE	Formalin Fixed and Parraffin Embbeded
g	Gram
GSSG	Glutathione disulfide
h	Hour

i.p.	Intraperitoneal
IC_{50}	Inhibitory Concentration (50%)
IHC	Immunohistochemistry
Kg	Kilogram
KV	Kilovolt
MHz	Megahertz
Min	Minute
mL	Mililitre
mRNA	Messenger Ribonucleic acid
n (N)	The Number of experimental unit per group (replicates)
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
°C	Centigrade
P	Probability Value of Test Statistic
p.p.m	Part per million
PCNA	Proliferating Cellular Nuclear Antigen
pH	Hydrogen ion concentration
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
rpm	Revolution per minute
RT-PCR	Real Time Polymerase Chain Reaction
TAE	Tris Acetate EDTA
TBE	Tris Borate EDTA
TBE	Tris base EDTA
TdT	Terminal deoxynucleotidyl transferase
TEM	Transmission Electron Microscopy
Tris-HCl	Tris-Hydrochloride
UV	Ultraviolet
w/v	Weight over volume
ZER	Zerumbone

CHAPTER I

INTRODUCTION

1.1 Introduction

Cancers are groups of cells that arise from a single cell (Bjerkvig *et al.,* 2005; Diehn *et al.,* 2009) and can be characterized as loss of normal growth regulation. The disease is widely recognized as one of the most formidable human afflictions (WHO, 2005). In Malaysia, the incidence of cancer is increasing. It is now the fourth leading cause of death among medically certified deaths. It is estimated that the annual incidence of cancer is 30, 000 cases (Lim, 2002). Liver cancer, especially hepatocellular carcinoma (HCC), is one of the most widespread malignancies in Malaysia (Lim and Halimah, 2004) and chronic liver infection is the main cause of HCC in this region (Cheng *et al.,* 2009). Hepatitis B and Hepatitis C viruses are the risk factors attributed to 80% of HCC globally (Shepard *et al.,* 2005). Other risk factors include toxin (aflatoxin) and exposure to certain chemical carcinogens such as diethylnitrosamine, polyaromatic hydrocarbon and acetyl aminofluorene (Loeb and Harris, 2008; Neumann, 2009). The pathogenesis of HCC is similar to other cancers. Hepatocarcinoma has also been suggested to be consequences of oxidative stress due to unremitting production of reactive oxygen species (ROS) which form covalent adducts with DNA bases, leading to genotoxicity and mutagenicity (Brown and Bicknell, 2001). The prognosis of advanced HCC remains poor and novel treatment and diagnosis strategies are urgently needed (Le Sheng *et al.,* 2009; Wons and Galle, 2010). Recent treatment regimes for liver cancer include percutaneous ethanol injection, transarterial chemoembolisation,

radiofrequency thermal ablation, liver resection and liver transplantation (Branco *et al.,* 2009; Gervais *et al.,* 2009). Liver transplantation is fraught with graft (liver organ) shortage, placing patient with HCC in the waiting list for a considerable length of time and facing the risks of not being able to receive transplants because of contraindications that developed during the waiting periods (Adam and Hoti, 2009; Taha *et al.,* 2007). Therefore, there is a dire need for an alternative treatment for liver cancer patients. Currently much attention has been placed on anticancer drugs of herbal origin (Calixto, 2000).

Plant-based medicines have significantly contributed to traditional treatment for cancers (Chang and Wu, 2001; Hartwell, 1982). Currently, tropical and sub-tropical countries with enormous biodiversity such as Malaysia (Cragg and Newman, 2005; Gratus *et al.,* 2009; Nobili *et al.,* 2009) are being focused as sources of medicinal plants. Zingiberaceae is a large family of perennial herbaceous plants. Members of the family have attracted continuous interest due to their culinary uses and their versatile biological and chemical properties (Krishnaswamy, 2008). *Zingiber zerumbet* Smith was reported to have a variety of biological properties and in Southeast Asia; it is traditionally used to treat various ailments including cancers (Burkill, 1966; Habsah *et al.,* 2000). *Z. zerumbet* also contains various phytochemical constituents, mainly essential oils with compounds such as zerumbone (ZER), kaempferol derivatives and humulene (Abdul *et al.,* 2008; Masuda *et al.,* 1991; Nakamura *et al.,* 2004). Zerumbone as the major constituent of *Z. zerumbet* has shown antiproliferative effects on different cancer cell lines (Abdelwahab *et al.,* 2010; Al-Zubairi *et al.,* 2010; Murakami *et al.,* 2004; Murakami *et al.,* 2002; Murakami *et al.,* 2004). This cytotoxicity of ZER has been reported to be selective on cancer cells (Hoffman *et al.,*

2002; Murakami *et al.,* 2002; Sakinah *et al.,* 2007). This compound was shown to induce redox cellular change and restore oxidative status differently in cancer and in normal cells (Hoffman *et al.,* 2002). The current study efforts to substantiate this claim, and show that ZER has antitumor properties in the liver cancer models. Zerumbone has been shown previously to have potential *in vivo* chemo-preventive properties on induced skin, cervix and colon cancers in rodent models (Abdelwahab *et al.,* 2009; Murakami *et al.,* 2004; Murakami *et al.,* 1999; Tanaka *et al.,* 2001). However, its effect on liver cancers is not known. Zerumbone also has other biological activities, to include anti-inflammatory, modulation of iNOS expression, TNF-α release, and antiHIV (Murakami *et al.,* 2003). Zerumbone also activates drug-metabolizing enzymes in normal rat liver cells (Nakamura *et al.,* 2004).

In this study, the antitumor mechanisms of ZER on hepatocarcinogenised rats and liver cancer (HepG2) and normal cell (WRL-68) lines were examined. The rat liver model was used. In this model, hepatocarcinogenesis was initiated with diethylnitrosamine (DEN) and promoted with 2-acetylaminofluorene (AAF). The human hepatoma HepG2 cells, a well-differentiated transformed cell line, are used as a model system for cultured hepatocyte-type cells to study liver cancers *in vitro* (Liu *et al.,* 2008).

It is well-known that most anticancer agents work through multiple mechanisms as suggested by their pleiotropic properties on cancer development. In fact the possible mechanisms of anticancer agents during the promotion stage are inhibition of genotoxic effects, inhibition of cell proliferation, modulation of cellular redox status (reduction-oxidation status of the cell), induction of apoptosis and inhibition of TNF-α

(Matés *et al.*, 2008). Cellular redox change can be part of signal transduction pathway during apoptosis. Bcl-2, a mitochondrial protein, has been shown to prevent cells from dying of apoptosis apparently by an antioxidative mechanism. For these reasons, one of the main objectives of this study is to determine the sequence of cellular signaling events that occur after modulation of the cellular redox state in the HepG2 cell line, with emphasis on the role of redox-initiated mitochondrial signaling. The effect of ZER on HepG2 was studied using the MTT assay, Hoechst stain, colorimetric assay of caspase-3, ROS assay, and lactate dehydrogenase aspartate aminotransferase, alanine transaminase and gamma-glutamyl transpeptidase determinations in HepG2-culture media with the intention of providing the first time practical evidence of the role of ROS in apoptosis induced by ZER on this cell line.

Therefore, the present study was designed to determine the potential of ZER in the treatment of liver cancers (Adam and Hoti, 2009; Befeler and Di Bisceglie, 2002) and evaluate ZER as an anticancer agent (Chang and Wu, 2001; Cragg and Newman, 2005; Guilford and Pezzuto, 2008; Jang *et al.*, 1997). Thus our study also aimed to determine the effectiveness of ZER as a chemopreventive and chemotherapeutic agent in experimental malignancy.

1.2 Hypothesis

It is hypothesized that zerumbone has antiproliferative effect on rat hepatocarcinoma and cytotoxic effect on the human cancer cell line (HepG2).

1.3 Aims of the Study

General Objective: To determine the anti-tumour effects of ZER on rat hepatocarcinoma and HepG2

Specific Objectives

To determine the effects of ZER on;

- body weight, liver weight and relative liver weight of hepatocarcinogenized rats.
- liver function parameters in hepatocarcinogenized rats.
- oxidative stress in DEN/AAF-induced rat hepatocarcinogenesis.
- cell proliferation, apoptosis and differential gene expression in DEN/AAF-induced rat hepatocarcinogenesis.
- serum and cytoplasmic alpha-fetoprotein in DEN/AAF-induced rat hepatocarcinogenesis.
- the liver cancer (HepG2) cell line *in vitro*.
- normal rats and human normal liver cells.

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