UNIVERSITI PUTRA MALAYSIA

ANTI-ANGIOGENIC AND ANTI-HEPATOCELLULAR CARCINOMA PROPERTIES OF ZERUMBONE EXTRACTED FROM ZINGIBER ZERUMBET (L.) SMITH

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By

NOZLENA BINTI ABDUL SAMAD

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Abstract of thesis presented to the senate of Universiti Putra Malaysia in fulfillment of the requirements for the degree of Doctor of Philosophy

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April 2015

Chairman: Ahmad Bustamam Abdul, PhD
Faculty: Institute of Bioscience

Zerumbone (ZER) extracted from *Zingiber zerumbet* is known to have anti-cancer properties; however, its mechanism in curbing liver cancer growth and spread is still not clear. Thus the objective of this study is determine the *in vitro* anti-cancer effect of ZER towards HepG2 cell line and the *in vivo* effect on induced rat hepatocellular carcinoma (HCC). The anti-cancer mechanisms investigated were apoptosis, anti-proliferation and anti-angiogensis. Zerumbone was shown to be toxic towards HepG2 cells with IC_{50} of 6.20±0.70 µg/mL and less toxic towards normal liver cells (WRL68) with IC_{50} of 61.00±0.40µg/mL. The study showed that ZER caused cell cycle arrest at the G2/M phase and apoptosis, demonstrated by chromatin condensation, cell shrinkage and formation of apoptotic bodies in the HepG2 cells in a time-dependent manner. Zerumbone also stimulated caspase-3 and -9 activities in the HepG2 cells, suggesting that the induction of apoptosis was via the mitochondrial pathway. The study employed the diethylnitrosamine-induced rat HCC model and the rat aortic ring to determine the effect of ZER treatment. The study showed that ZER significantly ($p<0.05$) inhibited microvessel outgrowth in the aortic ring model. Zerumbone at 12.5 µg/mL caused the most significant ($p<0.05$) 98±1.28% blood vessels inhibition compared with the control and inhibited endothelial tube formation at 96.00±0.72%. This study showed that ZER treatment decreases expression of VEGF, MMP-9 and Ki-67 in the rat HCC tissue as well as and inhibits neovascularization in the chick embryo. The treatment had also induced apoptosis in HCC. The ZER-treated liver tissues with HCC showed normal hepatocyte orientation, unlike the untreated livers, which showed pleomorphic hepatocytes and anaplastic appearance typical of HCC. It can be concluded from the study that the anti-cancer effect of ZER on the HepG2 cell line and HCC is multifaceted involving induction of cell cycle arrest, apoptosis, and suppression of VEGFR, VEGF, MMP-9 and Ki-67 proteins, leading to inhibition of angiogenesis. Since ZER was less toxic to the normal liver cells, this compound is a potentially effective anti-HCC agent, without significant side-effects and can be developed as a therapeutic regime either alone or in combination with other chemotherapeutic agents.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

KANDUNGAN ANTI-ANGIOGENESIS DAN ANTI-KARSINOMA HEPATOSEL BAGI ZERUMBON YANG DI EKSTRAK DARI *ZINGIBER ZERUMBET* (L.) SMITH

Oleh

NOZLENA BINTI ABDUL SAMAD

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Zerumbon (ZER) yang diekstrak daripada *Zingiber zerumbet* diketahui mempunyai sifat anti-kanser; bagaimanapun mekanisme dalam perencatan pertumbuhan dan perebakan kanser hati masih belum jelas. Objektif kajian ini ialah untuk menentukan kesan anti-kanser ZER *in vitro* terhadap titisan sel HepG2 dan kesan *in vivo* pada karsinoma hepatosel (HCC) tikus. Mekanisme anti-kanser yang diselidik ialah apoptosis, anti-pemproliferatan and anti-angiogenesis. Perubahan morfologi ditentukan melalui mikroskopi elektron imbasan. Zerumbon didapati toksik terhadap sel HepG2 dengan IC_{50} 6.20±0.40 µg/mL dan kurang toksik kepada sel hati normal (WRL68) dengan IC_{50} 61.00±0.04 µg/mL. Kajian ini menunjukkan ZER menyebabkan sekatan kitaran sel pada fasa G2/M dan apoptosis, yang ternyata sebagai pengenapan kromatin, pengecutan sel dan pembentukan jasad apoptosis pada sel HepG2 yang berlaku secara bersandarkan masa. Zerumbon juga merangsang aktiviti kaspase-3 dan -9 dalam sel HepG2, dimana ini menyarankan bahawa pengaruhan apoptosis adalah melalui arah laluan mitokondrion. Kajian ini telah menunjukkan bahawa ZER pada kepekatan 12.5µg/ml merencat pertumbuhan mikrovesel secara paling ketara ($p<0.05$) dalam model gegelang aorta serta merencat pembentukan tiub endotelium pada kadar 96.00±0.72%. Apoptosis dalam tisu hati terperlaku ZER ditentukan melalui assai TUNEL. Kajian ini menunjukkan yang perlakuan ZER telah mengurangkan penyataan protein VEGF, MMP-9 dan Ki-67 pada tisu HCC tikus dan juga merencat neopengvaskularan pada embrio anak ayam. Perlakuan ini juga telah mengaruhkan apoptosis dalam HCC. Tisu hati dengan HCC yang diperlaku ZER menunjukkan orientasi hepatosit yang normal, bukan seperti pada hati yang tidak terperlaku, yang menunjukkan hepatosit pleomorfik and tampilan anaplasia yang tipikal untuk HCC. Kesimpulan daripada kajian ini ialah, kesan anti-kanser ZER terhadap titisan sel HepG2 dan HCC adalah berserampang yang melibatkan pengaruhan sekatan kitaran sel, apoptosis dan penindasan protein VEGFR, VEGF, MMP-9 dan Ki-67, yang membawa kepada perencatan angiogenesis. Oleh kerana ZER kurang toksik terhadap titisan sel hati normal, maka sebatian ini adalah berpotensi berkesan sebagai agen anti-HCC, tanpa kesan sampingan yang ketara dan boleh dikembangkan sebagai regim terapeutik sama ada secara bersendirian atau gabungan dengan agen kemoterapi lain.

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I certify that a Thesis Examination Committee has met on 27 April 2015 to conduct the final examination of Nozlena binti Abdul Samad on his thesis entitled ñAnti-Angiogenic and Anti-Hepatocellular Carcinoma Properties of Zerumbone Extracted from *Zingiber zerumbet* (L.) Smithò 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 Doctor of Philosophy.

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TABLE OF CONTENTS

		Page
ABSTRACT		i
ABSTRAK		ii
ACKNOWLEDGMENTS		iii
APPROVAL		iv
DECLARATION		vi
LIST OF FIGURES		xii
LIST OF TABLES		xiv
LIST OF ABBREVIATIONS		xv
CHAPTER		
1	**INTRODUCTION**	**1**
	1.1 Introduction	1
	1.2 Aim and Objectives	3
	1.3 Hypothesis of The Study	3
2	**LITERATURE REVIEW**	**5**
	2.1 Natural Product of Medicinal Value	5
	2.2 Zingiberacea Family	6
	2.2.1 Botanical aspects of *Zingiber zerumbet*	6
	2.2.2 Traditional use of *Zingiber zerumbet*	8
	2.3 Zerumbone	8
	2.4 Synthetic Anti-Cancer Drugs Currently Used for Hepatocarcinoma Treatment	9
	2.4.1 Paclitaxel	9
	2.4.2 Doxorubicin	9
	2.5 Natural Compound Currently Used for Hepatocarcinoma Treatment	10
	2.5.1 Curcumin	10
	2.5.2 Resveratrol	10
	2.5.3 Silibinin	11
	2.5.4 Tanshinone IIA	11
	2.6 Cancer Biology	13
	2.7 The Cell Cycle	13
	2.8 Cell Death and Apoptosis	15
	2.9 Autophagy	17
	2.10 Liver Cancer	17
	2.10.1 Liver Cancer in Malaysia	17
	2.11 Cancer Treatment Method	18
	2.11.1 Surgery	18
	2.11.2 Radiation Theraphy	18
	2.11.3 Hormonal Therapy	19
	2.11.5 Anti-angiogenesis Theraphy	20
	2.12 Angiogenesis	20
	2.12.1 Types of Angiogenesis	22
	2.12.1.1 Sprouting Angiogenesis	22
	2.12.1.2 Intussusceptive Angiogenesis	22
	2.12.2 Angiogenesis Mediator	24

2.12.3 The Role of Angiogenesis in Solid tumor 24
2.12.4 Anti-angiogenesis Agents 26
2.12.5 Natural Anti-angiogenic Inhibitors 26
2.12.6 Suramin 28
2.13 Review Summary 28

3 **ANTIPROLIFERATIVE AND APOPTOTIC ACTIVITIES OF ZERUMBONE ON LIVER CANCER CELLS**
3.1 Introduction 29
3.2 Materials and Methods 29
3.2.1 ZER Preparation 29
3.2.2 Cell Culture 30
3.2.3 Growth Inhibition of Human Hepatocellula Carcinoma Cells 30
3.2.4 Cellular Morphology 31
3.2.5 Quantification of Apoptosis 31
3.2.6 Ultrastructure of HepG2 Cells 31
3.2.7 Cell Cycle 32
3.2.8 Annexin V-FITC Assay 32
3.2.9 DNA Content 32
3.2.10 Caspase As 33
3.2.11 Statistical Analysis 33
3.3 Results 34
3.3.1 Anti-proliferative Activity of ZER on HepG2 and WRL68 Cell Line 34
3.3.2 Effects of ZER on Hepatocellular Carcinoma Cell Line 40
3.3.3 Quantification of Apoptosis using AO/PI Double Staining 42
3.3.4 Ultrastructural of Hepatocellular Carcinoma Cell line treated with Zerumbone 45
3.3.5 Zerumbone Treatment on Cycle of Hepatocellular Cell Line 47
3.3.6 Induction of Apoptosis Hepatocellular carcinoma (HepG2) Cell Line by Zerumbone 49
3.3.7 Caspase Activity of Hepatocellular Carcinoma (HepG2) Cells Treated with Zerumbone 51
3.4 Discussion 53

4 **ZERUMBONE SUPPRESSES ANGIOGENESIS AND METASTASIS VIA INHIBITION OF NF-κB, MMP AND VEGF EXPRESSIONS IN HEPG2 CELLS AND VEGFR IN ENDOTHELIAL CELLS** **57**
4.1 Introduction 57
4.2 Materials and Methods 58
4.2.1 Animal Preparation 58
4.2.2 *Ex vivo* Rat Aortic Ring Assay 58
4.2.3 Cell Proliferation Inhibition Assay 58
4.2.4 Endothelial Cell Tube Formation Assay 59
4.2.5 Effect of Zerumbone on VEGF-Induced Tyrosine Phosphorylation of VEGFR- 59

	4.2.6	Chorioallantoic Membrane Assay	59
	4.2.7	Cell Migration Assay	60
	4.2.8	Human Cytokine Array	60
	4.2.9	Western Blot	60
	4.2.10	Statistical Analysis	61
4.3	Results		62
	4.3.1	*Ex vivo* Rat Aortic Ring Assay	62
	4.3.2	Cell Proliferation Inhibition	65
	4.3.3	Endothelial Cell Tube formation Assay	67
	4.3.4	Effect of Zerumbone on VEGF-Induced Tyrosine Phosphorylation of VEGFR-2	70
	4.3.5	Cell Migration Assay	71
	4.3.6	Chorioallantoic Membrane Assay	73
	4.3.7	Human Cytokine Array	75
	4.3.8	Western Blot	77
4.4	Discussion		78
5	**ANTI-ANGIOGENIC AND ANTI-PROLIFERATIVE EFFECT OF ZERUMBONE ON HEPATOCARCINOGENESIS IN RATS**		**81**
5.1	Introduction		81
5.2	Materials and Methods		83
	5.2.1	Animals	83
	5.2.2	Ethical Approval	83
	5.2.3	Zerumbone	83
	5.2.4	Preparation of Diethylnitrosamine	83
	5.2.5	Carcinogen Treatment	84
	5.2.6	Experimental Design	84
	5.2.7	Serum vascular endothelia growth factor	84
	5.2.8	Histopathology	84
	5.2.9	TUNEL Assay	85
	5.2.10	Immunohistochemistry of Liver Tissue	85
	5.2.11	Statistical analysis	86
5.3	Results		87
	5.3.1	Body and liver weight	87
	5.3.2	Serum Vascular Endothelial Growth Factor	88
	5.3.3	Histopathology	89
	5.3.4	TUNEL Assay	93
	5.3.5	Expression of Vascular endothelial Growth Factor and Matrix Metalloproteinase	97
	5.3.6	Expression of K-i67	100
5.4	Discussion		102
6	**GENERAL DISCUSSION, CONCLUSION AND FUTURE RESEARCH AND RECOMMENDATION**		**105**
6.1	General Discussion		105
	6.1.1	Anti-proliferative and Apoptosis Effects of Zerumbone	105
	6.1.2	Anti-angiogenesis Effect of Zerumbone	106
6.2	General Conclusion		108

6.3 Future Research and Recommendations 109
6.4 Limitation of The Studies 109

REFERENCES 110
APPENDICES 130
HPLC Result of ZER 130
Anti Proliferative Activity of ZER on HUVECs Cell Line 131
VEGF Standard Curve 132
Histopathology of Tissues 133
Cell Cycle Buffers 134
TUNEL Assay Solutions 135
Western Blot Buffers 136
Immunohistochemistry Solutions 138
BIODATA OF STUDENT 139
LIST OF PUBLICATIONS 140

LIST OF FIGURES

Figure		Page
2.1	*Zingiber zerumbet* plant.	11
2.2	The Cell Cycle.	23
2.3	Angiogenesis.	35
2.4	Sprouting and Intussusceptive Angiogenesis.	37
2.5	The Role of Angiogenesis in Solid Tumor Formation.	40
2.6	The Angiogenesis Pathways and the Examples of Its Inhibition.	43
3.1	Cell Proliferation Inhibition Activity of Zerumbone on Hepatocellular Carcinoma (HepG2) Cell Line.	58
3.2	Cell Proliferation Inhibition Activity of Zerumbone on Normal Liver (WRL68) Cell Line.	59
3.3	Cell Proliferation Inhibition Activity of Doxorubicin Hepatocellular Carcinoma (HepG2) Cell Line.	60
3.4	Cell Proliferation Inhibition Activity of Doxorubicin on Normal Liver (WRL68) Cell Line.	61
3.5	Hepatocellular Carcinoma (HepG2) Cell Line Treated Zerumbone at its IC_{50} Concentration (6.20 µg/mL).	64
3.6	Acridine orange/Propidium iodide-stained zerumbone-treated HepG2 cells after 72 h	67
3.7	Quantitative Analysis of AO/PI stained cells of untreated cells and ZER treated HepG2 cells	68
3.8	Morphology of Untreated and Zerumbone-treated Hepatocellular Carcinoma (HepG2) Cell Line.	70
3.9	Flow Cytometric Analysis of Zerumbone-treated Hepatocellular Carcinoma (HepG2) Cells After Staining with FITC-Conjugated Annexin-V and Propidium Iodide.	74
3.10	Caspases Activity in Hepatocellular Carcinoma (HepG2) Cells Treated with ZER after 12, 24, 48 and 72 H.	76
4.1	Rat Aorta Angiogenesis Assay.	94

4.2	Dose -Response Curve of Zerumbone on Rat Aortic Ring Assay.	99
4.3	Human Umbilical Vein Endothelial Cell (HUVEC) Tube Formation Inhibition by Zerumbone (ZER)	100
4.4	Dose Response Relationships of ZER on Tube Formation Assay	101
4.5	Phosphorylation of VEGFR-2 in Human Umbilical Vein Endothelial Cells treated with Zerumbone (ZER).	101
4.6	Effect of Zerumbone (ZER) on HepG2 Cells Migration.	103
4.7	Effects of Zerumbone (ZER) on Neovascularisation in Chick Chorioallantoic Membrane.	105
4.8	Differential Expression of Cytokines in Hepatocellular Carcinoma (HepG2) Cells treated with Zerumbone.	107
4.9	Expression of VEGF, MMP-9 and NF-əB Proteins in Zerumbone-treated Hepatocellular (HepG2) Cells.	108
5.1	Serum Vascular Endothelial Growth Factor (VEGF) Concentration of Rats with Hepatocellular Carcinoma and treated with Zerumbone (ZER) and Suramin.	127
5.2	Liver Tissues of Rats with Hepatocellular Carcinoma and treated with Zerumbone (ZER) and Suramin.	131
5.3	Liver Tissues of Rats with Hepatocellular Carcinoma Treated with Zerumbone (ZER) and Suramin.	135
5.4	Rat Liver Tissue Stained for Vascular Endothelial Growth Factor (VEGF).	137
5.5	Rat Liver Tissue Stained for Matrix Metalloproteinase-9 (MMP-9).	138
5.6	Rat Liver Tissue Stained for Ki-67.	140

LIST OF TABLES

Table		Page
2.1	Anti-hepatocarcinogenic effect of herbal compounds	20
2.2	Comparison between apoptosis and necrosis	26
3.1	Therapeutic Index	62
3.2	Cell cycle distribution of hepatocellular carcinoma (HepG2) cell line treated with zerumbone	72
4.1	Cytotoxic effect of zerumbone	97
5.1	Body and liver weights of zerumbone-treated diethylnitrosamine-induced hepatocellular carcinoma in rats.	126

LIST OF ABBREVIATIONS

%	Percentage
h	Hour/s
M	Molar
Ml	Milliliter
ζ g	Microgram
εL	Microliter
mM	Micromolar
Pg/ml	Pikogram/mililiter
rpm	Revolution per minute
v/v	Volume per volume
ANOVA	Analysis of variance
AO	Acridine orange
ATCC	American tissue culture collection
Bax	Bcl-2 associated X protein
Bcl-2	B cell lymphoma 2
BSA	Bovine serum albumin
$CaCo_2$	Calcium carbonate
CAM	Chick chorioallantoic membrane
CDK-2	Cyclin-dependent kinase 2
CDK-4	Cyclin-dependent kinase 4
DEN	Diethylnitrosamine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiotrheitol

EGF	Epidermal growth factor
eNOS	Endothelial nitric oxide synthase
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FFPE	Formalin-fixed paraffin-embedded
G_0	Gap 0 at cell cycle
G_1	Gap 1 at cell cycle
G_2/M	Gap 2/ mitosis at cell cycle
H3	Histone 3
H4	Histone 4
HCC	Human hepatocellular carcinoma
HepG2	Human hepatocellular carcinoma cells
HIFCS	Heat inactivated fetal calf serum
HRP	Horseradish peroxidase
HT29	Human colorectal adenocarcinoma cell
HUVEC	Human umbilical vein endothelial cells
IC_{50}	Half maximal (50%) inhibitory concentration
IL2	Interleukin 2
IL6	Interleukin 6
IL8	Interleukin 8
iNOS	Inducible nitric oxide synthase
IUPAC	International Union of Pure and Applied Chemistry
KI67	Protein associated with cell proliferation
LC_{50}	Lethal concentration, 50%
MCF-7	Human breast cancer cell

MMP	Matrix metalloproteinase
MMP-9	Matrix metalloproteinase 9
MTT	3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Nacl	Sodium chloride
NF-əB	Nuclear factor kappa B
PBS	Phosphate buffer saline
PCNA	Proliferating cell nuclear antigen
pH	A scale that measures how acidic or basic a substance
PI	Propidium iodide
PMSF	Phenylmethanesulfonylfluoride
PO_2	Oxygen partial pressure
PVDF	Polyvinylidene fluoride
PI	Propidium iodide
PS	Phosphotidylserine
ROS	Reactive oxygen species
RPMI	Roswell park memorial institute medium
Rtdt	Terminal deoxynucleotidyl transferase recombinant
SEM	Scanning electron microscope
TNF	Tumor necrosis factor
TBST	Tris buffered saline tween
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
WAF1	Cyclin dependant kinase interacting protein 1
WRL68	Human normal hepatic cells
ZER	Zerumbone

CHAPTER 1

INTRODUCTION

1.1 Introduction

Natural products refer to compounds that are derived from animals, plants or microorganisms. Since early civilization, natural products have played important roles in health care and prevention of diseases in humans and animals. Before the 19th century, natural products were the sole mean in the treatment diseases and injuries. Our ancestors chewed herbs to relieve pain and wrapped the leaves around the wound to facilitate healing. By the 19th century, the natural products in its original form began playing a secondary role in therapy when active therapeutic elements were isolated from medicinal plants. In 1806, morphine was isolated from *Papaver somniferum* (Wetzel *et al.*, 2010), atropine from *Atropa belladonna*, ziconotide from a cone snail and Taxol from the bark of the Pacific yew tree (Cragg and Newman, 2013). This was the beginning of evolution of natural products in modern medicine. Based on recent data (WHO), 80% of the world's population depend on traditional medicine (Koehn and Carter, 2005; Newman *et al.*, 2003). Approximately, 25% of the drugs prescribed today are derived from natural products (Zhang *et al*., 013). Natural products have also significantly contributed to the development of vaccines and anticancer drugs. Between 1981 to 2006 more than 100 anticancer drugs were developed and 47% of these were derived from natural products (Newman *et al*.,2003).

Cancer is a complex disease that develops from single damaged cell, subsequent to the accumulation of errors to its genes (Loeb, 2000). Manifestation of these genetic errors may possibly be the result of exposure of chemicals, viruses and physical assault to the cell (Karpinets and Foy, 2004). These noxious factors can influence or damage cellular pathways. Signaling pathways leading to cancer are numerous; therefore the biological profile of this disease would differ from one cancer patient to another depending on which pathway is affected by the cancer-causing agents (Chin and Gray, 2008).

Liver is a complex organ in the human body, perfoming approximately 500 functions daily for the maintenance of the organism (Maton *et al*., 1993). The liver is quite often affected by cancers and these diseases can originate in the liver itself or the result of metastasis. Liver cancer is the fifth most common type of cancers and the third leading cause of cancer-related death (Davis *et al*., 2008). The majority (80%) of reported liver cancer cases occur in developing countries. Among the countries with highest rates of liver cancers include central and Western Africa, Southeast Asia, China and Mongolia (Mokdad *et al*., 2014). Liver cancers are typically hypervascular tumours or carcinomas. Treatment of this type of carcinoma is difficult because most patients, especially those in less-developed countries, are diagnosed when the disease is already at an advanced stage (El-Serag and Rudolph, 2007). Furthermore, there is high incidence of recurrence, possibly metastasis after hepatic resection, with the disease becoming non-amenable towards therapy (Davis *et al*., 2008).

The failure and various side-effects of conventional medicine in treating cancers have led to the growing interest in the search for drugs from natural resources. Among advantages of drugs from natural products are that they are affordable and accessable to the majority of the world population that does not have access to modern conventional pharmacological treatments. Natural products are also claimed to be harmless and have minimal or no side-effect in comparison to synthetic drugs (Rates, 2001).

Angiogenesis is a process of new blood vessel formation. Inhibition of angiogenesis is considered one of the most promising strategies in treating a variety of illnesses including cancers (Adair, 2010). The inhibition of angiogenesis may potentially be a very effective way to treat and inhibit progression and spread of cancers. Angiogenesis is controlled through the balance between pro-angiogenesis and anti-angiogenesis factors, which are vital to the triggering of angiogenesis switch (Keshet and Ben-Sasson, 1999). Several signals that can trigger this switch include low partial oxygen pressure, pH and glucose levels (Kizaka *et al*., 2003). Anti-angiogenesis drugs are proven to boost anti-tumor activities of several conventional cytotoxic chemotherapeutic drugs (Folkman, 2002). However, different organs and tissues may express different angiogenesis receptors, which pose a great challenge in the development of effective anti-angiogenesis therapy, particularly with receptor-specific compounds, such as monoclonal antibodies. Moreover, the microenvironment of tumor site, for example the endothelium that is phenotypically distinctive for the organ, may influence the efficacy anti-angiogenesis. This phenomenon makes an agent that is therapeutically effective in one organ may not be effective in another (Kerbel, 2000).

Zerumbone (ZER) is a sesquiterpene phytochemical from a type of edible ginger known as *Zingiber zerumbet* (L.) Smith found abundantly in Southeast Asia (Murakami *et al.,* 2002). Zerumbone is currently being explored for its effects on cancers to include leukemia, cervical, colon and breast cancers. To date, there has been no report on the effect of ZER on anti-angiogenesis in liver cancers.

The current study was undertaken to determine the anti-angiogenesis properties as well as the anti-cancer effect of ZER in hepatocellular carcinoma. Previous studies in our laboratory showed that ZER retards cervical intraepithelial neoplasia (CIN) in cervical tissues of female BALB/C, induced prenatally with diethylstilbestrol to develop the cancer (Abdelwahab *et al.,* 2010). The anti-cancer properties of ZER were found to be equivalent to that of cisplatin, a commercial anticancer drug preferentially used in treating cervical cancer in humans (Abdelwahab *et al.,* 2010). Zerumbone also possesses anti-inflammatory activities (Sulaiman *et al.,* 2009), which is beneficial in the inhibition of angiogenesis. Zerumbone was also chosen for this study because of its traditional use in the treatment of several illnesses while possessing high anti-oxidant activities (Yob *et al.*, 2011). This study was conducted *in vitro* on HepG2 cells, *ex vivo* on isolated liver tissue and *in vivo* in a rat hepatocellular carcinoma model.

1.2 Aims and objective

General Objectives
To ascertain the anti-angiogenic and anti-cancer effects of ZER in rat hepatocellular carcinoma.

Specific Objectives

To determine the

anti-proliferative and apoptotic activity of ZER on a liver cancer (HepG2) cell line.

anti-angiogenesis mechanism of ZER using *in vitro*, *ex vivo* and *in vivo* assays

anti-angiogenesis and anti-proliferative effects of ZER in the rat hepatocellular carcinoma model.

1.3 Hypothesis of the Study

Zerumbone has anti-cancer effect through inhibition of angiogenesis.

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