



UNIVERSITI PUTRA MALAYSIA

**ANTITUMOR EFFECT OF ZERUMBONE ISOLATED FROM
LEMPOYANG (Zingiber zerumbet) ON HUMAN CERVICAL CANCER
CELLS AND MOUSE CERVICAL INTRAEPITHELIAL NEOPLASIA**

SIDDIG IBRAHIM ABDELWAHAB

IB 2009 10



**ANTITUMOR EFFECT OF ZERUMBONE ISOLATED FROM *LEMPOYANG*
(*Zingiber zerumbet*) ON HUMAN CERVICAL CANCER CELLS AND MOUSE
CERVICAL INTRAEPITHELIAL NEOPLASIA**

By

SIDDIG IBRAHIM ABDELWAHAB

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in
Fulfillment of the Requirement for the Degree of Philosophy of Doctor**

January 2009



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

Specially dedicated to,

Allah SWT, Prophet Mohamed (SAW)

My beloved parents

My wife

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

ANTITUMOR EFFECT OF ZERUMBONE ISOLATED FROM *LEMPOYANG* (*Zingiber zerumbet*) ON HUMAN CERVICAL CANCER CELLS AND MOUSE CERVICAL INTRAEPITHELIAL NEOPLASIA

By

SIDDIG IBRAHIM ABDEL WAHAB

January 2009

Chairman: Dr. Ahmad Bustamam Abdul, PhD

Faculty: Institute of Biosciences

Malaysia as a tropical country is a rich source of biologically active phytochemicals, which could be useful as an alternative to the current unsafe regimens of cancer treatment. This includes the use of cisplatin (CIS), the current chemotherapeutic drug to treat cervical cancer, the second most lethal cancer affecting women in Malaysia. Therefore, anti-tumor activities of zerumbone (ZER) were investigated in both *in vitro* and *in vivo* cervical cancer models. This natural compound was isolated from the edible plant *Zingiber zerumbet*, locally known as *Lempoyang*, through column chromatography and hydrodistillation methods. The chemical structure of ZER was confirmed using NMR. The cytotoxic effects of ZER were tested in human cervical cancer cell lines (HeLa) using MTT assay and compared concurrently to cisplatin. Zerumbone's induction of HeLa cancer cell deaths were quantified using AO/PI double staining and flow cytometry. Transmission and scanning electron microscopic analyses were done to evaluate ultra-morphological changes. The effect of ZER on caspase-3 and caspase-9 was evaluated colorimetrically in HeLa cells. The *in vivo* model of cervical intraepithelial neoplasia (CIN) was induced in pregnant female Balb/c mice using



Diethylstilboestrol (DES). Cervical tissues were stained with hematoxylin and eosin (H&E) and viewed under light microscopy and the *in vivo* antiproliferative properties of ZER was confirmed by the immunohistochemical staining of proliferating cellular nuclear antigen (PCNA) as a proliferation marker and the PCNA labeling index was obtained. Apoptosis (Bcl-2 & Bax) and G₂/M-cell cycle arrest (cdc25B, cyclinB1 and Chk2) associated proteins were investigated using immunohistochemistry. Moreover, RT-PCR was used to amplify mRNA of Bcl-2, Bax, c-myc and β -actin genes. The genetic material was obtained by laser capture microdissection microscopy (LCMM). No previous toxicological investigations have been carried out on this compound. Hence, acute, sub-acute and sub-chronic toxicity studies and ZER was evaluated for its behavioural, biochemical and histo-pathological effects. Findings of NMR coincide to the previously published data. However, ZER was able to exert an antiproliferative effect towards HeLa when isolated by both hydrodistillation and column chromatography, with an IC₅₀ of 20.30±1.1 μ M and 20.41±0.9 μ M ($p > 0.05$, student t-test, n=3), respectively. AO/PI-stained HeLa cells showed that ZER induced apoptosis in a time-dependent manner with insignificant statistical ($p > 0.05$) difference in necrosis between various doses of this compound. Moreover, flow cytometric evaluation of the effect of ZER on DNA content by cell cycle phase distribution revealed that the cell populations at G₀ and G₂/M phases were significantly different ($p < 0.05$) as compared to the untreated population. Antitumour activities of ZER were further confirmed by transmission and scanning electron microscopy investigations, which showed distinctive morphological changes corresponding to metaphasal arrest and the typical apoptosis. The colorimetric assay of caspase-3 and caspase-9 revealed a statistical significant difference between treated and untreated cells. *In vivo* model results disclosed that ZER

(16 mg/kg) has the capability to regress significantly ($p < 0.05$, χ^2 statistics) the proliferation of cervical intraepithelial neoplasia (CIN) from CIN3 to CIN1 resembling the anti-tumor effects of CIS 10mg/kg. Moreover, this antiproliferative property was further confirmed by the regression of the PCNA, an *in vivo* proliferation marker, which showed also a dose-dependent ($p < 0.05$) effect of ZER on the PCNA labeling index (PCNA positive nuclei). It has been found that ZER also modulated the ratio of Bcl-2 and Bax, which further supported the intonated levels of LCMM extracted and RT-PCR amplified mRNA of such proteins as well as c-myc oncogene, which was detected only in the CIN cancer group. Cervical tissues from female Balb/c mice treated with 16mg/kg of ZER, showed decreased levels of CyclinB1 and cdc25B immunoreactivity and associated with upregulation of Chk2 immunoexpression. Acute and subacute administration of ZER did not cause abnormalities on body weight, liver morphology or serum AST concentration. Moreover, sub-chronic study showed ZER did not modify significantly ($p > 0.05$) serum concentrations of AST, ALP, ALT and GGT. No histopathological changes were observed in the hepatic, renal, cardiac and gastrointestinal tissues. These histomorphological findings were supported by the insignificant differences ($p > 0.05$) between the mean lesion scores of hepatic and renal tissues. Collectively, results presented in this study demonstrated that ZER causes metaphasal blockage in HeLa cells, leading to growth inhibition and apoptosis, which was later confirmed to be through mitochondrial pathways. As ZER exhibits similar pharmacological activity to CIS, it possesses the potential to be developed as an antiproliferative agent for cervical cancer but producing less side effects, as the compound was shown to have no toxicological signs compared to the clinical complications of CIS.



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

**KESAN ANTIKANSER ZERUMBONE YANG DIPENCILKAN DARIPADA
LEMPLOYANG (*Zingiber zerumbet*) KE ATAS SEL KANSER SERVIKS MANUSIA
DAN INTRAEPITHELIAL NEOPLASIA SERVIKS MENCIT**

Oleh

SIDDIG IBRAHIM ABDEL WAHAB

Januari 2009

Pengerusi: Dr. Ahmad Bustamam, PhD

Fakulti: Institut Biosains

Malaysia merupakan sebuah negara yang kaya dengan sumber fitokimia yang aktif secara biologi yang mungkin berguna sebagai alternatif kepada rawatan kanser yang tidak selamat padamasa ini. Ini termasuklah, sisplatin (CIS), dadah kemoterapi pada masa kini untuk merawat kanser serviks, pembunuh kanser nombor dua di Malaysia. Oleh itu, aktiviti anti-kanser zerumbone telah diselidik secara *in vitro* dan *in vivo* dalam model kanser serviks. Hasil semulajadi dipencilkan dari tumbuhan *Zingiber zerumbet*, lebih dikenali dengan lempoyang, melalui kaedah kromatografi kolum dan penyulingan. Struktur kimia ZER disahkan melalui NMR. Kesan sitotoksik ZER diuji ke atas jujukan sel kanser serviks (HeLa) menggunakan kaedah MTT dan dibandingkan dengan sisplatin. Pengaruh kematian sel HeLa oleh ZER dikira menggunakan pewarnaan AO/PI dan flow cytometry. Analisis mikroskop elektron imbasan dan transmisi telah dijalankan untuk menilai perubahan morfologi yang kecil. Kesan ZER terhadap kaspase-3 dan kaspase-9 diuji secara kalorimetrik pada sel HeLa. Model *in vivo* untuk cervical intra-epithelial neoplasia (CIN) diaruh pada tikus Balb/c menggunakan Dietilstilbesterol



(DES). Tisu serviks diwarnakan menggunakan hematoxylin dan eosin (H &E) dan dilihat menggunakan mikroskop cahaya dan ciri-ciri antiproliferasi oleh ZER disahkan menggunakan pewarnaan immunohistokimia PCNA sebagai penunjuk proliferasi dan indeks penlabelan PCNA diperolehi. Protein yang berkaitan dengan apoptosis (Bcl-2 & Bax) dan G2/M-kitaran sel (cdc25B, cyclinB1 dan Chk2) diselidik menggunakan immunohistokimia. Sebagai tambahan, RT-PCR digunakan untuk amplifikasikan mRNA dari gen Bcl-2, bax, c-myc dan β -aktin. Bahan-bahan genetik pula didapati daripada mikroskop micro-pembedahan penangkap laser. Tiada kajian toksisiti telah dijalankan untuk kompaun ini. Oleh itu, kajian toksisiti akut, sub-akut dan sub-kronik oleh ZER dijalankan untuk melihat kesan kelakuan, biokimia dan histo-patologi. Penemuan menggunakan NMR adalah sama dengan kajian sebelumnya. Walau bagaimanapun, ZER yang dipencilkan menggunakan kedua-dua penyulihan hidro dan kromatografi kolum mampu untuk menunjukkan anti-proliferasi terdapat sel HeLa, dengan nilai IC50 masing-masing adalah 20.30 dan 20.41. Pewarnaan AO/PI terhadap sel HeLa menunjukkan ZER mengaruh apoptosis yang dipengaruhi oleh masa secara tidak signifikan ($p < 0.05$), berbeza dengan nekrosis untuk kepekatan dos kompaun yang berbeza. Tambahan pula, kajian flow cytometri untuk menentukan kesan ZER terhadap kandungan DNA oleh distribusi kitaran sel menunjukkan yang populasi sel pada fasa G0 dan G2/M berbeza secara signifikan berbanding dengan populasi tidak dirawat. Aktiviti anti-tumor oleh ZER disahkan menggunakan mikroskop electron imbasan dan transmisi yang menunjukkan perubahan morfologi yang ketara bersandarkan perencatan metafasa dan apoptosis. Kaedah kalorimetri kaspase 3 dan kaspase 9 menunjukkan perbezaan yang signifikan di antara sel rawatan dan sel tidak di rawat. Keputusan kajian model *in vivo* menunjukkan ZER (16 mg/kg) mampu untuk merencatkan secara signifikan

($p < 0.05$) proliferasi neoplasia intraepithelia serviks dari CIN3 dan CIN1 yang sama kesan anti-tumornya dengan CIS 10 mg/kg. Tambahan pula, ciri-ciri anti-proliferasi disahkan melalui pertumbuhan PCNA, penunjuk anti-proliferasi *in vivo* yang juga menunjukkan kesan ZER terhadap indeks penlabelan PCNA (nukleus positif dengan PCNA). Adalah didapati juga bahawa ZER mampu untuk mengubah nisbah Bcl-2 dan Bax yang seterusnya menyokong paras ekstrak LCCM dan mRNA amplifikasi RTPCR seperti protein-protein dan juga onkogen c-myc yang hanya boleh dikesan dalam kumpulan kanser CIN. Tisu serviks dari tikus betina Balb/c yang dirawat dengan 16 mg/kg ZER menunjukkan penurunan paras imun reaktif CyclinB1 dan cdc25B yang berkaitan dengan peningkatan pengawal aturan immuno ekspresi Chk2. Pengambilan akut dan sub-akut ZER tidak menyebabkan ketidak normalan terhadap berat badan, morfologi hati dan enzim (AST). Tambahan, kajian sub-kronik menunjukkan ZER tidak mengubah secara signifikan ($p > 0.05$) paras AST, ALP, ALT dan GGT. Penemuan histomorfologi ini disokong oleh tidak perbezaan secara signifikan di antara pemarkahan lesion purata untuk tisu hati dan buah pinggang. Tiada perubahan histopatologi dilihat pada tisu hati, buah pinggang, jantung dan usus. Secara keseluruhan, keputusan menunjukkan ZER mampu menyebabkan penyekatan metafasa pada sel HeLa, yang membawa kepada perencatan pertumbuhan dan apoptosis, yang kemudiannya disahkan melalui tapak jalan mitokondria. ZER yang menunjukkan ciri-ciri farmakologi yang sama dengan CIS, menunjukkan ia potensi untuk dibangunkan sebagai agen anti-proliferasi untuk kanser serviks tetapi menghasilkan kesan sampingan yang kurang, di mana kompaun tersebut tidak mempunyai tanda-tanda toksisiti benbanding dengan komplikasi CIS.

ACKNOWLEDGMENTS

First my praise to Almighty Allah for giving me the power and will to complete this study and peace be upon his final Prophet and Messenger Mohamed, SAW.

I would like to convey sincere gratitude to Dr. Ahmad Bustamam, the Chairman of my Supervisor Committee for his invaluable advice, guidance, constant support and encouragement. His enthusiasm and commitment to this research project is deeply appreciated and undoubtedly invaluable, where I shall always treasure his approach and philosophy to conduct scientific investigations in a simplified and systematic manner. I would like to extend my grateful thanks and appreciation to my co-supervisor, Associate Professor Dr. Muhamad Nazrul Hakim for his suggestion and advice during the course of this study.

I gratefully acknowledge the “Malaysian Technical Co-operation Programme (MTCP), UPM Graduate Fellowship and Malaysian National Cancer Council for giving me this opportunity and their financial support during the course of the PhD programme.

I am gratefully thanking all the staff of Bioscience for their constant assistance and friendship. Specially, Dr. Adel Sharaf and Mr. Syam Morali.

It is worth to mention my colleagues and friends from Sudanese community in UPM and Serdang area for their friendship and companion. Finally yet importantly, I would like to



extend my sincere appreciation to my lovely wife Dr. Manal Mohamed Elhassan and my daughters Roa and Ayah for their patience, sacrifices and moral support during the course of the study.



This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee are as follows:

Ahmad Bustamam Hj Abdul, Ph.D.

Lecturer
MAKNA-UPM Cancer Research Laboratory
Institute of Biosciences
Universiti Putra Malaysia
(Chairman)

Muhammad Nazrul Hakim, Ph.D.

Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

Prof. Dr. Mohd Azmi Laila, Ph.D.

Professor
Malaysian Technology Development Corporation
Ministry of Sciences, Technology and Innovation
Putrajaya, Malaysia
(Member)

HASANAH MOHD GHAZALI, PhD

Professor/Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 12 February 2009





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 UPM or other institutions.

SIDDIG IBRAHIM ABDELWAHAB
Date:



TABLE OF CONTENTS

DEDICATIONS	ii
ABSTRACT	iii
ABSTRAK	vi
ACKNOWLEDGEMENTS	ix
APPROVAL	xi
DECLARATION	xii
LIST OF TABLES	xvii
LIST OF FIGURES	xviii
LIST OF ABBREVIATIONS	xxiv

CHAPTER

1	INTRODUCTION	28
2	LITERATURE REVIEW	35
	2.1 History of Herbal Drugs	35
	2.2 Background of Herbal Medicine in Malaysia	40
	2.3 Plants derived anticancer agents	41
	2.4 <i>Zingiber zerumbet</i>	42
	2.5 Zerumbone	42
	2.5.1 Chemistry of Zerumbone	42
	2.5.2 Structural Relationship to Biological Activities	45
	2.5.3 Biological Activities of Zerumbone	47
	2.6 Anatomy and histology of cervix	49
	2.7 Cell Kinetics in the Transitional Zone	51
	2.8 Benign tumors	52
	2.9 Cervical Intraepithelial Neoplasia (CIN)	52
	2.10 Cervical invasive carcinoma	55
	2.10.1 Etiology	56
	2.10.2 Human Papillomavirus (HPV)	57
	2.10.3 Cigarette smoking	58
	2.10.4 Hormonal contraception	58
	2.10.5 Inheritance	59
	2.10.6 Diagnosis of Cervical Intraepithelial Carcinoma	59
	2.10.7 Cervical Cancer in Malaysia	61
	2.11 Apoptosis	62
	2.12 Cell Cycle	68
	2.12.1 Chk2, CyclinB1 & Cdc25B Proteins	68
	2.12.2 DNA Damage and Checkpoints	71
	2.12.3 Cell Cycle Target Inhibition and Anti-Cancer Drug Discovery	72
	2.13 Diethylstilboestrol	73
	2.13.1 Introduction	73
	2.13.2 The Use of DES <i>in vivo</i> Experiments	75
	2.14 Cisplatin	76



3	MATERIALS AND METHODS	80
3.1	Isolation and Characterization of ZII	80
3.1.1	ZER Structure Elucidation by NMR	80
3.2	<i>In vitro</i> anticancer Properties of ZER	81
3.2.1	Cell culture and MTT Cytotoxicity Assay for ZER isolated by Hydrodistillation and Column chromatography	81
3.2.2	Quantification of Apoptosis by Propidium Iodide, and Acridine Orange Double Staining	81
3.2.3	Flow cytometric Analysis of DNA Cell Cycle	83
3.2.4	Ultrastructural Effects of ZER on HeLa cell Line (Transmission Electron Microscopy)	83
3.2.5	Scanning Electron Microscopy (SEM) Evaluation	84
3.2.6	Caspase assays	85
3.3	<i>In vivo</i> Anticancer Properties of ZER	86
3.3.1	Cervical Intraepithelial Neoplasia (CIN) Model in Female Balb/C mice.	86
3.3.2	Hematoxylin and Eosin staining	89
3.3.3	Immunohistochemistry	89
3.3.4	Immunostaining of cervical tissues	92
3.3.5	PCNA Labeling Index (Proliferation Index)	94
3.3.6	Laser Capture Microdissection Microscopy and RNA Extraction	94
3.4	Toxicological Study	103
3.4.1	Animals	103
3.4.2	Acute Toxicity study	103
3.4.3	Sub-acute toxicity study	103
3.4.4	Sub-chronic Toxicity	104
3.4.5	Biochemical Tests	109
3.5	Statistical Analysis	112
4	RESULTS	113
4.1	¹³ C NMR and ¹ H NMR Analysis of ZER	113
4.2	Cytotoxic Effects of ZER on Cervical Cancer Cell Line (HeLa)	115
4.3	Quantification of Apoptosis by AO/PI.	117
4.4	Flow Cytometric Analysis of Cell cycle	120
4.5	Effects of ZER on Cervical Cell Line (HeLa) Using SEM	122
4.6	Effects of ZER on Cervical Cell Line (HeLa) Using TEM	124
4.6.1	Colorimetric Assay of Caspase-3 and Caspase-9	131
4.7	<i>In vivo</i> Anticancer Study	133
4.7.1	Histopathological Evaluation of the Regressive effects of ZER on CIN Induced by DES in Female Balb/c Mice.	133
4.7.2	Antiproliferative Effects of ZER on DES-CIN in Female Balb/c Mice Using Immunohistochemical detection of Retrieved PCNA Antigen.	139



4.7.3	<i>In Vivo</i> Investigation of ZER on DES-CIN in Female Balb/c Mice Using Immunohistochemical Detection of Retrieved Bcl-2/Bax Antigens.	145
4.7.4	<i>In Vivo</i> Effects of ZER on the Immuno-expression Regulatory Proteins of the G2/M Transverse (cdc25B & CyclinB1).	148
4.7.5	Effects of ZER on Immuno-expression of Chk2 (G2/M DNA Damage Checkpoint Regulatory Protein).	151
4.7.6	Analysis of mRNA Expression	153
4.8	Toxicity Study	158
4.8.1	Acute and Sub-Acute Toxicity Study.	158
4.8.1.2	General Observation of Mice in Acute and Sub-Acute Study.	158
4.8.2	Effect of Sub-acute Intra-peritoneal Administration of ZER on the Body Weight of Normal Female Balb/c Mice.	158
4.8.3	Effect of Acute and Sub-Acute Intraperitoneal Injection of ZER on Liver Function (AST) in Female Balb/c Mice.	161
4.8.4	Hepatic Histomorphological Effects of ZER	163
4.9	Sub-Chronic Toxicity Study	169
4.9.1	General Observation of Mice in Sub-Chronic Study	169
4.9.2	Effect of Sub-Chronic Intraperitoneal Administration of ZER on the Body Weight of Normal Female Balb/c Mice	169
4.9.3	Effect of Sub-Chronic Intra-peritoneal Administration of ZER on the Serum Level of AST, ALP, ALT and GGT in Female Balb/c Mice.	171
4.9.5	Hepatic and Renal Lesions Scoring	176
4.9.6	Histological Study	178
5	DISCUSSION	178
6	SUMMARY, CONCLUSION AND RECOMMENDATIONS	216
	REFERENCES	223
	APPENDICES	243
	BIODATA OF THE STUDENT	250
	LIST OF PUBLICATIONS	251



LIST OF TABLES

Table		Page
2.1	Morphological differences between apoptosis and necrosis.	67
3.1	Primary antibodies utilized in the detection of Bcl-2, Bax, PCNA, Cdc25B, Cyclin B1 and Chk2 expression.	91
3.2	RT-PCR conditions for the different genes in this study	101
3.3	The degree of severity in liver (Steven <i>et al.</i> , 2002).	106
3.4	The degree of severity in kidney	107
3.5	Lesion Scoring in Gastrointestinal Tract	108
4.1	¹³ C-NMR Analysis of ZER extracted and isolated from Zingiber zerumbet. Samples were submitted to The Laboratory of Natural Products, IBS, UPM for analysis.	114
4.2	Effects of ZER on the viability of HeLa cells. Cells were cultured in RPMI 1640 media maintained at 37°C and 5% CO ₂ .	116
4.3	Flow cytometric analysis of cell cycle distribution in HeLa cells which were treated with ZER (1C ₅₀) for 0, 24, 48, and 72 hr, <i>n</i> =3.	121
4.4	Statistical analysis of associating the response after treatment (CIN and ZER) with CIN grading.	138
4.5	The Antiproliferative Effect of ZER in DES-Induced CIN and Control Experimental Female Balb/c Mice (60 days old, <i>n</i> =6) using scoring of PCNA positive nuclei as The Proliferative Index.	144
4.6	Effect of Subacute Intraperitoneal injection of ZER on Body Weight of female Balb/c mice.	160
4.7	Effect of Acute and Sub-Acute Intraperitoneal Injection of ZER on Liver Function in Female Balb/c Mice.	162
4.8	Effect of Sub-chronic Intra-peritoneal Administration of ZER on Mean Lesion Scoring of Liver, Kidney and GIT of Female Balb/c Mice.	177

LIST OF FIGURES

Figure		Page
2.1	Methods for Obtaining Active Substances	39
2.2	Chemical Structure of Zerumbone (2, 6, 10-humulatrien-1-one), that belongs to the sesquiterpene family.	44
2.3	ZER Derivatives	46
2.4	Cervix and Transformation Zone.	50
2.5	Classification of Cervical Cancerous Lesions.	54
2.6	Mitochondrial Pathways of Apoptosis	64
2.7	The intrinsic and extrinsic pathways leading to apoptosis.	65
2.8	Cell cycle regulation at G2/M stage.	70
2.9	Diethylstilboestrol	74
2.10	The chemical structures of cisplatin and other platinating agents such as transplatin, carboplatin, oxaliplatin, and satraplatin.	78
2.11	Toxicities associated with treatment with platinating agents.	79
3.1	An animal model for cervical intraepithelial neoplasia in female Balb/c mice (Abdul et al., 2007).	88
3.2	Steps process to capture cells using LCMM	96
3.3	Extraction of cervical intraepithelial neoplastic tissues by laser capture microdissection microscopy.	99
4.1	Fluorescent micrograph of acridine orange and propidium iodide double stained cervical cancer cells lines (HeLa).	120
4.2	Percentages of apoptotic, necrotic and viable cells after ZER treatment	118
4.3	Surface ultra-structural characteristics of HeLa cells treated with and without ZER in a time dependent course (0, 24, 48 & 72 hours) cultured in RPMI1640 media maintained at 37°C and 5% CO ₂ .	123

4.4.1	Untreated cervical cancer cell line (HeLa), demonstrates the normal structure of HeLa cancer cell.	126
4.4.2	ZER-stimulated (24 hrs) cervical cancer cell line (HeLa), demonstrates the signs of early apoptosis: Cell shrinkage, chromatin condensation (Arrow) and integrity of plasma membrane (x6000).	127
4.4.3	ZER-stimulated cervical cancer cell line (HeLa) demonstrates the condensed cristae of mitochondria (MC) as a typical sign of the incidence of apoptosis.	127
4.4.4	ZER-stimulated (48 hrs) cervical cancer cell line (HeLa), demonstrates the signs of intermediate apoptosis: Cell shrinkage, chromatin condensation and membrane blebbing (White Arrow) (x6000).	128
4.4.5	ZER-stimulated (72 hrs) cervical cancer cell line (HeLa), demonstrate the signs of late apoptosis: Nuclear collapse, continuing blebbing and apoptotic body formation (Arrow) (x10000).	128
4.4.6	cisplatin treated cervical cancer cell line (HeLa), demonstrate the signs of apoptosis: Nuclear collapse, blebbing and apoptotic body formation with signs of necrosis such absence of nuclear membrane (x10000).	129
4.4.7	untreated cervical cancer cell line (HeLa), demonstrates the progression of cell cycle at G2/M which can be recognized by the absence of the nucleus and formation of bipolar centrimeres (CM) (x4600).	129
4.4.8	ZER-stimulated cervical cancer cell line (HeLa), demonstrates the ultrastructure of G2/M arrest cells. Condensed chromosomes (White Arrow) arrayed disorderly in the middle of cells indicating the dysfunction of mitotic spindle.	130
4.4.9	The colorimetric assay of caspase-3 and caspase-9 in human cervical cancer cell line treated and untreated with ZER (IC ₅₀). Cells were cultured in RPMI 1640 (75mL FLASK) media maintained at 37°C and 5% CO ₂ .	132
4.5.1	Light photomicrograph of normal cervical epithelial tissue from Balb/c mice female offspring (58 days old, n=6).	135

4.5.2	DES-induced cancerous cervical epithelial tissue from Balb/c mice female injected intraperitoneally with normal saline (Model +ve control, 58 days old, n=6).	135
4.5.3	Light photomicrograph of DES-induced cancerous cervical epithelial tissue from Balb/c female mice injected intraperitoneally with 10mg/kg of cisplatin (Control; 58 days old, n=6).	136
4.5.4	DES-induced cancerous cervical epithelial tissue from Balb/c mice female injected intraperitoneally with 16mg/kg of ZER (58 days old, n=6).	136
4.5.5	DES-induced cancerous cervical epithelial tissue from Balb/c mice female injected intraperitoneally with 8mg/kg of ZER (58 days old, n=6).	137
4.5.6	DES-induced cancerous cervical epithelial tissue from Balb/c mice female injected intraperitoneally with 4mg/kg of ZER (58 days old, n=6).	137
4.6.1	Immunohistochemical staining of cervical tissue from normal female Balb/c mice (58 days old, n=6) using PCNA mouse monoclonal antibody and ARK immunohistochemical kit. PCNA immunoreactivity was not observed in the nuclei (Black arrow) (200X).	140
4.6.2	Immunohistochemical staining of cervical cancer tissue, from female Balb/c mice (58 days old, n=6) treated with 4mg/kg of ZER, using PCNA mouse monoclonal antibody and ARK immunohistochemical kit.	140
4.6.3	Immunohistochemical staining of cervical cancer tissue, from female Balb/c mice (58 days old, n=6) treated with 8mg/kg of ZER, using PCNA mouse monoclonal antibody and ARK immunohistochemical kit.	141
4.6.4	Immunohistochemical staining of cervical cancer tissue, from female Balb/c mice (58 days old, n=6) treated with 16mg/kg of ZER, using PCNA mouse monoclonal antibody and ARK immunohistochemical kit.	141
4.6.5	Immunohistochemical staining of cervical cancer tissue, from female Balb/c mice (58 days old, n=6) treated with 10mg/kg of cisplatin, using PCNA mouse monoclonal antibody and ARK immunohistochemical kit.	142
4.6.6	Immunohistochemical staining of cervical cancer tissue, from	142

	female Balb/c mice (DES induced; 58 days old, n=6), with ARK immunohistochemical kit with the primary antibody (PCNA mouse monoclonal antibody).	
4.6.7	Immunohistochemical staining of cervical cancer tissue, from female Balb/c mice (58 days old, n=6), with ARK immunohistochemical with missing the primary antibody (PCNA mouse monoclonal antibody).	143
4.7	Immunohistochemical staining of cervical cancer tissue, from female Balb/c mice (58 days old, n=6) treated with 16mg/kg of ZER (Right) showing down-expression of Bcl-2.	146
4.8	Immunohistochemical staining of cervical cancer tissue, from female Balb/c mice (58 days old, n=6) treated with 16mg/kg of ZER (Left) showing an overexpression of Bax.	146
4.9	Immunohistochemical staining of normal cancer tissue, of female Balb/c mice (58 days old, n=6) showing (Left) low immunoreactivity of Bax and (Right) high immunoreactivity of Bcl2 (Black arrow) (200X).	147
4.10	Immunohistochemical staining of cervical cancer tissue, from female Balb/c mice (58 days old, n=6) treated with 10mg/kg of cisplatin (Right) showing an overexpression of Bax.	147
4.11	Cervical cancer tissue stained with Cyclin B1 antibody using ARK immunohistochemical kit.	149
4.12	Cervical cancer tissue stained with cdc25B Ab using ARK immunohistochemical kit. (Right photo) Balb/c mice (58 days old, n=6) cervical tissues from DES induced CIN, note nuclear staining of tumor cells.	150
4.13	Cervical cancer tissue stained with Chk2 antibody using ARK immunohistochemical kit. Right: Balb/c mice (58 days old, n=6) cervical tissues from DES induced CIN, note Low intense of nuclear immunoeprression of Chk2 in cancer animals.	152
4.14	Effect of ZER on the expression of Bcl-2 gene of CIN induced female Balb/c mice. RTPCR products were analyzed using 2% agarose gel.	155
4.15	Effect of ZER on the expression of Bax gene of CIN induced female Balb/c mice. RTPCR products were analyzed using 2% agarose gel.	156

4.16	Effect of ZER on the expression of c-myc (218bp) gene of CIN induced female Balb/c mice. RTPCR products were analyzed using 2% agarose gel.	157
4.17.1	Liver of control mice (received 5% (v/v) ethanol + distilled water), showing normal histology in the centrilobular region of liver parenchyma. Magnification: 200X.	164
4.17.2	Liver of mice treated with a single dose of 20mg ZER /kg after 24 hours showing normal histology in the parenchyma.	165
4.17.3	Liver of mice treated with a single dose of 100mg ZER /kg after 24 hours showing normal histology in the parenchyma.	165
4.17.4	Liver of mice treated with a single dose of 250mg ZER /kg after 24 hours showing normal histology in the parenchyma.	166
4.17.5	Liver of mice treated with ethanol 5% (v/v) showing focal inflammation in liver parenchyma.	166
4.17.6	Liver of mice treated with 20mg ZER /kg daily until day 14. Liver shows few hepatocytes undergoing degenerative changes and focal inflammation in liver parenchyma.	167
4.17.7	Liver of mice treated with 100mg ZER /kg daily until day 14. Liver shows few hepatocytes undergoing degenerative changes (arrows) in the periportal area.	167
4.17.8	Liver of mice treated with 250mg ZER/kg daily until day 14. Liver shows few hepatocytes undergoing degenerative changes (arrows) in liver parenchyma.	168
4.18	Effect of sub-chronic intraperitoneal administration of ZER (90 days) on the body weight of normal female Balb/c mice.	170
4.19	Serum concentration of AST in female Balb/c mice of different treatment groups (Control, 10 & 100 mg/kg).	172
4.20	Serum concentration of ALP in female Balb/c mice of different treatment groups (Control, 10 & 100 mg/kg).	173
4.21	Serum concentration of ALT in female Balb/c mice of different treatment groups (Control, 10 & 100 mg/kg).	174
4.22	Serum concentration of GGT in female Balb/c mice of different treatment groups (Control, 10 & 100 mg/kg).	175



4.23	Liver of female Balb/c mice treated with 5% v/v (EtOH/Distilled water) showing normal architecture. The hepatocytes (H) are radiating normally outward the central vein (C) and no abnormal histopathological lesions (score 0) were observed in this group.	180
4.24	Liver of female Balb/c mice liver treated with 10 mg ZER /kg. The hepatocytes reserve the healthy feature with few normal binuclear cells.	181
4.25	Liver of female Balb/c mice liver treated with 100 mg ZER /kg. The hepatocytes reserve the normal architecture. Hepatocellular arrays remain regular with mild intraradial congestion.	182
4.26	Kidney of female Balb/c mice kidney treated with 5% v/v (EtOH/Distilled water) showing the normal architecture. Renal tissue reveals no pathological lesions with healthy tubules and intact basement membrane	183
4.27	Kidney of female Balb/c mice kidney treated with 10mg/kg ZER shows normal architecture.	184
4.28	: Kidney of female Balb/c mice kidney treated with 100mg/kg ZER shows normal architecture. Note the tubule is normal with intact basement membrane, and a mild hyperemia is observed.	185
4.29	(a) Myocardial tissue of a control mouse showing normal architecture. (b) Heart of a mouse treated sub-chronically with 10mgZER/kg showing normal myocyte. (c) Heart of a mouse treated sub-chronically with 100mgZER/kg showing normal architecture and normal coronary vessels.	186
6.1	Proposed scheme for the mode of action of ZER.	221

LIST OF ABBREVIATIONS

%	Percentage
μl	Microlitre
0.05	Level of Significance (Type 1 error)
10 ⁶	1000,000
200X	Two Hundred Times
Abs	Absorbance
ACUC	Animal Care and Use Committee
ALP	Alkaline phosphatase
ALT	Alanine Aminotransferase
ANOVA	Analysis of Variance
AO	Acridine Orange
AST	Aspartate aminotransferase
ATCC	American Type Culture Collection
B.W.	Body weight
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
Bp	Basepair
cDNA	Complementary DNA
CDNB	1-chloro-2,4-dinitrobenzene
CIN	Cervical Intraepithelial Neoplasia
CIS	Cisplatin
cm	Centimeter