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

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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

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SIDDIG IBRAHIM ABDEL WAHAB

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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 IC_{50} of 20.30±1.1 μM and 20.41±0.9 μM ($p>0.05$, 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 G_0 and G_2/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 keapda Senat Universiti Putra Malaysia sebagai memenunhi keperluan untuk ijazah Doktor Falsafah

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

Oleh

SIDDIG IBRAHIM ABDEL WAHAB

Januari 2009

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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. Hasilan 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. Pengaruhan 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 microskop 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 immunihistokimia. 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. Pewarnaa 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 aopotosis. 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 memalui 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 ekstraks 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 immun 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 histo morfologi ini disokong oleh tidak perbezaan secara signifikan di antara pemarkahan lesion purata untuk tisu hati dan nuah pinggang. Tiada perubahan histopatologi dilihat pada tisu hati, buah pinggang, jantung dan usus. Secara kesuluruhan, 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-proliferatisi untuk kanser serviks tetapi menghasilkan kesan sampingan yang kurang, di mana kompaun tersebut tidak mempunyai tanda-tanda toksisiti benbanding dengan komplikasi CIS.

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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:

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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 UPM or other institutions.

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SIDDIG IBRAHIM ABDELWAHAB
Date:

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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