UNIVERSITI PUTRA MALAYSIA

CHARACTERIZATION OF DENTATIN ISOLATED FROM CLAUSENA EXCAVATE AND ITS POTENTIAL USE FOR TREATMENT OF HUMAN BREAST AND PROSTATE CANCERS

ISMAIL ADAM ARBAB ISHAG

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By

ISMAIL ADAM ARBAB ISHAG

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

June 2013

DEDICATIONS

Adherence, effort, and dedications were fundamental elements for the completion of my doctoral dissertation, but even more was the support of my patient family.

To the souls of my Parents,

To my dearest wife Bdria Abdalla Hassan and the three greatest projects of my life: my sons Abdalmnam, Almotman and my daughter Matab, today I dedicate them this important professional achievement because without their presence, support and comprehension I would have not achieved my goal. I love you.

To the soul of my special brother, Ahmad Adam Arbab, Whom I have a lot of respect and appreciation for his support and guidance of me.

Also Dedicated to:

My sisters,,,,,,,,

My brothers,,,,,

Our families,,,,,,

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

To my supervisory committee members, for their overwhelming academic and moral support

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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By

ISMAIL ADAM ARBAB ISHAG

June 2012

Chairman: Ahmad Bustamam Abdul, PhD

Institute: Institute of Bioscience

To date, there has been no literature reported on the mechanism of dentatin and its effects on breast and prostate cancers. Hence, anti-cancer effect of dentatin was investigated towards breast (*in vivo* and *in vitro*) and prostate (*in vitro*) cancers. Ethnopharmacologically, *Clausena excavata* Burm. F., has been used as folk medicines in the eastern of Thailand for the treatment of cancer. Dentatin (DTN) was isolated from this plant via bio assay guided approach and its apoptosis mechanism was investigated. With respect to MCF-7 cells of breast, DTN induced cytotoxicity was observed using MTT assay. Acridine orange/Propidium iodide staining was used to detect the early apoptosis cells. High content screening (HCS) was used to observe the nuclear condensation,

cell permeability, mitochondrial membrane potential (MMP) and cytochrome c release. Apoptosis was confirmed by using clonogenic assay, DNA laddering and caspase 3/7 and 9 assays. Reactive oxygen species formation, Bcl-2/Bax expressions and cell cycle arrest also has been investigated. The involvement of NF-kB was analyzed using HCS assay. Significant increase in chromatin condensation in the cell nucleus was observed in the fluorescent analysis. The apoptosis was confirmed by reduced colony of cells in clonogenic assay and increased cellular DNA breaks on treated cells observed as ladder. Treatment of MCF-7 cells with DTN encouraged apoptosis with cell death-transducing signals that reduced the MMP by down-regulation of Bcl-2 and up-regulation of Bax, triggering the cytochrome c release from mitochondria to cytosol. The released cytochrome c triggered the activation of caspase 9 and then the executioner caspase 3/7. The DTN treatment significantly arrested MCF-7 cells at G0/G1 phase ($p < 0.05$). The ROS was significantly found to be elevated. Moreover the DTN significantly blocks the induced translocation of NF-kB from cytoplasm to nucleus. This part of the study was set to investigate anti-proliferative potential of dentatin (a natural coumarin isolated from *Clausena excavata* Burm.F) against prostate cancer and to delineate the underlying mechanism of action. Treatment with dentatin dose-dependently inhibited cell growth of PC-3 and LNCaP prostate cancer cell lines, whereas it showed less cytotoxic effects on normal prostate epithelial cell line (RWPE-1). The inhibitory effect of dentatin on prostate cancer cell growth was due to induction of apoptosis as evidenced by Annexin V staining and cell shrinkage. We found that dentatin mediated

accumulation of reactive oxygen species (ROS) and downregulated expression levels of anti-apoptotic molecules (Bcl-2, Bcl-xL and Survivin), leading to disruption of mitochondrial membrane potential (MMP), cell membrane permeability and release of cytochrome c from the mitochondria into the cytosol. These effects were associated with induction of caspase-9,-3/7activities and subsequent DNA fragmentation. In addition, we found that dentatin inhibited TNF-α-induced nuclear translocation of p65, suggesting dentatin as a potential NF-κB inhibitor. Acute toxicity tests by intraperitoneal administration of up to 1 g/kg in rats did not show any biochemical, anatomical, or histopathological signs of toxicity, suggesting dentatin is relatively tolerable *in vivo*. An *in vivo* study was conducted to determine the effect of dentatin (DTN) on LA-7 cell-induced rat mammary tumor. In this study, we evaluated for the first time the anti-tumor potential of dentatin (30mg/kg LD and 60 mg/kg HD body weight), orally administered for four weeks against LA7-induced mammary carcinogenesis in SD rats. After the first tumors appearance, the thirty rats were divided into five groups (n=6). The first group comprised untreated normal healthy rats and served as the normal negative control group (NNC), while the second group comprised rats induced to develop mammary gland tumor and served as the positive control. This group of rats received a single dose of 1 ml of soy oil and was ascribed as mammary tumor control (MTC). The third group of mammary gland tumor-bearing rats was treated weekly with 30/kg mg (low dose) DTN dissolved in 1 mL soy oil. As a result, this group was assigned the group DTN-LD. The fourth group also comprised the mammary gland tumor-bearing rats

and each rat received 60 mg/kg (high dose) DTN dissolved in 1 mL soy oil and assigned the group DTN-HD. Also, the fifth group comprised rats with mammary gland tumor that treated with 10 mg/kg TAM dissolved in 1 mL soy oil and assigned as the TAM group. The results suggest that DTN has better effect on the tumor compared to TAM, which promoted apoptosis in the rat mammary gland tumor. However, the DTN-HD showed a more prolonged effect suggesting that DTN could be a vital future drug in the chemotherapy of breast cancers. Hence, further studies are warranted to further investigate and develop a drug delivery system for DTN in the treatment of cancers. Together, results presented in this study demonstrated that the DTN inhibited the proliferation of MCF-7, PC-3 and LNCaP cells, leading to the cell cycle arrest and programmed cell death, which was confirmed to be through the mitochondrial pathway with the involvement of NF-kB signaling pathway. The *in vivo* study suggests that DTN reduced oxidative stress, inhibited proliferation, induced mitochondria-regulated apoptosis, therefore, minimizing LA-7-induced carcinogenesis in rat mammary glands. Thus, we suggest that dentatin may have therapeutic value in breast and prostate cancer treatment worthy of further development.

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

PENCIRIAN DENTATIN DIASINGKAN DARIPADA CLAUSENA EXCAVATA DAN POTENSI UNTUK RAWATAN PAYUDARA MANUSIA DAN KANCER PROSTAT

Oleh

ISMAIL ADAM ARBAB ISHAG

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Sehingga kini, tidak ada sastera dilaporkan pada mekanisme dentatin dan kesannya terhadap payudara dan kanser prostat. Oleh itu, kesan anti-kanser dentatin disiasat ke arah payudara (*in vivo* dan *in vitro*) dan prostat (*in vitro*) kanser. Ethnopharmacologically, *Clausena excavata* Burm. F., telah digunakan sebagai ubat tradisional di timur Thailand untuk rawatan kanser. Dentatin (DTN) telah diasingkan daripada tumbuhan ini melalui pendekatan berpandu bio assay dan mekanisme apoptosis yang telah dikaji. Berkenaan dengan MCF-7 sel-sel payudara, DTN disebabkan cytotoxicity diperhatikan menggunakan asai MTT. Acridine oren / Propidium iodida mengotorkan telah digunakan untuk mengesan sel-sel apoptosis awal. Pemeriksaan kandungan yang tinggi (HCS) telah digunakan untuk melihat pemeluwapan nuklear, ketelapan sel, potensi membran mitokondria (MMP) dan cytochrome c dibebaskan. Apoptosis telah disahkan

dengan menggunakan clonogenic assay, laddering DNA dan caspase 3/7 dan 9 ujian. Reaktif oksigen pembentukan spesies, ungkapan Bcl-2/Bax dan kitaran penangkapan sel juga telah disiasat. Penglibatan NF-kB dianalisis menggunakan HCS assay. Peningkatan yang ketara dalam pemeluwapan chromatin dalam nukleus sel diperhatikan dalam analisis pendarfluor. Apoptosis itu disahkan oleh dikurangkan koloni sel-sel dalam clonogenic assay dan meningkat DNA rehat sel pada sel-sel dirawat diperhatikan sebagai tangga. Rawatan MCF-7 sel dengan DTN digalakkan apoptosis dengan sel isyarat kematian transducing yang mengurangkan MMP oleh turun-peraturan Bcl-2 dan up-peraturan Bax, mencetuskan c cytochrome pelepasan dari mitokondria untuk cytosol. Cytochrome dikeluarkan c mencetuskan pengaktifan caspase 9 dan kemudian algojo yang caspase 3/7. Rawatan DTN ketara ditangkap MCF-7 sel pada G0/G1 fasa ($p < 0.05$). ROS nyata didapati meningkat. Selain itu DTN ketara menghalang translokasi teraruh daripada NF-MB dari sitoplasma dengan nukleus. Ini sebahagian daripada kajian yang telah ditubuhkan untuk menyiasat potensi anti-proliferatif daripada dentatin (a coumarin semulajadi diasingkan daripada Clausena excavata Burm.F) terhadap kanser prostat dan untuk menggambarkan mekanisme yang mendasari tindakan. Rawatan dengan dentatin dos dependently menghalang pertumbuhan sel PC-3 dan LNCaP prostat garisan sel kanser, sedangkan ia menunjukkan kesan kurang sitotoksik pada normal prostat garis sel epitelium (RWPE-1). Kesan yg melarang dentatin pada prostat pertumbuhan sel kanser adalah disebabkan oleh induksi apoptosis seperti yang dibuktikan oleh Annexin V mengotorkan dan pengecutan sel. Kami

mendapati bahawa pengumpulan pengantara dentatin spesies oksigen reaktif (ROS) dan tahap ungkapan downregulated molekul anti-apoptotic (Bcl-2, Bcl-xL dan survivin), yang membawa kepada gangguan potensi membran mitokondria (MMP), membran sel kebolehtelapan dan melepaskan sitokrom c dari mitokondria ke dalam cytosol. Kesan-kesan yang berkaitan dengan induksi caspase-9, -3/7activities dan pemecahan DNA berikutnya. Di samping itu, kami mendapati dentatin yang menghalang TNF-α yang disebabkan translokasi nuklear P65, menunjukkan dentatin sebagai potensi NF-κB inhibitor. Ujian ketoksikan akut oleh pentadbiran intraperitoneal sehingga 1 g / kg pada tikus tidak menunjukkan sebarang tanda-tanda biokimia, anatomi, atau histopathological ketoksikan, dentatin mencadangkan agak boleh diterima dalam vivo. Suatu kajian vivo telah dijalankan untuk menentukan kesan dentatin (DTN) pada LA-7 sel yang disebabkan oleh tumor tikus susu. Dalam kajian ini, kita dinilai buat kali pertama potensi anti-tumor dentatin (30mg/kg LD dan 60 mg / kg berat badan HD), secara lisan ditadbir selama empat minggu terhadap LA7 yang disebabkan oleh susu karsinogenesis dalam SD tikus. Selepas kemunculan tumor pertama, tiga puluh tikus telah dibahagikan kepada lima kumpulan (n = 6). Kumpulan pertama yang terdiri dirawat tikus yang sihat dan berkhidmat sebagai kumpulan kawalan negatif normal (NNC), manakala kumpulan kedua terdiri tikus disebabkan untuk membangunkan tumor kelenjar susu dan berkhidmat sebagai kawalan positif. Ini kumpulan tikus yang menerima dos tunggal 1 ml minyak soya dan telah disifatkan sebagai kawalan tumor susu (MTC). Kumpulan ketiga kelenjar susu tikus tumor-bearing dirawat mingguan dengan 30/kg mg (dos

rendah) DTN dibubarkan dalam 1 mL minyak soya. Hasilnya, kumpulan ini telah ditugaskan kumpulan DTN-LD. Kumpulan yang keempat juga terdiri daripada kelenjar susu tikus tumor-bearing dan setiap tikus menerima 60 mg / kg (dos tinggi) DTN dibubarkan dalam 1 mL minyak soya dan diberikan kumpulan DTN-HD. Selain itu, kumpulan itu terdiri daripada lima tikus dengan kelenjar susu tumor yang dirawat dengan 10 mg / kg TAM dibubarkan dalam 1 mL minyak soya dan ditugaskan sebagai keputusan Kumpulan.Elemen TAM mencadangkan DTN yang mempunyai kesan yang lebih baik pada tumor berbanding TAM, yang dinaikkan pangkat apoptosis dalam tikus susu kelenjar tumor. Walau bagaimanapun, DTN-HD menunjukkan kesan yang lebih berpanjangan mencadangkan bahawa DTN boleh menjadi dadah masa depan penting dalam kemoterapi kanser payudara. Oleh itu, kajian selanjutnya diperlukan untuk terus menyiasat dan membangunkan sistem penyampaian ubat untuk DTN dalam rawatan kanser. Bersama-sama, keputusan yang dibentangkan dalam kajian ini menunjukkan bahawa DTN yang menghalang percambahan MCF-7, PC-3 dan sel-sel LNCaP, yang membawa kepada penangkapan kitaran sel dan kematian sel diprogram, yang telah disahkan melalui laluan mitokondria dengan penglibatan NF-kB isyarat laluan. Dalam kajian vivo mencadangkan DTN yang mengurangkan tekanan oksidatif, menghalang percambahan, mendorong apoptosis mitokondria dikawal selia, oleh itu, mengurangkan LA-7-disebabkan karsinogenesis dalam kelenjar susu tikus. Oleh itu, kami mencadangkan dentatin yang mungkin mempunyai nilai terapeutik dalam payudara dan prostat rawatan kanser layak pembangunan selanjutnya.

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ISMAIL ADAM ARBAB
June 2013

I certify that a Thesis Examination Committee has met on 11 June 2013 to conduct the final examination of Ismail Adam Arbab Ishag on his thesis entitled ‘‘Characterization of Dentatin Isolated from *Clausena excavata* and its Potential Use for Treatment of Human Breast and Prostate Cancers’’ 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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This thesis was 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 were as follows:

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DECLARATION

I 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, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

ISMAIL ADAM ARBAB ISHAG

Date: 11 June 2013

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

%	Percentage
µl	Microlitre
0.05	Level of Significance (Type 1 error)
10^6	1000,000
20X	Twenty Times
40X	Fourty Times
100	One 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
CC	Column Chromatography
cm	Centimeter
CO_2	Carbon dioxide

CMC	Carboxymethyl cellulose sodium salt
DAB	3, 3' –diaminobenzidine
DEN	Diethylnitrosamine
DCF	Dichlorofluorescein
DCFH-DA	2,7-dichlorofluorescin diacetate
DI-MS	Direct Infusion-mass spectra
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DTN	Dentatin
EDTA	Disodium Ethylene Diaminetetracetate
EtOH	Ethanol
FFPE	Formalin Fixed and Parraffin Embbeded
FITC	Fluorescein Isothiocyanate
g	Gram
h	Hour
HBSS	Hank's balanced salt solutions
H&E	Hematoxylin and eosin
HCS	High content screening
HPLC	High performance liquid Chromatography
i.p.	Intraperitoneal
IBS	Institute of Bioscience
IC_{50}	Inhibitory Concentration (50%)
IR	Infrared

Kg	Kilogram
KV	Kilovolt
LD_{50}	50% lethal dose
mg	Miligram
MeOH	Methanol
MHz	Megahertz
Min	Minute
mL	Mililitre
MMP	Mitochondrial membrane potential
MPTP	Mitochondrial membrane transition pores
mRNA	Messenger Ribonucleic acid
MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
n (N)	The Number of experimental unit per group (replicates)
NF-*K*b	Nuclear factor-kappa B
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NMR	Nuclear Magnetic Resonance
OD	Optical density
^{o}C	Centigrade
P	Probability Value of Test Statistic
p.p.m	Part per million

PBS	Phosphate buffer saline
PARP	Poly(ADP-ribose) polymerase
pH	Hydrogen ion concentration
RNA	Ribonucleic acid
Rpm	Revolution per minute
RTCA	Real time cell analyzer
S.D.	Standard deviation
SD	Spraque Dawley
S.E.M.	Standard error of the Mean
TAE	Tris Acetate EDTA
Tris-HCl	Tris-Hydrochloride
TLC	Thin layer Chromatography
TNF-α	Tumour necrosis factor alpha
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling assay
UPM	Universiti Putra Malaysia
UV	Ultraviolet
v/v	Volume over volume
Vs	Versus
w/v	Weight over volume

CHAPTER 1

INTRODUCTION

1.1 Introduction

Cancer is a disease having complex problems that continues to intrigue researchers in the fields of plant chemistry, medicine and ethnopharmacology (Arnold, 2002). It is a malady characterized by abnormal uncontrollable cell growth (Kim, 2001; Sierra *et al*., 1995). It is also a hereditary ailment which represents a principal cause of human demise worldwide. Cancer is exceedingly well-known as one of the most severe mankind pain and still remains as a major threat to the lives of human beings (Conolly, 1998).

To date, there are more than one hundred types of diseases classified under cancer, each depending on the type of tissue being affected and the primary cause of this uncontrollable cell division, whether it is a genetic factor, viral infection or a combination of both (Dinshaw *et al*., 2006). In Malaysia, the occurrence of cancer is on the rise (Jemal *et al*., 2011; Lim, 2002). According to statistics reported by MAKNA (National Cancer Council), cancer ranked fourth leading to death amongst the medically recorded and certified deaths (Lim, 2002). Almost 70,000 new cancer cases were diagnosed among Malaysians in the West part of Malaysia between 2003 and 2005 (Sulaiman, 2010).

Breast cancer, specifically invasive ductal carcinoma (IDC), is the ultimate prevalent type of malignancies widespread in the world with remarkable annual incidence (Bachman *et al*., 2004). Breast cancer is considered as the most frequent cancer (2^{nd}) after lung cancers, amongst 17 most common cancers reported in Malaysia (18 per cent). It ranked 13^{th} by type among top 50 causes of death in Malaysia and 10^{th} by age. Deaths due to breast cancer in Malaysia attained 1,716 or 1.68% of total deaths. The age adjusted Death Rate is 15.83 per 100,000 of population ranks Malaysia 100 in the world (Arbab *et al*., 2013; Jemal *et al*., 2011).

Despite the presentation of supplementary efficacious remediation, Rates of mortality due to cancer of breast are increasing (Parkin *et al*., 2001) and it is considered as a deep-seated illness, certainly for hormone-sensitive cancerous tumors, which may be managed by successive endocrine therapy (Goldhirsch *et al*., 2005). Approximately, 80% of all breast cancers are (hormone-sensitive) invasive ductal carcinomas (Axelsson *et al*., 1995). Therefore, expressed estrogen and/or progesterone receptors (ER and/or PgR) are more possibly to comply with endocrine treatment (Macpherson *et al*., 2000).

The foretelling for breast cancer is mostly relying on the stage of the ailment at diagnosis. Five-year survival rates range from 84% for early disease to just 18% for advanced cancers (Sainsbury *et al*., 2000). Therefore, the goal of treatment also depends on the cancer stage at diagnosis (Parkin *et al*., 2001). Early breast cancer (EBC) is potentially curable and therefore, the primary treatment aim is to

prevent recurrence and prolong overall survival without causing complications (Mikeljevic *et al*., 2003). As treatments for advanced breast cancer are essentially palliative, goals in this setting are to achieve sustainable duration of response towards treatment and maintenance of patients' quality of life with minimum of treatment-related toxicity (Fallowfield *et al*., 2003).

Prostate cancer on the other hand is one of the principal reasons of deaths in men global spread with considerable mortality rate due to the progressive behavior of androgen-independent cells, which become insensitive to hormone ablation therapy (Fitzpatrick *et al*., 2009; Quinn and Babb, 2002). This disease starts when mutated cells in the prostate begin splitting and expanding without control. The resulted cancerous tumors can distribute to other parts of the body murdering ordinary tissues. The ranking of prostate cancer in Malaysia is in the sixth position amongst the ten most frequent cancers in males (Quinn and Babb, 2002).

Despite these difficulties, few progresses have been made to determine the morphological lesions that may act as potential precursor lesions. The most likely precursor lesion leading towards prostate cancer is prostatic intraepithelial neoplasia (PIN) (Qian *et al*., 1995). Unfortunately, the cellular and molecular pathways that contribute to the genesis and progression of prostate cancer remains poorly understood till today.

Plants are considered as the oldest source of pharmacologically active bio-compounds that contribute most significantly in disease treatments throughout mankind history (Rates, 2001). There are presently about 200,000 to 550,000 species of plants, as estimation on Earth Around the world (Borris, 1996; Raven, 1988). Comparatively small part representing about (1 to 10%) of these is utilized to day as nutrition by mankind and also as food by other animal species. It has been estimated that above 20% of these plant species are used for medicinal targets (Moerman *et al*., 1996). Of the 90 anticancer drugs commercially available prior 1982 in US and worldwide approved anticancer drugs between 1984and 1995, 62% are of natural origin (Cragg *et al*., 1999). Malaysia is one of the sub-tropical countries blessed with vast biodiversity and current efforts are concentrating on herbal plants as the source for modern medicine (Sajise and Ahmad, 2007).

Plant-derived drugs remain an important resource, especially in developing countries, to combat serious diseases. A number of medicinal plants containing bioactive compounds are included in our daily diet or used as natural medicine for treatment of several diseases (Krief *et al*., 2005; Taylor *et al*., 2001). Scientific experiments have verified the anticancer properties of many of these plants and their bioactive compounds elucidated (Mauli *et al*., 1997; Normile, 2004). Bioassay offers advantage in the standardization and quality control of heterogeneous botanical products. To achieve significant application to its usefulness, today's analysis of natural products must incorporate bioassays. Bioassays play important roles in the discovery of bioactive agents in natural

products (Mukherjee *et al*., 2001). To this, the use of guided fractionation of crude extracts towards isolation of pure bioactive compounds has been the primary aim to the use of bioassays for screening.

The Rutaceae are herbs, shrubs, and trees with glandular punctate, commonly with strong smelling herbage comprising of nearly 150 genera and 1,500 species that are further characterized by the common occurrence of spines and winged petioles (Carlsen and Weismann, 2007). Members of this family have been essential in providing many natural products of interest in the fields of phytochemistry and pharmacology. They have attracted continuous interest due to their needs in households with regards to their versatile chemical and biological properties (Arbab *et al*., 2012a; Bergman and Pelcman, 1990).

Clausena excavata Burm. F. is a wild shrub, belonging to the Rutaceae family (Arbab *et al*., 2012a; Taufiq-Yap *et al*., 2007). Considerable work has been done on the phytochemistry of *Clausena excavata* in recent years, with many previously unidentified secondary metabolites now currently being reported by phytochemists (Kongkathip *et al*., 2010). In respect to this, a large number of secondary metabolites, mainly alkaloids, coumarins and few limonoids have been isolated from different parts of this plant, using different techniques of extraction and purification during the last 20 years (Zhi, 2006). The structures of these compounds have also been elucidated using different spectroscopic methods (Arbab *et al*., 2011; Shier, 1983). Dentatin is one promising bio-compounds originally isolated from the roots of *Clausena excavata.* It is a secondary

metabolite that belongs to the coumarin class (Arbab *et al*., 2011; Mowat and Murray, 1973).

Coumarins are naturally occurring benzopyrene derivatives (Kostova, 2005, 2006). Several coumarins have been identified from natural sources, especially green plants. Coumarins have attracted intense interest in recent years because of their diverse pharmacological properties, their cytotoxic effects being the most extensively examined. Therefore, these coumarins which characterized by valuable cytotoxic properties perform as an exploitable source of new anticancer agents, which might provide solutions in solving the problem of side-toxicity and resistance original sin. These natural compounds have served as valuable leads for further design and synthesis of more active anti cancer analogues.

To date, there has been no detailed investigation reported elsewhere on dentatin regarding its anticancer activities towards breast and prostate cancers. No previous study had reported the importance of dentatin neither as anti-cancer nor to its mechanistic of action for possible treatment of human breast and prostate cancers. Therefore, this current study provided detailed investigations to the characterization and potential use of dentatin, isolated from *Clausena excavata* for treatment of human breast and prostate cancers.

This study was divided into two principle stages, each of which was further divided into sub stages. Stage I demonstrated the isolation of the bioactive compound as New Chemical Entity. In this respect, the main objective was to

achieve a bioassay guided fractionation of the crude extract of the roots and leaves of *Clausena excavata*, using several chromatographic solvent systems. The active phytochemical pure compound present in the plant was further identified and characterized. *In vitro* assays provided evidence of apoptosis induction to the phytochemical compound under investigation.

Stage II further demonstrated the phytochemical compound as potential New Molecular Entity. The main objective in this part of the research was to establish the probable mechanism of action of the compound as potential use for treatment of human breast and prostate cancers. This includes both *in vitro* and *in vivo* investigations of the compound to look into the possible apoptotic mechanism of action and to intervene cancer progression of induced mammary gland tumors in *Sprague Dawley* rats, respectively.

This current investigation has unraveled the potential capability of the phytochemical, isolated and later identified, of having potential anti cancer activities towards prostate and breast. The information provided through this investigation will be useful for future documentation of the compound intended as New Chemical Entity, in turn shall provide the basis to conduct human Clinical Trials Phases I and II, in a near future.

1.2 Aims of the Study

General Objective

To isolate a phytochemical metabolite, dentatin (DTN) from *Clausena excavata* and to determine its affects on treatment of human breast and prostate cancer cells compared to normal cells and its potentiality as anti cancer towards induced rat mammary gland tumors.

Specific Objectives

1. To isolate and purify a bioactive phytocompound (**Dentatin, DTN)**, from *Clausena excavata* using a developed bioactivity guided fractionation and existing techniques of HPLC and LCMS.

2. To perform structural elucidation and characterization of DTN using spectral analysis techniques of NMR, IR, DIMS and UV.

3. To study the probable mechanistic properties of DTN as anti-cancer towards human breast and prostate cancer cell lines, involving molecular techniques and protein assays (*in vitro*).

4. To investigate *in vivo* anticancer efficacy of DTN in female Sprague Dawley rats induced with breast mammary gland tumors.

5. To further use this study as a basis to doccument DTN as a New Potential Chemical Entitry, in the course to prepare for human clinical trials.

1.3 The Hypothesis of the Research

The current study could provide useful information in solving the existing problems to treat human breast and prostate cancers in Malaysia as the compound under investigation could be an effective anti-cancers for these cancers.

The phytocompound dentatin is expected to eliminate breast and prostate cancer cells through the induction of apoptosis, a programmed cell death, without affecting normal breast and prostate cells. Investigating the basic mechanism of the compound as anti-cancer suggest possible apoptosis cell death induction through cellular mitochondria involving signaling pathways of proteins implicated in breast and prostate cancer pathogensis. This compound, useful as anticancer for breast and prostate, is the first to be discovered in this world today, which eventually provides more avenues to investigate further, its usefulness as a probable anticancer drug for treatment.

The current study hypothesized that dentatin has cytotoxic effect on human breast cell lines MCF-7 and prostate cell lines, PC-3, LNCaP, and anti-proliferative effect on induced tumors of rat mammary glands.

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