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CYTOTOXICITY ACTIVITY AND MECHANISM OF ACTION OF ARBORININE, AN ACRIDONE ALKALOID FROM *Glycosmis pentaphylla* (RETZ.) DC.

BY

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A thesis submitted in fulfilment of the requirement for the degree of Master in Pharmaceutical Sciences (Pharmaceutical Chemistry)

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ABSTRACT

Cancer is one of the deadliest disease and the number of case being increasing over the time. Anticancer treatments are believed to have the ability to induce cell death via apoptosis to prevent the proliferation of cancer cell. Plant-based anticancer is expected to receive a huge public attention due to its potential on cancer therapy and availability of sources. Arborinine which is an acridone alkaloid found exclusively in Rutaceae family has been shown to inhibit proliferation of a wide variety of cancer cell lines. It was isolated from a Malaysian Rutaceae, *Glycosmis pentaphylla* (Retz.) DC. The present study is aimed to isolate the arborinine and to investigate its cytotoxicity activity with the mechanism of action on human mammary gland adenocarcinoma (MCF-7) cancer cell line. Isolation of arborinine was conducted from the leaves of *G. pentaphylla* via acid-base extraction and column chromatographic techniques. The structure of arborinine was authenticated by comparing its spectroscopic data with that of published reports. Cytotoxicity activity of arborinine was conducted towards selected cell lines MCF-7, human non-small cell lung carcinoma; A549 and H1299 through 3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide colorimetric assay (MTT) assay. TdT-dependent dUTP-biotin nick end-labelling (TUNEL) and caspase 3/7 assay were used to investigate the apoptosis mechanism induced. Involvements of cytochrome c and bax protein were further evaluated through western blotting to determine the mechanism of action. The result showed that 0.1295 g (12.16 %) of arborinine was yielded from 1.0653 g of alkaloidal extract. Arborinine showed the most significant inhibitory activity on MCF-7 with IC_{50} of (10.50 ± 0.58 µM) compared to A549 and H1299 cancer cell lines at all tested concentrations with IC_{50} of (70.3 ± 1.77 µM) and (75.0 ± 0.29 µM) respectively. Apoptotic nuclei were identified by the presence of dark brown staining and activation of caspase-3 and -7 which further confirmed that the mode of cell death induced by arborinine was via apoptosis. However, the apoptosis triggered by arborinine was not associated with the expression of bax and cytochrome c. The data presented suggest that arborinine can be a potential lead for further investigation in the development of a new anticancer agent against breast cancer.

خلاصة البحث

السرطان هو أحد أكثر الأمراض فتكا وحالات الإصابة به في تزايد مستمر. لدى العلاجات المضادة للسرطان القدرة على قتل الخلايا عن طريق موت الخلايا المبرمج لمنع انتشار الخلايا السرطانية، كما هو مثبت بحثيا، ومن المتوقع أن تتلقى العلاجات المضادة للسرطان النباتية الأصل اهتماما كبيرا من العامة بسبب إمكاناتها في العلاج وتوافر المواد الخامة. القلويد الأكريدوني أربورنين (Arborinine) موجود حصرا في العائلة النباتية روتاكيا (Rutaceae) وقد تبين أنه فعال ضد انتشار مجموعة كبيرة من خطوط الخلايا السرطانية، وقد تم عزل الأربورنين من روتاكيا ماليزية وهي نبتة الجليكوسميس بينتافيلا *Glycosmis pentaphylla* (Retz.) DC. هدفت هذا الدراسة لعزل الأربورينين والتحقيق في سميتها الخلوية مع آلية العمل على السرطانة الغدية للغدة الثديية (MCF-7). تم عزل الأبرورينين من أوراق نبتة الجليكوسميس بينتافيلا بالاستخلاص الحمضي القاعدي طرق الكروماتوغرافيا العمودية. تم التأكد من بنية مركب الأبرورينين عن طريق مقارنة البيانات الطيفية مع البيانات المنشورة. تم قياس نشاط السمية الخلوية للأربورينين ضد خطوط خلايا مختارة وهي MCF-7، وسرطان الرئة البشري ذو الخلايا غير الصغيرة (A549، و H1299). تم هذا القياس بفحص MTT اللوني [3-(4,5)-تايوزول ثنائي الميثيل-2-yl)-2,5-بروميد رباعي الزوليوم]. تم استخدام اختبار TUNEL (التوسيم الذيلي بالصدعات بواسطة dUTP-بيوتين المعتمد على TdT) واختبار كاسبيس 3/7 للتحقيق في آلية موت الخلايا المبرمج المستحث. تم أيضا تقييم تداخلات السيتوكروم c وبروتين الباكس بواسطة طريقة لطخة ويسترن لتحديد آلية العمل. أظهرت النتائج أنه تم الحصول على 0.1295 غم (12.16٪) من الأبرورينين من 1.0653 غم من المستخلصات القلوية. أظهر الأربورينين أقوى نشاط تثبيطي على MCF-7 بمعدل IC_{50} بلغ 10.50±0.58 ميكرومتر مقارنة مع خطوط الخلايا السرطانية A549 و H1299 في جميع التركيزات المفحوصة بمعدل IC_{50} البالغ 70.3±1.77 ميكرومتر لـ A549 و 75.0±0.29 ميكرومتر لـ H1299. تم التعرف على الأنوية الميتة بفعل موت الخلايا المبرمج من خلال وجود تلطيخ بني داكن وتفعيل كاسبيس-3 و -7 وأكد ذلك أن آلية عمل الأربورينين المميت للخلايا كان عن طريق موت الخلايا المبرمج. ومع ذلك، لم يكن موت الخلايا المبرمج الناجم عن أربورينين مرتبطا بالتعبير الخلوي لباكس والسيتوكروم c. تشير البيانات المقدمة إلى أنه بامكان الأربورينين أن يكون مركبا واعدا للمزيد من الدراسات في تطوير عامل جديد مضاد لسرطان الثدي.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master in Pharmaceutical Sciences (Pharmaceutical Chemistry).

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DECLARATION

I hereby declare that this thesis is the result of my own investigation, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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This thesis is dedicated to all who have been by my side during this meaningful journey.

May Allah SWT bless us here and hereafter.

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LIST OF ABBREVIATIONS

^{1}H NMR	Proton Nuclear Magnetic Resonance
AC	Acetone
AIDS	Acquired Immune Deficiency Syndrome
AIF	Apoptosis-inducing factor
APS	Ammonium persulfate
ASR	Age-standardized Rate
ATP	Adeno-sine Triphosphate
ATR-FTIR	Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy
Bad	Bcl-2-associated death promoter
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2 associated X protein
Bcl-2	B-cell lymphoma 2
Bcl-XL	B-cell lymphoma-extra large
BCR-ABL	Breakpoint cluster region Abelson kinase
BH	Bcl-2 homology
Bid	BH3 interacting-domain death agonist
Bim	Bcl-2-like protein 11
BME	Beta Mercarptoethanol
BSA	Bovine Serum Albumin
$C_{16}H_{15}NO_4$	Arborinine (molecular formula)
CARD	Caspase Recruitment Domains
caspase	Cysteine-dependent aspartate-specific proteases
CDKs	Cyclin-Dependant Kinases
CLL	Chronic Lymphocytic Leukaemia
CO_2	Carbon dioxide
DAB	Diaminobenzidine
DCM	Dichloromethane
DD	Death Domain
ddH_2O	Double distilled water
DED	Death Effector Domain
DEVD	aspartic acid-glutamic acid-valine-aspartic acid
DISC	Death Inducing Signalling Complex
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EA	Ethyl acetate
ER	Endoplasmic Reticulum
FADD	Fas-associated protein with death domain
FBS	Foetal Bovine Serum
GC-MS	Gas Chromatography-Mass Spectrometry
H_2O	Water
HCL	Hydrocholoric acid
IARC	International Agency for Research on Cancer
IC_{50}	50 % inhibitory concentration

IDCR	Ionic Detergent Compatibility Reagent
IGFBP	Insulin-like growth factor-binding protein
IH	Intermittent Hypoxia
IMS	Intermembrane Space Proteins
IRS1	Insulin receptor substrate 1
MEF	mouse embryo fibroblasts
MeOH	Methanol
MIC	Minimum inhibitory concentration
MOMP	Mitochondrial Outer Membrane Permeabilization
MTT	3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide
$NaNO_3$	Sodium azide
NaOH	Sodium hydroxide
NCI	National Cancer Institute
NCR	National Cancer Registry
Noxa	Known as Phorbol-12-myristate-13-acetate-induced protein 1
NSCLC	non-small cell lung cancer
Omi	Known as HtrA2 (HtrA serine peptidase 2)
PARP	polyADPribose polymerase
PBS	Phosphate Buffer Saline
PE	petroleum benzene
PTP	Permeability Transition Pore
PVDF	Poly Vinylidine Flouride
R_f	Retention factor
rTdT	Terminal Deoxynucleotidyl Transferase, Recombinant
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	SDS-Polyacrylamide Gel Electrophoresis
Smac	Second mitochondria-derived activator of caspases
Streptavidin HRP	Horseradish peroxidase-labelled streptavidin
tBID	Truncated BH3 interacting-domain
TBS	Tris Buffered Saline
TEMED	N,N,N',N-tetramethylethylenediamine
TLC	Thin Layer Chromatography
TNFR	Tumor Necrosis Factor Receptor
TRAIL	TNF-Related Apoptosis Inducing Ligand
TUNEL	TdT-dependent dUTP-biotin nick end-labeling
UV-VIS	Ultraviolet-Visible Spectrophotometry
WHO	World Health Organization

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND

Cancer is one of the deadliest disease and the number of case being increasing over the time. Cancer can be observed by its abnormal cell growth where, there are too much cell division yet there were too little cell death at one time. National Cancer Registry (NCR) Ministry of Health Malaysia presented data with the highest frequency of cancer suffered by women in Malaysia, was breast cancer (Zainal Ariffin & Nor Saleha, 2011). The treatment for breast cancer takes several factors into consideration having the standard treatment modality with surgery, radiation therapy, chemotherapy and targeted therapy. These treatments, were believed could lead to years painful side effects like hairfalls, peripheral numbness of limbs, fatigue, hair loss, lack of appetite, insomnia, constipation, nausea or vomiting and others. In other word, current cancer drugs still give lots of side effects on normal cell alongside with its beneficial effect in killing the cancer cells, hence the importance of conventional therapies may decline. Thus, the scientists and scholars are excessively looking forward for alternative via natural based anticancer and chemoprentive substances in order to find the best preventive measures that might yield fewer undesireable side effects.

There are variety of cancer treatments and part of it are the usage of anticancer agents which exhibit the mechanism of cell death via apoptsis as one of the way to prevent the proliferation of cancer cell. Plant-based anticancer agent is expected to received a huge public attention due to its potential on cancer therapy, availability of sources and might be safer compared to the existing drugs.

Glycosmis pentaphylla (Retz.) DC. is one of the plants containing alkaloid that possess potential therapeutic value as an anticancer agent. Recently, the interest to develop model for anticancer agents from alkaloids of Rutaceous family was increased among the reseachers. It might be due to their consistent ability to interact with DNA or with systems involved in the control of the DNA topology repair and replication. Arborinine **(3)**, an acridone alkaloid extracted from the Rutaceous species, *Ruta graveolens* L. has demonstrated best in inhibiting the proliferation of HeLa, MCF-7 and A431 (Rethy et al., 2007). Nevertheless, the study on anticancer properties of arborinine **(3)** is still lacking especially from Malaysian Rutaceous species.

Thus, this research is aimed to investigate the anticancer properties of arborinine **(3)** extracted from Malaysian Rutaceous species, namely *G. pentaphylla* (Retz.) DC. and the mode of cell death induced as well as the mechanism of action of this compound on selected cancer cell lines. Cytotoxicity assay was employed to determine the antiproliferative activity of arborinine **(3)**. Following that, TdT-dependent dUTP-biotin nick end-labelling (TUNEL) and Caspase-3/7 assay method were done to study the mode of cell death induced by arborinine **(3)**. Further investigation on proteins expression involved under the apoptosis mechanism was employed by western blotting technique. This study was expected to provide information on the antiproliferative activity, mode of cell death induced and the mechanism of action of arborinine **(3)**. Promising findings on the anticancer properties of arborinine **(3)** would be very useful as the platform for future study as a new compound that might be introduced as a single or combination treatment for cancer.

1.2 RESEARCH QUESTIONS

(1) Does arborinine play a role as an antiproliferative agent towards cancer cells?

(2) What is the mechanism of growth inhibition or cell death induced by arborinine?

(3) If the mode of cell death is via apoptosis, what is the mechanism of action and proteins involved?

1.3 OBJECTIVES

This study was embarked on the following objectives:

(1) To isolate and authenticate the arborinine, an acridone alkaloid from *G. pentaphylla*.

(2) To determine the antiproliferative activity of arborinine on cancer cell lines.

(3) To investigate the mode of cell death induced and the mechanism of actions of arborinine in cancer.

1.4 HYPOTHESES

Arborinine, an acridone alkaloid from *G. pentaphylla* is a new potent antiproliferative agent which induce apoptosis in various types of cancer cell lines.

CHAPTER TWO

LITERATURE REVIEW

2.1 NATURAL PRODUCT AS ANTICANCER AGENT

Natural product, especially from plants, has been used to treat various diseases in past years. Natural compounds of diverse structures, isolated from plant sources have been considered prototypes, leads or heads of series and later structural modification via semi synthesis process has generate structural analogues with greater pharmacological activity and therapeutic possibilities with fewer side effects (Gordaliza, 2007). Naturally occurring compounds in fruits and vegetables played a role in the development of effective anticancer agents (Shoeb, 2006). Currently, 60 % of the anticancer used like camptothecins, bryostatins and anthracyclines are derived from natural sources which are plants, marine organisms and microorganisms, respectively (Cragg & Newman, 2005). Moreover, 92 anticancer drugs commercially available in US and worldwide approved anticancer are originated from nature (Newman & Cragg, 2007).

Paclitaxel or taxol is most well-known example of natural product-based cancer drug. It was isolated from the bark of *Taxus brevifolia* or known as Pacific yew tree (American Chemical Society, 2003). Its molecular formula is $C_{47}H_{51}NO_{14}$ and its molecular weight is 853.9 Da (Ding & Zhang, 2016).

In previous years, various parts of *Taxus brevifolia* and other *Taxus* species were used to treat non-cancerous and cancerous case by Native American Tribes and Indian people (Ayurveda system) respectively (Cragg & Newman, 2005). It is one of the current drug that being prescribed to patient suffering with breast cancer, either to

be taken alone or in combination with other drugs. Moreover, it was used to treat AIDS-related kaposi sarcoma, lung cancer and ovarian cancer (Shoeb, 2006). It targets the cell's microtubules, binding to the microtubule assembly, blocking the segregation of chromosomes and slowing cell division and growth of the cancer cells.

2.2 CANCER

Cancer is known as a collection of diseases with uncontrolled growth as common features. Cancer was characterised by unregulated cell growth that invading or metastasizing sorrounding tissues (King, 2000). Cancer occurs due to a loss of balance between cell division and cell death (Wong, 2011). Later, it transforms healthy cell to cancerous one as the cells failed to respond to nomal regulatory process. The cancer cell is differ from normal cell in a way that it ables to ignore the cell death signal, influence and evade the immune system as well as less specialized, making them dividing so fast.

Cancer is generated through multistep of molecular mechanisms resulting from accumulation of errors in the vital reguatory pathway named carcinogenesis (King, 2000). One of the main errors is the DNA mutation which acquired from parents or cancer-causing environmental exposures leading to genetic changes. It affects the genes that driving the cancer; proto-oncogenes, tumour suppressor genes and DNA repair genes. Any alteration involving these genes may cause cells to divide in uncontrolled manner.

There are two factors that can cause cancer namely internal and external factors. The internal factors are including heredity, immunology and hormones whereas the external factors are chemicals, viruses, diet and radiation. Many factors

interact to each other to disturb the cell division making cancer become a complicated disease to treat (Jones, 2002).

2.2.1 Epidemiology of Cancer

Cancer is a deadliest disease and becomes major cause mortality worldwide. Referring to the data from GLOBOCAN project which was produced by International Agency for Research on Cancer (IARC), it was estimated that 14.1 million new cancer cases and 8.2 million cancer related death were occurred worldwide (World Health Organization [WHO], 2017). Breast cancer is the most frequently diagnosed cancer alongside with lung cancer regardless the gender in less developed countries. In females, it was estimated that 1.7 million new cases and 521, 900 deaths associated with breast cancer occurred in 2012. In total, breast cancer alone accounts for 25 % of all cancer cases and 15 % of all cancer deaths among female worldwide (Torre et al., 2015).

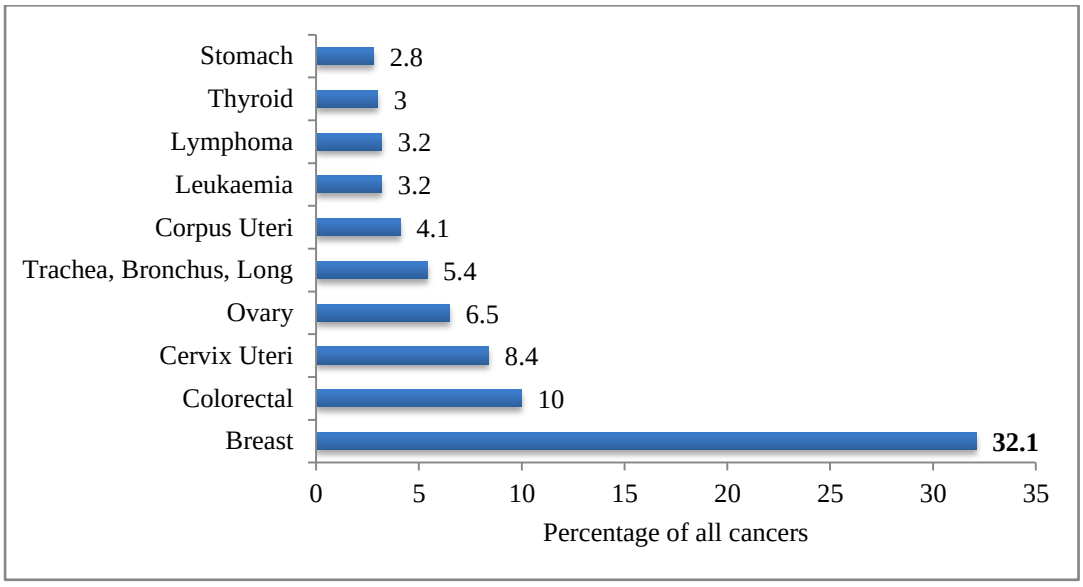

Figure 2.1: Most frequent cancers in female, Malaysia. (Zainal Ariffin & Nor Saleha, 2011)