UNIVERSITI PUTRA MALAYSIA

CHARACTERISATION OF PLANT DERIVED DAMNACANTHAL AND NORDAMNACANTBAL INDUCED CYTOTOXICITY ON HUMAN HT29 COLON ADENOCARCINOMA CELL LINE

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By

KHOR TIN OO

Thesis Submitted in Fulfilment of the Requirements for the Degree of Master of Science in the Faculty of Food Science and Biotechnology
Universiti Putra Malaysia

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DEDICATION

Dedicated to my beloved choon, my parents & young sister

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science.

CHARACTERISATION OF PLANT DERIVED DAMNACANTHAL AND NORDAMNACANTHAL INDUCED CYTOTOXICITY ON HUMAN HT29 COLON ADENOCARCINOMA CELL LINE

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Chairman: Associate Professor Abdul Manaf Ali, Ph.D.

Faculty : Food Science and Biotechnology

Nordamnacanthal and damnacanthal are two anthraquinones isolated from the roots of *Morinda elliptica*. They were found to exhibit cytotoxic activity against HT29 human colon adenocarcinoma cells. The cytotoxic concentrations of damnacanthal and nordamnacanthal that inhibited 50% growth (IC_{50}) of HT29 were 17 µg/ml and 7 µg/ml respectively. For the comparative purposes, the IC_{50}s of several cytotoxic drugs against HT29 were also determined. The inhibition effect of nordamnacanthal was found to be comparable to etoposide (IC_{50} = 7 µg/ml), cisplatin (IC_{50} = 5 µg/ml) and doxorubicin (IC_{50} = 6 µg/ml). The compound was found to be less active than methotrexate (MTX) (IC_{50} < 0.05 µg/ml) and leunase (IC_{50} = 2 µg/ml). On the other hand, the cytotoxic effect of damnacanthal was less active as compared to all cytotoxic drugs. However both compounds were found to be less toxic against non-cancerous fibroblast 3T3 cells with the IC_{50}s of 30 µg/ml (damnacanthal) and 21 µg/ml (nordamnacanthal) respectively. Furthermore, damnacanthal and nordamnacanthal were found to induce apoptosis on HT29 cells at their IC_{50} concentration as

demonstrated by conventional agarose gel electrophoresis and also morphological alterations. DNA laddering was obtained after 12 hours of treatment by both compounds in a dose-independent but time-dependent fashion. Both compounds also caused cell death with apoptotic features such as cell shrinkage, membrane blebbing, nuclear fragmentation, and the presence of apoptotic bodies. In addition, caspase-3 was found to be activated during the execution of apoptosis induced by these compounds. This caspase activation was inhibited by a peptide based general caspase inhibitor, benzyloxycarbonyl-Val-Ala-Asp (0Me) fluoromethylketone (Z-VAD-FMK). In conclusion, this study demonstrates the potential antitumor activites of damnacanthal and nordamnacanthal.

Abstraks tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains.

PENCIRIAN SITOTOKSIKSITI YANG DIARAHKAN OLEH DAMNACANTHAL DAN NORDAMNACANTHAL DARI TUMBUHAN KE ATAS JUJUKAN SEL ADENOKARSINOMA USUS MANUSIA, HT29

Oleh

KHOR TIN OO

Januari 2001

Pengerusi: Profesor Madya Abdul Manaf Ali, Ph.D.

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Nordamnacanthal dan damnacanthal merupakan dua jenis antrakuinon yang diasingkan daripada akar *Morinda elliptica*. Mereka didapati menunjukkan aktiviti sitotoksik ke atas jujukan sel adenokarsinoma kolon manusia, HT29. Kepekatan sitotoksik damnacanthal dan nordamnacanthal yang dapat merencat pertumbuhan sel HT29 sebanyak 50% (IC_{50}), adalah masing-masingnya 17 µg/ml dan 7 µg/ml. Untuk tujuan perbandingan, IC_{50} bagi beberapa jenis dadah sitotoksik ke atas HT29 juga ditentukan. Kesan perencatan nordamnacanthal didapati agak setara dibandingkan dengan etoposid (IC_{50} = 7 µg/ml), sisplatin (IC_{50} = 5 µg/ml) dan doksorubisin (IC_{50} = 6 µg/ml). Sebatian tersebut didapati kurang aktif berbanding dengan methotrexate (MTX) (IC_{50} < 0.05 µg/ml) dan leunase (IC_{50} = 2 µg/ml). Sebaliknya, kesan sitotoksik damnacanthal adalah kurang aktif berbanding dengan kesemua dadah sitotoksik. Walau bagaimanapun, kedua-dua sebatian itu didapati kurang aktif ke atas jujukan sel fibroblas bukan-kanser, 3T3 dengan IC_{50} 30 µg/ml (damnacanthal) dan 21 µg/ml

(nordamnacanthal). Selain daripada itu, damnacanthal dan nordamnacanthal didapati mengarahkan apoptosis terhadap sel HT29 pada IC_{50} masing-masing sebagaimana yang ditunjukkan oleh elektroforesis gel agaros konvensyenal dan juga perubahan morfologi. "Penanggaan DNA" diperolehi 12 jam selepas dirawat oleh kedua-dua jenis sebatian dalam bentuk "bebas dos" tetapi "bergantung kepada masa". Kedua-dua sebatian juga menyebabkan kematian sel dengan ciri-ciri apoptosis seperti pengecutan sel, "membrane blebbing", fragmentasi nukleus, dan kehadiran "jasad apoptotik". Selain daripada itu, "caspase-3" didapati diaktifkan semasa apoptosis diarahkan oleh kedua-dua jenis sebatian. Pengaktifan itu adalah sensitif terhadap perencat caspase umum, benziloksikarbonil-Val-Ala-Asp (0Me) fluorometilketon, (Z-VAD-FMK). Sebagai kesimpulan, hasil pengajian ini menunjukkan potensi antikanser damnacanthal dan nordamnacanthal.

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I certify that an Examination Committee met on 12th January 2001 to conduct the final examination of Khor Tin Oo on his Master of Science thesis entitled "Characterisation of Plant Derived Damnacanthal and Nordamnacanthal Induced Cytotoxicity on Human HT29 Colon Adenocarcinoma Cell Line" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations 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.

Name: Khor Tin Oo
Date: 27/2/2001

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LIST OF ABBREVIATIONS

IC_{50}	Inhibition concentration at 50%
%	percentage
nm	nanometer
mg	milligram
µg	microgram
ml	milliliter
rpm	rotation per minute
mM	millimolar
UV	ultraviolet
bp	base pairs
EDTA	ethylenediamine tetraacetic acid
PBS	phosphate buffered saline
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
AO	acridine orange
PI	propidium iodide
OsO_4	osmium tetraoxide
SEM	scanning electron microscope
TEM	transmission electron microscope
ATCC	American Type Culture Collection
MTX	methotrexate
Z-VAD-FMK	benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone
pNA	p-nitroaniline
Ala	alanine
Val	valine
Asp	aspartic acid

CHAPTER I

INTRODUCTION

Throughout the history of civilization, several diseases have challenged human health. Leprosy was the most dreaded disease in ancient times. In Medieval and Renaissance Europe the scourge was the bubonic plaque or Black Death. Then in the 19th century, a major killer often associated with extreme suffering was the White Death, or tuberculosis. With the advances achieved in the 20th century in microbiology and pharmacology, many of the infectious diseases that formerly killed a large population have been overcome. However, in this century cancer becomes an increasing problem in developing as well as developed countries. Statistics have shown that one person gets cancer every 30 seconds while a person dies of cancer every 50 seconds. Each year cancer affects at least nine million people worldwide and kills five million. In Malaysia, cancer is the fifth major cause of death in government hospitals and the estimated cancer incidence is about 150 per 100,000. Meanwhile, the estimated number of new cancer cases in Malaysia per year is around 27,000 (Malaysia's Health, 1996).

Chemotherapy is one of the four major approaches used by physicians to destroy cancer cells selectively. Others included surgery and radiotherapy for treatment of localized tumors and immunotherapy, which aim to increase patient's own resistance to the cancer (Joseph and Joan, 1988).

Since the first recorded clinical trial of a chemotherapeutic agent took place in 1942, the field of chemotherapy has grown tremendously. Nowadays, emphasis has been placed on chemotherapy as a form of treatment for cancer patients instead of the use of surgery or radiotherapy. The objective of chemotherapy is to treat diseases without seriously harming the patient by the use of chemicals. Several potential chemotherapeutic agents have been discovered serendipitously while others have been discovered through large-scale experimental screening. Tropical rain forests including the one in Malaysia stores a large chemical diversity. Some of these natural products can be isolated and may become chemotherapeutic agents. Out of 12,000 species of higher plants in Malaysia, more than 1000 species are said to have therapeutic properties and currently being used in the local traditional medicine system (Said, 1995). Goniothalamin, a secondary metabolites isolated from the leaves and roots of *Goniothalamus spp.* has been shown to possess a potent antitumor activity in DMBA induced rat mammary tumor and human breast cancer cell lines (Zauyah and Azimahtol, 1992). Study by Ali *et al.* in 1996 showed that the fruits of *Cerbera manghas* exhibited antitumor activity against HeLa cell line with cytotoxic dose at 50%, CD_{50} value at 1 μg/ml. For the reason, recently a few private research institutions as well as local universities have started the program to prospect for drugs from plants.

Since there are more than 100 different types of cancer, screening for potential antitumor compounds is very important. For large scale antitumor drug screening, *in vivo* and *in vitro* models are used. Established human tumor cell lines are used in preliminary screening for potential antitumor drugs. This rational

approach is fairly inexpensive, rapid and capable of demonstrating high sensitivity (Shier, 1991).

Once certain compounds have demonstrated cytotoxicity against tumors in tissue culture or in small animals, the study of their actual mechanism of action is also very important. Many closely related derivatives could be synthesized by knowing their mode of action. Some of these derivatives have been great improvements over the original compounds in treating many types of cancer.

The aim of this research is to evaluate further the cytotoxic potential of two anthraquinone compounds, nordamnacanthal and damnacanthal on human colon cancer cell line, HT 29.

The objectives of this study are:

i) to determine the cytotoxicity of damnacanthal and nordamnacanthal on human colon adenocarcinoma HT29 cells.

ii) to study the effects of damnacanthal and nordamnacanthal on HT29 cells in terms of proliferation, morphological changes and the mode of cell death induced by the compounds.

iii) To identify the mode of action of damnacanthal and nordamnacanthal.

CHAPTER II

LITERATURE REVIEW

2.1 What is Cancer?

Cancer or a malignant tumor is also called neoplasm in the scientific or medical term. Neoplasm, meaning a new growth results from an inheritable change in a cell (or cells) which allow them to escape from many of the normal homeostatic mechanisms that control proliferation. When any of the dividing cells undergo this type of changes they are said to be transformed. Transformation may be triggered in a number of ways, including exposure to chemicals, certain viruses, and radiation. The basis of transformation is probably a mutation (a change in the primary structure of DNA) but it is likely to be influenced by epigenetic events (shifts in gene expression) (Evans, 1991).

2.1.1 Classification of Tumors

Tumors are classified based on a number of criteria including their behavior, their appearances and their origin. Basically they are two types of tumors, benign and malignant which differ in their behavior. Table 1 shows the major differences between benign and malignant cells.

Table 1: Major differences between benign and malignant cells

Feature	Benign	Malignant
Cytoplasm	Slight basophilia	Marked basophilia
Mitotic figures	Few and normal	Many and abnormal
Nucleus	Predominantly normal	Pleomorphic
Nucleoli	Little altered	Often swollen
Tissue structure	Usually normal	Dyplastic/anaplastic
Functions	Usually normal	Lost or deranged
Capsule	Usually intact	Often lacking
Metastasis	Never	Often
Local invasion	Rare	Common
Fatalities	Rare	Common

(Evans, 1991)

2.1.2 Characteristic Features of Tumor Cells

A summary of some of the features possibly altered in tumor cells is provided in Table 2. Many of these changes reflect alterations in cell metabolism/behavior without any readily obvious direction of change. Therefore it is difficult to define any universal tumor cell characteristic.

2.2 Molecular Basis of Cancer

It has been realized for many years that cancer has a genetic component and at the level of the cell it can be said to be a genetic disease. The genetic injury may be acquired in somatic cells by environmental agents or inherited in the germ-line. The clonal progeny of single genetically damaged progenitor cell will develop as tumor. Recently, the involvement of specific genes has been demonstrated at the molecular level. These specific genes are usually the targets of genetic damage and can be classified into three classes as growth-promoting proto-oncogenes, the growth-inhibiting tumor suppressor genes, and genes that regulate apoptosis (Cotran *et al.*, 1994).

2.2.1 Oncogenes

The term oncogenes are used to describe any gene sequence whose products are associated with neoplastic transformation. Many oncogenes causing human cancer are mutated versions of normal cellular genes that control growth