



**EVALUATING CYTOTOXICITY POTENCY OF GLUCOMORINGIN
FROM THE SEEDS OF *Moringa oleifera* Lam AGAINST PROSTATE
CANCER CELL LINE, PC-3**

By

NURUL ASHIKIN BINTI ABDUL KARIM

**Thesis Submitted to the School of Graduate Studies, Universiti
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Doctor of Philosophy**

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in fulfilment of the requirement for the degree of Doctor of Philosophy

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November 2019

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Institute : Bioscience

Inhibition of several protein pathways involved in cancer cell regulation is a necessary key in the discovery of cancer chemotherapy. *Moringa oleifera* Lam is often used in traditional medicine in several countries for the treatment of various illnesses. The plant was also found to contain bioactive compounds with therapeutic potential against various cancer cells. This study was carried out to determine the cancer cell inhibition of compounds from natural origins, specifically from the seeds of *M. oleifera*, where glucomoringin can be found abundantly and might carry the potential to inhibit the proliferation of prostate cancer cells.

The crude seed extracts were obtained from water, acetone, chloroform, ethanol, methanol, and hexane. The water extract produced the highest crude yield (21.78%). The crude extracts were initially screened for phytochemical content, followed by cell viability tests using MTT assay against MCF 7, HepG2, DU 145, and PC-3 cell lines. Crude hexane, acetone and chloroform, were found to have coumarin *and* fat. Crude ethanol contains coumarin, quinone and fat. Crude methanol contains only saponin, coumarin and quinone, and finally, crude water contains saponin, alkaloid and coumarin. The crude water extract showed an inhibition (IC₅₀) value with a time-dependent manner at 24, 48, and 72 hours of treatment. The crude water extract showed low IC₅₀ against MCF 7 (39.95 µg/ml), Hep G2 (20.9 µg/ml), DU 145 (32.12 µg/ml), and PC-3 (4.12 µg/ml) at 72 hours respectively. The crude water extract was chosen to be use in the experiment because it was the most effective against the cell line that shows the lowest IC₅₀ value as well as giving the highest crude yield All IC₅₀ values are significant compared with control after analysis using one sample T-test (**P <0.05**)

The crude water extract was used for further for compound isolation, which yielded 9.43% of glucomoringin (GMG). Subsequently, the compound was

activated with myrosinase enzymes to yield its active compound, glucomoringin isothiocyanate (GMG-ITC), followed by characterization using high performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR) spectroscopy analyses. The isolated GMG-ITC was screened for its cytotoxicity, employing MTT assays against all the cell lines mentioned previously, and PC-3 was found to be more selective with IC₅₀ of the isolated compound is 3.5 µg/ml. All IC₅₀ values are significant compared to control after analysis using one sample T-test (P <0.05). The IC₅₀ value of GMG-ITC was then used in the following studies, including morphological observation of apoptosis via AO/PI and TUNEL assays on the PC-3 cell line. The cell showed clear apoptotic body characterisation under phase contrast analysis, as well as colour changes with membrane blebbing and chromatin condensation in the AO/PI staining, followed by dark brown colour staining in the TUNEL assay due to nuclear DNA fragmentation of the cancer cells.

The cytotoxic potency study was further performed with Annexin V-FITC, cell cycle analysis, mRNA quantification, and protein signalling. In Annexin V-FITC, the apoptosis cell activity increased significantly (P < 0.05) from 24, 48, to 72 hours of treatment compared to the control by GMG-ITC. The apoptotic cell percentage increased significantly (P<0.01) from 21.35% (24 hours), followed by 34.47% (48 hours) and finally 72.9% (72 hours) after treatment. The compound was observed to arrest the cell cycle at G₂/M phase, which is an important phase in apoptosis. mRNA quantification indicates Nrf2 expression was downregulated significantly (P<0.05), but p53, Bax, and p21 were upregulated by the treatment of the GMG-ITC against PC-3 cell lines. The expression of p53 can be linked with a blockage of cell cycle progression at G₂/M phase and the upregulation of Bax, caspase-3, while Bcl2 and Nrf2 were downregulated. Finally, protein analysis employing cell signalling immunoassay studies by Multiplex analysis revealed the modulation of apoptotic proteins after the treatment of the cancer cells with GMG-ITC. The expression of proteins in the analysis; JNK, Bad, Bcl2, and p53 were significantly upregulated (p<0.01) compared to control. It indicates that early apoptosis expression was triggered by the protein signalling induced by the treatment. In this study, apoptosis induction and DNA fragmentation processes were observed in treated PC-3 cells. These phenomena suggest that the bioactive compound from *M. oleifera* seed could be useful for future cytotoxic agent against prostate cancer.

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

PENILAIAN POTENSI SITOTOKSISITI OLEH GLUKOMORINGIN DARI BIJI *M. oleifera* Lam TERHADAP SEL SELANJAR KANSER PROSTAT PC-3

Oleh

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Perencatan beberapa laluan protein yang terlibat dalam pengawal aturan sel kanser adalah kunci keperluan dalam penemuan kemoterapi kanser. *Moringa oleifera* sering digunakan dalam ubatan tradisional oleh beberapa negara dalam rawatan beberapa penyakit. Tumbuhan ini juga didapati mengandungi sebatian bioaktif dengan potensi terapeutik terhadap pelbagai sel kanser. Kajian ini dijalankan untuk menentukan penghalang sel kanser yang berkesan daripada unsur semula jadi, khususnya biji benih *M. oleifera* di mana glukomoringin boleh didapati dengan banyaknya yang mungkin membawa potensi untuk menghalang percambahan sel kanser prostat. Kajian ini dilakukan menggunakan pendekatan *in vitro* dan molekul.

Ekstrak mentah tumbuhan diperolehi daripada air, aseton, kloroform, etanol, metanol dan heksana di mana ekstrak air memberikan hasil ekstrak tertinggi (21.78%). Ekstrak mentah pada mulanya disaring untuk-, kandungan fitokimia dan viabiliti sel menggunakan ujian MTT terhadap MCF 7, HepG2, DU 145 dan sel PC 3. Ekstrak heksana, aseton dan kloroform didapati mengandungi kumarin dan lemak. Ekstrak etanol mengandungi kumarin, quinon dan lemak. Ekstrak metanol hanya mengandungi saponin, kumarin dan kuinon dan terakhir sekali, ekstrak air mengandungi saponin, alkaloid dan kumarin. Ekstrak air mentah menunjukkan nilai perencatan yang stabil (IC50) dengan kadar berturutan pada masa 24, 48 dan 72 jam rawatan. Ekstrak itu menunjukkan nilai IC50 rendah terhadap MCF 7 (39.95 µg / ml), Hep G2 (20.9 µg / ml), DU 145 (32.12 µg / ml) dan PC 3 (4.12 µg / ml) pada 72 jam. Semua nilai IC50 adalah signifikan selepas dianalisis menggunakan satu sampel T-test ($P < 0.05$)

Ekstrak air mentah digunakan untuk kajian selanjutnya yang melibatkan penghasilan ekstrak, 21.78% dan pengasingan kompaun yang menghasilkan 9.43% glukomoringin (GMG). Kompaun telah diaktifkan dengan enzim myrosinase untuk menghasilkan kompaun aktif, glukomoringin isotiosianat (GMG-ITC) diikuti oleh pencirian menggunakan analisis kromatografi cecair prestasi tinggi (HPLC) dan analisis spektroskopi resonans magnetik nuklear (NMR)

Kemudian GMG-ITC yang terisolasi telah digunakan untuk kajian sitotoksisitinya menggunakan ujian asai MTT terhadap semua sel-sel yang telah disebutkan dan PC 3 didapati paling selektif dengan IC₅₀ (3.5 µg / ml). Kesemua nilai IC₅₀ adalah signifikan selepas dianalisis menggunakan satu sampel T-test (P <0.05). Nilai IC₅₀ GMG-ITC kemudiannya digunakan dalam kajian berikut termasuk pemerhatian morfologi apoptosis melalui ujian AO/PI dan TUNEL pada sel PC 3. Sel ini menunjukkan ciri-ciri tubuh apoptosis yang jelas di bawah analisis kontras fasa serta perubahan warna dengan membran dan penggumpalan kromatin dalam pewarnaan AOPI diikuti oleh warna coklat gelap di dalam TUNEL asai disebabkan oleh pemecahan DNA nuklear sel kanser.

Kajian potensi sitotoksiti selanjutnya dilakukan dengan Annexin V-FITC, analisis kitaran sel, kuantifikasi mRNA dan isyarat protein. Dalam kajian Annexin V-FITC, sel-sel apoptosis meningkat dengan ketara (P<0.05) dari 24, 48 hingga 72 jam rawatan berbanding dengan sel kawalan oleh GMG-ITC. Peratusan sel apoptotik meningkat dengan ketara (P<0.001) dari 21.35% (24 jam), diikuti oleh 34.47% (48 jam) dan akhirnya 72.9% pada (72 jam) rawatan. Kompaun itu didapati menghentikan kitaran sel pada kitaran G2/M yang merupakan fasa penting dalam apoptosis. Pengkuantifikasi mRNA menunjukkan ekspresi Nrf2 adalah berkurang dengan ketara (P<0.05) tetapi p53, Bax dan Parp 6 menjadi meningkat setelah rawatan GMG-ITC dilakukan terhadap sel PC 3. p53 boleh dihubungkan dengan pengurangan perkembangan kitaran sel pada fasa G2/M dan pengawalan Bax, caspase-3 sementara Bcl2 dan Nrf2 telah direncatkan dengan ketara (P<0.01). Akhirnya, analisis protein yang menggunakan kajian immuno asai isyarat set dilakukan oleh analisis Multiplex mendedahkan modulasi protein apoptotik selepas rawatan set kanser dengan GMG-ITC. Ekspresi JNK, Bad, Bcl2 dan p53 adalah protein yang dikawal. Ia menunjukkan ekspresi apoptosis awal yang berlaku oleh isyarat protein yang terlibat disebabkan oleh rawatan yang berlaku. Dalam kajian ini, induksi apoptosis dan pemecahan DNA dapat diperhatikan dalam PC 3 yang dirawat. Fenomena dari kajian menunjukkan bahawa sebatian bioaktif dari biji *M. o/eifera* boleh digunakan untuk kegunaan kajian sitotoksik terhadap kanser prostat pada masa depan.

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LIST OF ABBREVIATIONS

ADP	Adenosine 5'-diphosphate
Ala	Alanine
AO/PI	Acridine Orange/Propidium Iodide
Arg	Arginine
<i>bp</i>	Base pair
BP	Base peak
C	Carbon
CDKs	Cyclin-Dependent Kinase
CDCL3	Deuterated chloroform
CGM	Complete growth medium
CO ₂	Carbon dioxide
DAPI	4',6-diamidino-2-phenylindole
DHA	Hydrogen donor; hydrogen; hydrogen acceptor
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
DU 145	Prostate adenocarcinoma cell line
ELISA	Enzyme-linked immunoassay
FBS	Foetal Bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GMG	Glucomoringin
GMG-ITC	Glucomoringin isothiocyanate
GIs	Glucosinolates
Gly	Glycine
GSH	Glutathione
H	Hydrogen
H ₂ O	Water
HepG2	Human hepatocyte carcinoma cell line
IC ₅₀	Inhibitory concentration at 50%
ITC	Isothiocyanate
IU	International unit
Lys	Lysine
MAPK	Mitogen-activated protein kinase
Mg ²⁺	Magnesium ion
mRNA	Messenger RNA
MCF-7	Human breast adenocarcinoma
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl Bromide tetrazolium
MW	Molecular weight
MYR	Myrosinase
NOP	Nucleoside diphosphates
NMPs	Nucleoside monophosphates
NF-κB	Nuclear Factor kappa B
NMR	Nuclear Magnetic Resonance
O ₂	Oxygen
PARP	ADP-ribose polymerase

PBS	Phosphate buffered saline
PE	Petroleum ether
Phe	Phenylalanine
PC3	Human Prostate adenocarcinoma cell line
PCR	Polymerase chain reaction
RLU	Relative Luminescence Unit
ROS	Reactive Oxygen Species
RNA	Ribonucleic acid
RP	Ribosomal proteins
RPMI	Roswell Park Memorial Institute
rRNA	Ribosomal RNA
RT-PCR	Reverse transcription-polymerase chain reaction
TLC	Thin-layer chromatography
Tris-HCl	Tris-hydrochloride
TdT	Terminal deoxynucleotidyl transferase
TUNEL	Terminal deoxynucleotidyl transferase dUTP labeling nick end
UV	Ultraviolet
WHO	World Health Organisation
Globocan	Global cancer incidence, mortality and prevalence

CHAPTER 1

INTRODUCTION

1.1 Research Background

Cancer can be described as a disease caused by aberrant cells that grows uncontrollably that it evades, erodes, then affects or destroys normal cells or tissues. According to the Malaysian National Cancer Institute (2016), there are about 12 common types of cancer that occurs among citizens nationwide. Five types of cancers that most commonly occur in males (shown in Table 1.1) include colorectal, lung, nasopharynx, lymphoma, and prostate cancer. The disease can attack young children and adults, regardless of race, however, men who are more than 50 years old are more at risk of developing prostate cancer.

Table 1.1: Most common cancers among males in Malaysia

Types of Cancer	Incidence(%)
Colorectal	16.3
Lung	15.8
Nasopharynx	8.1
Lymphoma	6.8
Prostate	6.7

(Source: Malaysian National Cancer Registry Report ,2016)

When a cell grows abnormally, but kept under control by the internal immune system, it may not cause any harm. The cell can be called benign or non-cancerous cells. But when the cell starts to replicate, forming lumps or growths, it will potentially lead to the formation of cancerous or malignant cells. Cancerous cells usually spread to the surrounding tissues, cause cellular destruction, and facilitates the development of other tumors (Chen et al., 2015; Pal et al., 2014). Malignant or cancerous cells can migrate to vascular or lymphatic systems as well as grow anywhere inside the body or organ, thus causing various types of cancers which are life threatening (Roomi et al., 2015). In Malaysia, the number of cancer cases increased by 7.96% within a span of four years (Ministry of Health Malaysia, 2013). It is one of the most common diseases that contribute to the mortality rate among the Malaysian population, with report cases stating 44.6% (8123) in males and 55.4% (1096) in females (Malaysian National Cancer Registry Report, 2016). Cancer has been a global burden in recent years. In cancer treatments, the most common way to treat cancers are through surgery, chemotherapy, and radiation. Most cancer patients will undergo surgery

followed by chemotherapy. Side effects are oftentimes reported by patients undergoing chemotherapy treatment, which include hair loss, fatigue, bruises, and hormonal problems (Zagouri et al., 2015). Medicinal plants that contains phytochemicals such as alkaloid, polyphenols and flavonoids provide a significant contribution in combating cancer. The use of medicinal plants for disease treatment have been reported in the past. Natural plant sources played an important role in discovering alternative medicine that helps prevent cancer. It was reported that around 250,000 plant species are believed to have anti-cancer properties. Medicinal plants are regarded to have a high potential for novel drug discovery, nutraceuticals, and also provides food supplements (Jeeshna and Paulsamy, 2011; Anwar, et al., 2007). The plant compounds, podophyllotoxin led the discovery of cancer treatment drugs for lung cancer and testicular. (Clarke et al., 2008). Paclitaxel and docetaxel were discovered from natural plant compound in Pacific Yew tree used in cancer treatments (Steams and Wang, 2011). These compounds were derived from plants and are utilized as natural anti-cancer agents in the market. Paclitaxel was reported to have cognitive side effects on cancer patients (Smith et al., 2016). In fact, ovarian cancer survivors were also found to have early menopause, as well as diminished ovarian function which is mainly caused by procarbazine and alkylating agents (Overbeek et al., 2017). Diminished ovarian function can lead to a lot of other effects on the patients. Due to the increasing number of cancer cases, it is important to discover more potential chemotherapeutic agents against cancer. Therefore, it is crucial to study medicinal plants and their natural compounds to counter the lack of natural anticancer treatment drug effectively.

1.2 Problem statements

Glucosinolates, which derived from isothiocyanates (ITC) such as benzyl isothiocyanate (B-ITC), allyl isothiocyanate (Al-TC), and phenylethyl isothiocyanate (PE-ITC), have been reported for their protective effects against cancer cells. The ITCs had shown cytotoxic effects towards breast, liver and lung cancer via modulation of MEK/ERK, PI3K/AKT and Nrf2, intrinsic and extrinsic apoptosis pathway. However, lack of study had been done on glucomoringin isothiocyanate (GMG-ITC) against prostate cancer cell line to determine its cytotoxic effect. Isothiocyanates {ITCs} contains anti-cancer agents that may provide the solution for natural anti-cancer treatment with less side effect for prostate cancer treatment. This study will provide more facts on the cytotoxic potency of glucomoringin isothiocyanate (GMG-ITC) to induce apoptosis in prostate cancer cell and determine the pathways involved to inhibit tumor or cancer progression.

1.3 Justification of Study

Cancer is well known as one of the deadly diseases at present, which it can be categorized as death sentence due its consequences or series of events, and stages, which some cancer patients have to go through. The current treatment did not promise an escape from the devastating disease due to its high cost of treatments and side effects of modern drugs. Cytotoxic compound or therapeutic compound discovered from natural sources are usually highly abundance and readily available. The compound will also be cheap to produce with no or less side effect. Thus, it will benefit the societies either in preventing cancer or treatment of cancer. The discovery of cytotoxic potential of the compound from natural resources may give hope or opportunities in treatment of cancer especially for the most common cancer among the societies.

1.4 Hypothesis

1.4.1 Null Hypothesis

GMG-ITC does not show cytotoxicity potency through the induction of apoptosis and cell cycle arrest in PC-3 cells.

1.4.2 Alternative Hypothesis

GMG-ITC shows cytotoxicity potency through the induction of apoptosis and cell cycle arrest in PC-3 cells.

1.5 Objectives of research

General objective is to evaluate cytotoxic potency of glucomoringin isolated from *Moringa oleifera* Lam against prostate cancer cell line, PC-3

Specific objectives

1. To assess cytotoxic effects of *M. oleifera* Lam crude seeds extract on several cancer cell lines
2. To elucidate glucomoringin isolated from crude water seed extract of *M. oleifera* Lam
3. To evaluate the cytotoxicity potency of glucomoringin through the induction of apoptosis and cell cycle arrest of PC-3 cells.

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