UNIVERSITI PUTRA MALAYSIA

PUTATIVE APOPTOSIS EFFECT OF MOMORDICA CHARANTIA LINN. EXTRACTS IN HUMAN LUNG CANCER CELL LINE A549

SIROSHINI A/P K THIAGARAJAN

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By

SIROSHINI A/P K THIAGARAJAN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

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

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January 2019

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Lung cancer is the leading cause of cancer related deaths worldwide comprising about 40% occurring in developing countries. Formerly traditional medicines were the major forms of cancer treatment prior to chemotherapeutic drugs. *Momordica charantia* or known as bitter melon is an edible fruit that has been used traditionally for cancer treatment. In this study, non-small cell lung cancer cells (NSCLC), A549 as an *in vitro* model to assess the apoptosis inducing effect of two variations Chinese (C) and Indian (I) bitter melon. The inhibitory effect of the hot aqueous (HA) and cold aqueous (CA) extracts was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The pro-apoptotic and derangement effect in A549 cells was observed under a fluorescence microscope using Hoechst 33358 (H33358) staining. The role of reactive oxygen species (ROS), caspase-3/7 and p53 was observed by examining the activity in the treated cells. Both hot and cold aqueous extraction of the bitter melons treated on NSCLC resulted a significant ($p<0.05$) decrease in cell viability and induced apoptotic cell death. H33358 staining showed that the crude extracts induced the typical nuclear apoptotic morphology and derangement of filamentous-actin. The apoptosis of NSCLC cells was accompanied by the increase in ROS, caspase-3/7 and p53 expression. Further study using flowcytometry also confirmed the apoptosis activity suggesting the results obtain were aligned with the intrinsic mitochondria apoptosis pathway. Generally all crude water-soluble extracts exhibited apoptosis via the same pathway. Among the crudes extracts, Chinese bitter melon hot aqueous extract (CHA) showed a significant ($p<0.05$) anti-cancer activity to cisplatin acting as a positive control. CHA also increased the Caspase 3/7 activity by 1.6 folds while 5 folds in ROS activity. With CHA significantly ($p<0.05$) increasing the apoptotic activity when compared to CCA, IHA, and ICA, CHA may induce the intrinsic apoptotic pathway due to their rich bioactive chemical constituents as shown in the Liquid Chormatography-Mass Spectrometry (LC-MS) result. These findings propose that the anti-proliferative

effect of CHA at inhibitory concentration, IC_{50} of 32.5±0.18µg/ml was associated with apoptosis by regulating mitochondria destruction by increasing caspase-3/7 activity. CHA also induces p53-dependent apoptosis of A549 in a ROS-dependent manner subjecting to 34.5% apoptotic cells.

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

PENYELIDIKAN KEBOLEHAN APOPTOSIS DARIPADA *MOMORDICA CHARANTIA* KEATAS KANSER PARU-PARU CELL A549

Oleh

SIROSHINI A/P K THIAGARAJAN

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Kanser paru-paru adalah penyebab utama kematian diantara jenis kanser di seluruh dunia dan merupakan punca utama kematian sebanyak 40% di negara-negara membangun. Penggunaan ubat tradisional adalah salah satu jenis rawatan kanser sebelum kewujudan ubat kemoterapi. Momordica charantia atau dikenali sebagai peria adalah buah yang boleh dimakan dan digunakan secara tradisional untuk rawatan kanser. Dalam kajian ini, kita telah menggunakan sel-sel kanser peparu sel kecil, A549 sebagai model in vitro untuk menilai kesan apoptosis daripada dua variasi peria Cina (C) dan India (I). Kesan perencatan ekstrak panas (HA) dan sejuk (CA) telah dinilai dengan assai 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT). Kesan pro-apoptosis dalam sel A549 diperhatikan dibawah mikroskop pendarfluor menggunakan pewarnaan Hoechst 33358 (H33358). Peranan spesies oksigen reaktif (ROS), caspase-3/7 dan p53 diperhatikan dengan mengkaji aktiviti dalam sel-sel yang dirawat. Kedua-dua ekstrak peria panas dan sejuk pmengakibatkan penurunan ketara dalam daya hidup sel dan mengaruh kematian sel apoptotik. Pewarnaan H33358 menunjukkan bahawa ekstrak mengaruh perubahan morfologi, apoptotik nukleus tipikal dan penyusuanan filamen-actin. Parameter apoptosis ini diiringi oleh peningkatan ROS, caspase-3/7 dan ekpressi p53. Kajian lanjut menggunakan “flowcytometry” juga mengesahkan aktiviti apoptosis sejajar dengan laluan apoptosis mitokondria intrinsik. Secara umumnya semua ekstrak larut air menunjukkan apoptosis melalui laluan yang sama. Di antara semua ekstrak, ekstrak peria air panas Cina (CHA) menunjukkan aktiviti anti-kanser yang kuat apabila dibandingkan dengan cisplatin yang bertindak sebagai kawalan positif. CHA juga meningkatkan aktiviti caspase 3/7 sebanyak 1.6 lipatan manakala 5 lipatan dalam aktiviti ROS CHA meningkat dengan ketara apabila dibandingkan dengan CCA, IHA, dan ICA pada aktiviti apoptosis, CHA dicadangkan mengaruhkan apoptosis intrinsik disebabkan oleh kewujudan kandungan-kandungan bahan kimia bioaktif yang dicadangkan dalam “Liquid Chromatograpy-Mass Spectrometry” (LC-MS). Penemuan ini menunjukkan

bahawa kesan anti-proliferatif CHA pada kepekatan perencatan, IC50 of of 32.50 ± 0.18µg/ml dikaitkan dengan apoptosis dengan mengawal kemusnahan mitokondria, peningkatan caspase-3/7 dan aktiviti ROS yang menyebabkan 34.5% sel apoptosis dalam sel-sel kanser peparu manusia yang melalui laluan intrinsik apoptosis.

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I certify that a Thesis Examination Committee has met on 10 January 2019 to conduct the final examination of Siroshini a/p K Thiagarajan on her thesis entitled "Putative Apoptosis Effect of *Momordica charantia* Linn. Extracts in Human Lung Cancer Cell Line A549" 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 Master of Science.

Members of the Thesis Examination Committee were as follows:

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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 Master of Science. The members of the Supervisory Committee were as follows:

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This is to confirm that:

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Supervisory Committee :Dr. Yong Yoke Keong

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LIST OF ABBREVIATIONS

AIF	Apoptosis inducing factor
ATCC	American Type Culture Collection
DCFH-DA	2',7'- dichlorodihydrofluorescein diacetate
DIABLO	Direct IAP Binding protein with Lo Pi
DISC	Death-inducing signaling complex
FADD	Fas-associated death domain
FasL	Fas Ligand
FBS	Fetal Bovine Serum
g	Gravitational force
hrs	Hours
IARC	International Agency of Research for Cancer
LC-MS	Liquid Chromatography Mass Spectometry
min	Minute
MTT	3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide
NCSM	National Cancer Society Malaysia
NSCLC	Non-Small Cell Lung Carcinoma
PBS	Phosphate buffered saline
PS	Phosphatidylserine
ROS	Reactive Oxygen Species
rpm	Rotation Per Minute
RPMI	Roswell Park Memorial Institute
SCLC	Small Cell Lung Carcinoma
Smac	Second mitochondira-derived activator of caspase

TNFR1	Type 1 TNF receptor
TRADD	TNF receptor-associated death domain
WHO	World Health Organization
CHA	Chinese Hot Aqueous
CCA	Chinese Cold Aqueous
IHA	Indian Hot Aqueous
ICA	Indian Cold Aqueous

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CHAPTER 1

INTRODUCTION

1.1 Research background

Cancer is a form of silent disease characterized by an uncontrolled and unscheduled cell proliferation. Cancer is a type of heterogeneous disease in which the normal cells are able to escape the cell regulation cycle resulting in formation of a tumor (Demir et al., 2015). This can be triggered due to several factors for instance, the environmental and genetic factor. Environmental factors such as tobacco, infections, alcohol consumption and obesity contributed 90 to 95 percent towards the risk of cancer. Whereas, the genetic and hereditary factors have contributed about 5 to 10 percent towards the risk of cancers (Anand et al., 2008). These causative agents may act simultaneously to promote carcinogenesis initiating cancer formation (Demir et al., 2015).

In Malaysia, cancer is one of the major life-threatening diseases which affect both the genders of all age group. Based on the International Agency of Research for Cancer [IARC] in the year 2015, it is stated that there were massive increase in the number of cancer cases which was from 32,000 in the year 2008 to 37,400 in 2012 (IARC, 2015). New cancer cases that are reported worldwide have risen to 18.1 million in 2018 (IARC, 2018). It is also expected that the number of cancer cases will approximately rise to 43.8 million by 2023 if no proper action is taken immediately (IARC, 2018). In 2012, cancer is reported being the second utmost common root of death in private hospitals in Malaysia (IARC, 2015). There was an estimate of 9.6 million deaths from cancer till August 2018 (Global Cancer Observatory, 2018). According to the cancer incidence and mortality rate by Globacan statistics, the five most common cancers in Malaysia was breast, colorectal, cervix, uterine and prostate cancer, while worldwide was breast, prostate, lung and cervix uteri cancers (IARC, 2015).

Lung cancer is one of the leading cancer cases in Malaysia which occurs due to various internal and external factors. Some of the factors include the environmental factor, daily lifestyle and genetic factor. Carcinoma which is commonly known as the lung tumor cells undergoes rapid cell division leading to accumulation of these abnormal cells on the lung tissue itself. Lung tissue can eventually spread to other parts of the body causing different types of cancers. In addition, treatment such as surgery, chemotherapy and radiation therapy can eventually decrease the growth of these abnormal cells.

Despite the tremendous advancement of the technologies and treatments in cancer, the mortality rate due to cancer has not been decreased since the past 50 years. Treatment modalities such as chemotherapy, radiotherapy and

surgeries for cancer have improved the patient's survival rate (Basch et al., 2016). However, these treatments are expensive (Basch et al., 2016) and cause several serious side effects to the body and destroy normal or rapidly dividing cells or tissues. As a result, other serious medical complications such as bone marrow suppression, hepatic, cardiac, pulmonary, renal toxicities and ocular problems (Basch et al., 2016) may develop. Besides, some chemotherapeutic drugs may develop resistance thereby decreasing the efficacy of the drug itself (Anand et al., 2008).

1.2 Problem statement

There is no cure for cancer to date (Vliet et al., 2013). Therefore, research on natural products particularly medicinal plants will be a good attempt as a potential source of drugs to cure various diseases including cancer. Cancer can be prevented, suppressed or delayed by natural bioactive components or synthetic products. Therefore, natural products performs vital role in treatment and prevention of human diseases prevention for example an anticancer drug discovery and the developmental process (Tang & Du, 2014).

Plants have the cure to diseases and in addition to that, most have been emphasizing on characteristics and analysis of many plants based on their medicinal values (Tiwari et al., 2011). Bioactive components can be found in almost all parts of the plants (Watson & Preedy, 2011). The main difference between bioactive compounds and pharmaceutical drugs is the method of isolation and purification level. The pharmaceutical drugs have the highest purity as they contain mono components of artificial chemicals while the bioactive compounds are from partially purified extracts which contain a mixture of natural chemicals (Watson & Preedy, 2011).
Furthermore, plants have been used for medicinal purposes since ancient civilization times and the chemical constituents were isolated when modern chemistry developed (Watson & Preedy, 2011). Many drugs have been produced eventually by studying the underlying mechanism of action which is present in the plants. Therefore, the chances of discovering novel bioactive compounds to treat various diseases become greater (Palombo, 2009). Moreover, plants exert lesser side effects as compared to the synthetic drugs (Watson & Preedy, 2011).

Primary metabolites such as nucleotides, carbohydrates, lipids and amino acids play vital metabolic roles in nutrition and reproduction. While these compounds are not important for cell survival they contribute to the interactions of cells with the environment and protection against biotic and abiotic stress (Patil, Pagare, Patil & Sidhu, 2015). Medicinal plants are the richest biosource of drugs and these plants have been used as traditional and modern medicine in development of food supplements and synthetic drugs (Tiwari et al., 2011).

Approximately 80% of people living in developing countries use medicinal plants for their health care (Palombo, 2011). An example of anticancer drug derived from the medicinal plant is vinblastine which is obtained from the plant alkaloid (Cancer Research UK, 2015). Besides, medicinal plants consist of a mixture of various chemical compounds which increase the quality of health (Gurib-Fakim, 2006). Active plant ingredients such as flavonoids, alkaloids and phenols in medicinal plants are useful to human (United States Department of Agriculture, 2014). Malaysia in the year 2005 and 2006, there was an increase in usage of the medicinal plants by 3.7% and 7.8%, respectively as compared to the previous years (Globinmed, 2015). The product of natural origin which is derived from mammals, microbes and plants are known as natural products (Du & Tang, 2014). Natural products have a lower toxicity and higher absorption and metabolism in the body as compared to the newly synthesized chemical compounds (Du & Tang, 2014).

1.3 Significance of study

Momordica charantia known as bitter melon is widely used as an alternative and complementary medicine traditionally. This fruit can be easily harvested in Africa, America, China, India, Thailand and other domestication. Being able to be cultivated in many regions, bitter melon comes in different variations such as the Chinese and Indian bitter melon. Generally, bitter melon is used in culinary but also grown as ornamental and with the known medicinal properties it is widely used as folk medicine (Behera et al., 2010). Bitter melon can act as an anti-diabetic, antitumor, anti-ulcer, hypoglycemic and many other analgesic properties (Raghavan, 2015).

Generally, the bitter gourd has many medicinal assets whereas it acts as an anti-diabetic, hypoglycemic, antitumor, anti-oxidant, anti-ulcer, analgesic properties and many others (Raghavan, 2015). It is often consumed with water either hot or cold depending on the individual preferences. Therefore, with alleged folkloric use in anti-tumor agent, different variation of bitter melon with different extraction method is a logical undertaking in the search for new anti-cancer drugs which will be elaborated clearly in the foregoing discussion.

1.4 Objectives

1.4.1 General objective

In this research, whole fruit extract of Chinese and Indian *M. charantia* was used to determine the cytotoxic effect on human NSCLC cell line, A549. Although there are many studies on Momordica genus, but only a few studies were reported on *M. charantia* Chinese and Indian variation by using hot and cold water extraction. Therefore, the study aims to investigate the effectiveness of the extracts on the intrinsic pathway of apoptosis.

1.4.2 Specific objectives

The specific objectives of this study are to:

1. Characterize the compounds using liquid chromatography-mass spectrometry (LC-MS) present in cold and hot aqueous extraction of Chinese and Indian bitter melon.
2. Determine and compare the effect of hot and cold aqueous extraction of Chinese and Indian bitter melon extracts on viability, morphological changes, and the apoptosis event using flowcytometry of A549 cell line.
3. Investigate the effect of hot and cold aqueous extraction of Chinese and Indian bitter melon extracts on the morphological changes of actin cytoskeleton, adheren junction and mitochondria in A549 cells.
4. Compare the effect of hot and cold aqueous extraction of Chinese and Indian bitter melon extracts on the Reactive Oxygen Species (ROS) scavenging activity, the expression of signaling molecules (caspase-3/7) and tumor suppressor (p53) gene expression in A549 cells.

1.5 Hypothesis

1. There will be many compounds that can be screened from the LC-MS qualitative analysis where the possible active compound is identified.
2. Hot and cold aqueous extraction of Chinese and Indian bitter melon extracts reduces the cell viability of lung cancer cell line A549, with visible mitochondria disruption suggesting apoptosis.
3. Actin cytoskeleton and adherens junction in A549 cells will be disrupted by cold and hot aqueous extraction of Chinese and Indian bitter melon extracts.
4. Hot and cold aqueous extraction of Chinese and Indian bitter melon extracts increases ROS activity, the caspase-3/7 activity and p53 signaling molecules in A549 cells.

1.6 Overview of the study (framework)

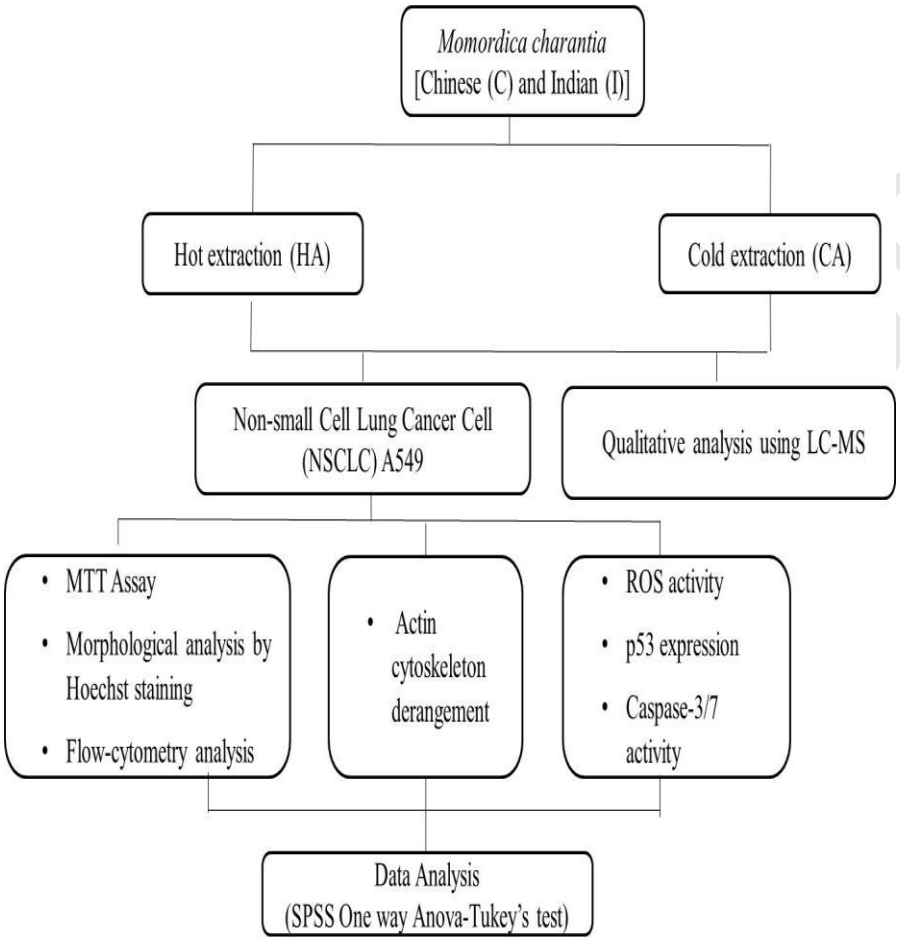

Figure 1.1: **Conceptual Framework of the apoptosis inducing effect of *Momordica charantia* Linn. (Chinese and Indian Bitter Melon) extracts in human lung cancer cell line A549**

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BIODATA OF STUDENT

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Subsequently, she enrolled herself for Foundation in Science for one year and been offered for a full three years course of Bachelors of Science (Hons) Biomedical Science in Universiti Tunku Abdul Rahman, Kampar Campus. She was under an educational loan of Perbadanan Tabung Pendidikan Tinggi Nasional (PTPTN).

After 3 and a half years she graduated successfully with his produced thesis on Anti-adipogenesis effect on 3T3-L1. Then, she was offered to pursue her postgraduate study in Master of Science in Human Physiology at Universiti Putra Malaysia, Selangor, Malaysia under the main supervision of Dr. Hasnah Binti Bahari.

LIST OF PUBLICATIONS

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