CHEMOPREVENTION STUDY OF *Luffa aegyptiaca* Mill (Petola Bantal) SEEDS ETHANOLIC EXTRACT ON BREAST CANCER CARCINOMA CELLS (MCF-7)

BY

HABIBAH BINTI HASSAN

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International Islamic University Malaysia

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ABSTRACT

Breast cancer is a major health challenges in worldwide including Malaysia. It is the commonest malignancy among women. Although there are several of cancer treatments; however, these current treatments have consequences to the cancer patient such as recurrence of cancer, drug resistance and severe side effects. Discovery of alternative treatment which have minimal or almost no side effect is needed for cancer treatment. Traditional practitioners in India used the powder form of *Luffa aegyptiaca* seeds (LAS) in treating breast cancer. The old folks in Malaysia also used the powder form of LAS to treat the breast cancer patient. Thus, the present study is aim to determine the chemopreventive effect of LAS extract on breast carcinoma cell (MCF-7) by determining IC_{50} value, antiproliferative effect, apoptosis induction and cell cycle arrest via flow cytometric analysis as well as molecular signalling pathway of related genes expression with regard to apoptosis induction and cell cycle arrest by using qPCR analysis. MCF-7 cells were treated with various concentrations of ethanol extract of LAS. IC_{50} value of 80 µg/mL was determined after 72h of LAS treatment on MCF-7 cells and its anti-proliferative effect was assessed for 24, 48 and 96 hours using TBEA method. LAS extract demonstrated inhibitory activity in cell growth in a dose-dependent and time-dependent manner. The effect of LAS extract on apoptosis induction and cell cycle arrest indicated that it inhibits the growth of MCF-7 cells by inducing apoptosis and cell cycle arrest in G_0/G_1 phase. Meanwhile, gene expression analysis of qPCR assay revealed that LAS extract induced apoptosis through the down-regulation of *Bcl-2*, whereas *Bax cytochrome c*, *caspase-9* and *caspase-7* were up-regulated in MCF-7 cells. Cell cycle arrest was associated with down-regulation of *CDK2* with subsequent up-regulation of *p21* and *cyclin E*. Overall, the research finding provide new insight that *Luffa aegyptiaca* seeds (LAS) is a promising candidate of chemopreventive agents on MCF-7 cells.

خلاصة البحث

إن سرطان الثدي يعد من التحديات الصحية الرئيسية في جميع أنحاء العالم، بما في ذلك ماليزيا، وهو السرطان الأكثر شيوعا بين النساء. على الرغم من وجود العديد من العلاجات للسرطان؛ فإن العلاجات الحالية لا تخلو من السلبيات تجاه مرضى السرطان، مثل رجوع السرطان، والمقاومة الدوائية، والآثار الجانبية الحادة. هناك حاجة إلى اكتشاف علاج بديل بآثار جانبية ضئيلة أو بلا أي تأثير جانبي لعلاج السرطان. استخدم الأطباء الشعبيون في الهند مسحوق بذور ثمرة) في علاج سرطان الثدي. وفي ماليزيا أيضًا استخدم الماليزيون القدامى مسحوق بذور *Luffa aegyptiaca* الليف (ثمرة الليف لعلاج مرضى سرطان الثدي. ولذلك فقد هدف هذا البحث إلى تحديد التأثير الوقائي الكيميائي لمستخلص ، والتأثير المضاد لتكاثر الخلايا، IC_{50}) من خلال تحديد قيمة MCF-7 بذور ثمرة الليف على خلايا سرطان الثدي (وتحفيز موت الخلايا المبرمج، وتوقف الدورة الخلوية عبر تحليل التدفق الخلوي وكذلك مسار الإشارات الجزيئية للتعبيرات . تم علاج qPCR الجينية ذات الصلة المتعلقة بتحفيز موت الخلايا المبرمج وتوقيف الدورة الخلوية باستخدام تحليل للكمية 80 IC_{50} بتركيزات مختلفة من المستخلص الإيثانولي لبذور ثمرة الليف. تم تحديد قيمة MCF-7 خلايا وتم تقييم تأثيره المضاد للتكاثر الخلوي MCF-7 ميكروغرام/مل بعد 72 ساعة من العلاج بالمستخلصات على خلايا . أظهرت مستخلصات بذور ثمرة الليف نشاطا مثبطا لنمو TBEA لمدة 24 و 48 و 96 ساعة باستخدام طريقة الخلايا معتمدة على الجرعة والوقت. أشار تأثير المستخلصات في تحفيز موت الخلايا المبرمج وتوقيف الدورة الخلوية إلى . وفي الوقت G0/G1 عن طريق تحفيز موت الخلايا المبرمج وتوقيف الدورة الخلوية في مرحلة MCF-7 منع نمو خلايا أن المستخلصات قد سببت موت الخلايا المبرمج من خلال التنظيم qPCR نفسه كشف تحليل التعبير الجيني لتحليل فقد تم تنظيمها caspase-7، و caspase-9، و *Bax cytochrome c*، أما بالنسبة لـ Bcl-2 التخفيضي لـ مع التنظيم الرفعي اللاحق لـ *CDK2*. ارتبط توقف الدورة الخلوية بالتنظيم التخفيضي لـ MCF-7 رفعيا في خلايا . أعطت بشكل عام نتائج هذا البحث رؤية جديدة مفادها أن بذور ثمرة الليف مرشح واعد ليكون *cyclin E* و *p21* أحد عوامل الوقاية الكيماوية لخلايا MCF-7.

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 of Health Sciences.

……..……
Ridhwan Abdul Wahab
Supervisor

...……..……
Alfi Khatib
Co-supervisor

I certify that I have 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 thesis for the degree of Master of Health Science.

……
Qamar Uddin Ahmed
Internal Examiner

……
Wan Amir Nizam Wan Ahmad
External Examiner

This thesis was submitted to the Department of Biomedical Science and is accepted as a fulfilment of the requirement for the degree of Master of Health Science

……
Hanani Ahmad Yusof @ Hanafi
Head, Department of Biomedical Science

This thesis was submitted to the Kulliyyah of Allied Health Science and is accepted as a fulfilment of the requirement for the degree of Master of Health Science.

……
Suzanah Abdul Rahman
Dean, Kulliyyah of Allied Health Science

DECLARATION

I hereby declare that this thesis is the result of my own investigations, 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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LIST OF ABBREVIATIONS

ATCC	American type culture collection
Apaf-1	Apoptotic protease activating factor 1
BLAST	Basic local alignment search tool
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
BID	BH3-interacting-domain death
Caspase	Cysteine aspartic acid protease
CDKs	Cyclin-dependent kinases
CDKI	Cyclin dependent kinases inhibitors
cDNA	complementary DNA
CGM	Complete growth media
CIP/KIP	CDK Interacting protein/kinase inhibitor protein
CO_2	Carbon dioxide
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimetyl sulphoxide
E	Efficiency
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
IC_{50}	Inhibition concentration (reduces the effect by 50%)
IAP	Inhibitor of apoptosis
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC	Guanine-cytosine (DNA base pairing)
KIP	Kinase inhibitory proteins
LAS	*Luffa aegyptiaca* seeds
mRNA	messenger RNAs
MIQE	Minimum information for quantitative polymerase chain reaction publication experiments
MCF-7	Breast cancer cells
NCBI	National Center for Biotechnology Institute
PBS	Phosphate buffer saline
PCD	Programmed cell death
PCR	Polymerase chain reaction
PI	Propidium Iodide
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RT	Real-Time

LIST OF SYMBOLS

α	Alpha
β	Beta
Δ	Delta
Cq	Quantification cycle
Ct	Threshold cycle
g	Gram
G	gravity
G	Gap
M	Mitosis phase
S	Synthesis phase
µL	Microliter
µg/mL	Microgram per millilitre
°C	Degree Celsius
%	Percent
-	To
>	More than
<	Less than
±	Plus-minus
x	Times
=	Equal to
*	Statistical significance denotation

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND OF STUDY

Cancer is the second leading cause of mortality globally and one of the major public health challenges of the 21st century. The cancer burden continues to grow rapidly worldwide due to aging and growing population (Jemal *et al.*, 2011), lifestyle and socioeconomic changes (Sankaranarayanan *et al.*, 2014). According to estimates from the International Agency for Research on Cancer (IARC), the global burden of cancer is estimated to increase to 18.1 million incidence and 9.6 million mortality in 2018 (Bray *et al.*, 2018). The cancer incidence is estimated to rise from 6.1 million to 10.7 million while mortality is estimated to rise from 4.1 million to 7.5 million in 2008 to 2030 in Asia (Sankaranarayanan *et al.*, 2014). As in Malaysia, the cancer incidence was 86.9 in males and 99.3 in females per 100,000 populations from 2007 until 2011 while the cancer mortality have risen from 11.3% to 12.6% in 2007 to 2016 [National Cancer Registry (NCI), 2018].

Breast cancer has become one of the serious malignant diseases throughout the world and the sharp rising of breast cancer gives burden to the world (IARC, 2013). It is the most commonly diagnosed cancer and the leading cause of cancer death among women [(Comsa *et al.*, 2015; Dahlui *et al.*, 2011; Yip *et al.*, 2006)]. In 2011, more than 508,000 women in the world have died due to breast cancer (Ghoncheh *et al.*, 2015). According to Bray *et al.* (2018) approximately 2.1 million newly diagnosed of female breast cancer cases were estimated in 2018, accounting for almost 1 in 4 cancer cases among women worldwide. In most Asian countries, the prevalence of

breast cancer was reported as increasing [(Abdullah *et al.*, 2013; Hirabayashi & Zhang, 2009; Medina *et al.*, 2010; Park *et al.*, 2011; Sim *et al.*, 2006; Takiar & Srivastav, 2008)]. A report from the College of Radiology, Academy of Medicine of Malaysia indicated breast cancer as the most common cancer as well as the most common female cancer in all races ages of 20 years in Malaysia from 2003 until 2005 (Din *et al*., 2014). It is likely to occur for all major ethnic groups in Malaysia which include Malays, Chinese and Indians (Yip *et.al*, 2006).

Currently, the treatment for cancers are varies. It includes surgery, radiotherapy, chemotherapy, immunotherapy, hormonal therapy and anticancer drugs. Although the survival of the cancer patients has increased due to the rapid advancement of treatment (Miller *et al.*, 2016), however, this current treatment has consequences to the cancer patient such as recurrence of cancer, drug resistance and severe side effects (Rayan *et.al*, 2017). In addition, drug resistance acquired by cancer cells has led to the treatment failure, which results in tumour recurrence and metastasis (Prieto-Vila *et al.*, 2017). Hence, the new therapeutic approach is seriously needed to improve the quality of health of the patients. Therefore, cancer chemoprevention can be the most realistic and promising approach nowadays because it uses natural product to either suppress, delaying or reversing the process of carcinogenesis. It is another important perspective for easing this alarming public health challenges (Cragg & Pezzuto, 2015). Chemoprevention was widely known in the scientific research field for many years in the East while it has emerged in the recent years in the West (George & Albert, 2014).

Rapid developments of the diseases have brought to the exploration of natural products as alternative strategies for cancer treatment. Humans have used natural

products such as plant, marine organisms, and microorganisms as source of medicines for disease treatment since pre historic times (Yuan *et al.*, 2016). Natural products exist in large diversity in nature may have the chance to stimulate various physiological pathways which can be promising treatment for diseases like cancer (Mitra & Dash, 2018). In most countries, natural product especially plants have a common alternative for cancer therapy and it is reported that more than 3000 plants globally possess anticancer properties (Seca & Pinto, 2018). Plant derived anticancer agent such as vinblastine and vincristine isolated from *Catharanthus roseus* (Kumar *et al*, 2013) were the first drugs to approach clinical use for cancer treatment (Shoeb, 2006). Many plant derived agents are promising anticancer agents because they are commonly nontoxic to normal cells and demonstrate anticancer activity on cell death through different mechanisms such as apoptosis and cell cycle arrest (Pfeffer & Singh, 2018).

Apoptosis is a form of programmed cell death. It is also called as "cellular suicide". Apoptosis plays a crucial role in development and aging as well as homeostasis in long-lived mammals (Pfeffer & Singh, 2018). Apoptosis maintains balance in the body, eliminates unnecessary cells growth and eradicate pre-cancerous cells and virus-infected cells. Apoptosis machinery is complex and involves multiple signalling pathways. Apoptosis is induced through caspase-mediated extrinsic or mitochondria-mediated intrinsic pathways (Pistritto *et al.*, 2016). Induction of apoptosis in cells is arguably the most effective defence in fighting cancer (Sun *et al.*, 2004). Therefore, a better understanding of apoptosis signalling pathway by potential chemopreventive agent is reported to improve the effectiveness of cancer treatment (Bode & Dong, 2004).

Cell cycle is an ordered sequence of events in which a cell undergoes DNA replication with chromosomal segregation thereby assuring the duplicated genetic information is divided equally to two daughter cells (Sherr & Bartek, 2016). Cell cycle plays an important role in cell proliferation and growth processes as well as cell division after DNA damage (Schwartz & Shah, 2005). Faulty in cell cycle affects in deregulated DNA replication and mitosis which is a vital cause for cancer (Geleta *et al.*, 2016). There are four phases in the cell division cycle; G1, S, G2 and M phase. Cell cycle events are controlled by cyclin-dependent kinases (CDKs), a family of serine/threonine kinases which are regulated by induction or degradation of cyclin proteins (Choi *et al.*, 2001). Cyclin overexpression and cyclin-dependent kinases inhibitors (CDKIs) inactivation result in loss of checkpoint integrity which contributes to cancer (Brown & Gelger, 2018). Thus, targeting cell cycle checkpoints signalling pathway by chemopreventive agent will provide another approach of cancer chemoprevention.

Luffa aegyptiaca Mill. (*L. aegyptiaca*) (syn *Luffa cylindrica* (L.) J. M. Roem) commonly called loofa, sponge gourd, vegetable sponge, bath sponge or dish cloth gourd. This plant is part of the cucumber family and it is grown for its valuable fruit in several tropical countries (Oboh & Aluyor, 2009). *L. aegyptiaca* has been shown to possess medicinal and nutritional values. It is reported to have good phytochemical agents that have potential chemopreventive properties (Mhya & Mankilik, 2014). Nassr-Allah *et al.* (2009) has shown luffin, ribosome-inactivating protein from seeds of *L. aegyptiaca* induced cell death by apoptosis in melanoma and Ehrlich ascites tumour cells. Additionally, the luffin has been shown to be effective against insects, parasites, protozoa and fungi growth (Azeez *et al.*, 2013). *L. aegyptiaca* has been

identified to demonstrate promising pharmacological actions such as anti-inflammatory, anti-fungal, anti-allergy, anti-asthmatic, analgesic and sedative (Partap *et al.*, 2012), anti-diabetic (Patel *et al.*, 2012), anti-microbial and anti-cancer (Bulbul *et al.*, 2011). However, there is limited scientific research on this part of the plant especially seeds. Therefore, the mechanism of cell death induced by apoptosis and cell cycle arrest corresponding to the biologically active substances from *L. aegyptiaca* seeds are important to be studied for the new chemopreventive agent development.

1.2 PROBLEM STATEMENT

The breast cancer burden in Malaysia is increasing from year to year. A report from IARC (2018), the highest incidence and prevalence of cancer in Malaysia is breast cancer. Although the current breast cancer therapy for patient varies, however, drug resistance, cancer recurrence and severe side effects (Rayan *et al*., 2017) still occur which lead to the treatment failure. Besides, the increasing cancer care cost is one of the challenges in breast cancer therapy. Therefore, new therapeutic approaches are necessary for effective cancer therapy.

The recent success of various clinical trials in fighting cancer suggests that chemoprevention is one of the most significant strategies in the cancer growth control (Tanaka *et al*., 2012). Exploration of effective chemopreventive agents with least possible side effects and low cost as well as possessing multi-target mechanisms of action on cancer is seriously needed. Thus, this study was aimed to investigate the chemopreventive effect of *L. aegyptiaca* seeds on breast cancer cells through *in vitro* analysis and its mechanisms towards cell death induced by apoptosis and cell cycle arrest.

1.3 RESEARCH OBJECTIVES

1.3.1 General Objectives

The main aim of this research is to acquire a better understanding on chemo preventive effect of *Luffa aegyptiaca* seeds (LAS) on breast cancer cell line (MCF-7) at the molecular level. In order to achieve the main idea of the research, there are a few specific objectives needed to be outlined.

1.3.2 Specific Objectives

i. To determine the inhibitory concentration [IC_{50}] of *L. aegyptiaca* seeds (LAS) 80% ethanolic extract and its anti-proliferative effects on breast cancer cell line (MCF-7).

ii. To determine the effects of the LAS 80% ethanolic extract on induction of apoptosis and cell cycle arrest on breast cancer cell line (MCF-7) using flow cytometer.

iii. To investigate the molecular signalling pathways with regards to apoptosis and cell cycle arrest of related genes expression of the LAS 80% ethanolic extract on breast cancer cell line (MCF-7) by quantitative polymerase chain reaction (qPCR) assay.

1.4 RESEARCH QUESTIONS

i. What is the concentration of the LAS extract that can cause 50% inhibition of cell growth and its inhibition on proliferation of MCF-7 cells?

ii. Can LAS extract trigger apoptosis induction on MCF-7 cells upon treatment at [IC_{50}]?