ANTICANCER STUDY OF *NEOLAMARCKIA CADAMBA* (KALEMPAYAN) LEAVES EXTRACT ON BREAST CANCER CARCINOMA CELLS (MCF-7)

BY

SHAKIRAH BINTI RAZALI

A thesis submitted in fulfilment of the requirement for the degree of Master of Health Science

Kulliyyah of Allied Health Science
International Islamic University Malaysia

DECEMBER 2019

ABSTRACT

Breast cancer is a leading cause of cancer-related death in women. Despite the advancement of breast cancer treatments, patients are suffered with side effects and other consequences such as cancer recurrence and resistance to treatments. An effective strategies are needed of which resources from the natural plants have been used for alternative approach as anti-cancer agents against breast cancer. The present study is an attempt to evaluate anti-cancer activities of *Neolamarckia cadamba* leaf (NCL) extract on the breast cancer carcinoma cells (MCF-7). MCF-7 cells were treated with various concentration of ethanolic NCL extracts. The concentration of NCL extract that caused 50% of cell growth inhibition [IC_{50}] in MCF-7 cells was determined after 72 hours of treatments, and its anti-proliferative effect was assessed upon treatment for 24, 48, 72 and 96 hours using TBEA method. NCL extract demonstrated inhibitory activity in cell growth at IC_{50} value of 0.206 mg/ml, in dose-dependent and time-independent manner. The effect of NCL extract on apoptosis induction and cell cycle arrest were analysed using flow cytometer. The flow cytometric analysis indicated that NCL extract 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 NCL extract induced apoptosis through the down-regulated 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-regulated of *CDK2* with subsequent up-regulated of *p21* and *cyclin E*. Overall, our data demonstrated that the NCL extract exerts anti-cancer effect on MCF-7 human breast cancer cells through induction of apoptosis and cell cycle arrest. The present study suggests that NCL extract may be an important alternative of anti-cancer agent candidate in breast cancer treatment.

خلاصة البحث

إن سرطان الثدي هو السبب الرئيسي لمعظم الوفيات المرتبطة بالسرطان لدى النساء. على الرغم من تقدم علاج سرطان الثدي، ما زال المرضى يعانون من آثاره جانبية وعواقب أخرى مثل تكرار السرطان ومقاومة العلاج. هناك حاجة ملحة إلى استراتيجيات فعالة للموارد التي تستخلص من النباتات الطبيعية في الأساليب البديلة كعوامل مضادة للسرطان ضد سرطان الثدي. الدراسة الحالية عبارة عن محاولة لتقييم الأنشطة المضادة للسرطان باستخدام خلاصة أوراق الكادامولا (NCL) *Neolamarckia cadamba* على خلايا سرطان الثدي (MCF-7) . تم علاج خلايا MCF-7 بتركيزات مختلفة من مستخلصات NCL الإيثانولية. تم تحديد تركيز مستخلص NCL الذي تسبب بتثبيط 50 ٪ من نمو الخلايا [IC_{50}] ضد خلايا-MCF 7بعد 72 ساعة من العلاج، وتم تقييم تأثيره المضاد للتكاثر على العلاج لمدة 24 ، 48 ، 72 و 96 ساعة باستخدام TBEA . أظهر مستخلص NCL نشاطا مثبطا في نمو الخلايا بقيمة 0.206 IC50 ملغرام / مل ، بطريقة تعتمد على الجرعة ومستقلة عن الوقت. تم تحليل تأثير مستخلص NCL على تحريض موت الخلايا المبرمج وتوقف دورة الخلية باستخدام تعداد تدفق الكريات. أشار تحليل التدفق الخلوي إلى أن مستخلص NCL يمنع نمو خلايا MCF-7 عن طريق تحفيز موت الخلايا المبرمج وتوقف دورة الخلية في مرحلة G1 / G0 وفي الوقت نفسه ، كشف تحليل التعبير الجيني باستخدام qPCR لمستخلص NCL أن موت الخلايا المبرمج قد نجم من خلال *Bcl-2* ، بينما تم تنظيم *Bax* و *cytochrome c* *caspase-9* و *caspase-7* في خلايا MCF-7 . ارتبط توقف الدورة الخلوية بتخفيض *CDK2* الذي تم تنظيمه بشكل متزامن مع إعادة التنظيم لـ *p21* و *cyclin E* . بشكل عام ، أظهرت بياناتنا أن مستخلص NCL يمارس تأثيرًا مضادًا للسرطان على خلايا سرطان الثدي البشري MCF-7 من خلال تحفيز موت الخلايا المبرمج والقبض على دورة الخلية. تشير الدراسة الحالية إلى أن مستخلص NCL قد يكون بديلاً هامًا كعامل مضاد للسرطان في علاج سرطان 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 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.

……..
Mohammad Syaiful Bahari Abdull Rasad
Internal Examiner

……
Azman Senin
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.

Shakirah binti Razali

Signature …….. Date ……..

INTERNATIONAL ISLAMIC UNIVERSITY MALAYSIA

DECLARATION OF COPYRIGHT AND AFFIRMATION OF FAIR USE OF UNPUBLISHED RESEARCH

ANTICANCER STUDY OF *NEOLAMARCKIA CADAMBA* (KALEMPAYAN) LEAVES EXTRACT ON BREAST CANCER CARCINOMA CELLS (MCF-7)

I declare that the copyright holder of this thesis are jointly owned by the student and IIUM.

By signing this form, I acknowledged that I have read and understand the IIUM Intellectual Property Right and Commercialization policy.

Affirmed by Shakirah binti Razali

…….. ….....

Signature Date

ACKNOWLEDGEMENT

In the name of Allah The Most Merciful and The Most Compassionate. All praise and gratitude is due to Allah and may peace and blessing be upon the Prophet Muhammad s.a.w and his family and his companions. Firstly, the most gratitude to Allah for always being with me, facing the challenges and barriers in my life as a servant and as a student. Helping me in whatever situation I face.

Alhamdulillah. It was a very long journey for me in order to complete my master study and to finish up my thesis. Gaining new experiences and kind of challenges to me for improving myself. Knowing and meeting various kinds of people and learnt something from them.

With my gratitude, I would to thank to my beloved family, father, mother, kak long, kak ngah, Amy and Syukur who are always supporting me, stay with me through thick or thin situations, give me courage and lots of advices. I would like to express my great appreciation to my supervisor, Assoc. Prof. Dr. Ridhwan Abdul Wahab, who is willingly to accept me as his student, as well as my co-supervisors, Prof. Dr. Dato' Ridzwan Hashim (Department of Biomedical Science) and Assoc. Prof. Dr Alfi Khatib (Department of Pharmacy) for their helping, support, guide and advice throughout the completion of this work. A special thanks to Dr Azir Salleh who make this study possible and have been a great help throughout the study.

Thanks to my gorgeous team mates, Sr Ina, Sr Tara, Sr Habibah, Sr Faizah, ICRACU staff, Bro Syahril, Bro Wali, Bro Saiful, Sis Norrul, Sis Aini, Sis Aini, Sis Syuhada, Sis Puteri and also to my juniors Nazihah, Syikin, Amanina, Syafiqah, Mislia, Khadijah, Naemah, I learnt a lot from them. InshaAllah may Allah help us. Once again, we glorify Allah for His endless mercy on us one of which is enabling us to successfully round off the efforts of writing this thesis. Alhamdulillah.

Last but not least, this study was financially supported by Fundamental Research Grant Scheme (FRGS 16-046-0545) and IIUM Research Initiative Grant (RIGS 15-085-0085).

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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
mRNA	messenger RNAs
MIQE	Minimum information for quantitative polymerase chain reaction publication experiments
MCF-7	Breast cancer cells
NCBI	National Center for Biotechnology Institute
NCL	*Neolamarckia cadamba* leaves
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 has become a major health problem that causes morbidity and mortality in millions of people worldwide. The number of cancer cases and deaths is expected to increase rapidly, as in relation to the increase of population, age and unhealthy lifestyle (Torre et al., 2015). It is estimated by 2030 approximately 26 million new cancer cases and 17 million cancer deaths per year globally (Thun, DeLancey, Center, Jemal, & Ward, 2009). In Asia alone, the incident of cancer cases and death in 2008 to 2030 are estimated to rise, 6.1 million to 10.7 million of cancer cases and 4.1 million to 7.5 million of death cancer (Sankaranarayanan, Ramadas, & Qiao, 2014). About 103,507 new cancer cases in Malaysia were diagnosed in the period of 2007 until 2011 as recorded by the Malaysian National Cancer Registry (Azizah, Nor Saleha, Noor Hashimah, Asmah, & Mastulu, 2016).

Breast cancer is the second most common of cancer type in women of developed and developing countries (Torre et al., 2015). This disease affects both genders, but mainly in women that exponentially rise with age (Lukong, 2017). It is rare in the male that approximately accounts for 1% of all cancer cases (Madeira et al., 2011). Breast cancer has become the leading cause of morbidity and cancer-related death. Malaysia is one of the Asian countries with the increase prevalence of breast cancer cases (Azizah et al., 2016; Sue, Yeonju, Daehee, En-Joo, & Keun-Young, 2011; Medina, Laudico, Mirasol-Lumague, Brenner, & Redaniel, 2010; Hirabayashi & Zhang, 2009; Takiar & Srivastav, 2008; Sim et al., 2006).

Surgery, radiotherapy, chemotherapy and anticancer drugs have been used in treating breast cancer patients. The advances of current treatments and detecting method have reported to improve and increase the survival rate (Blumen, Fitch, & Polkus, 2016; Bodai, 2015). Unfortunately, the consequences of these treatments left patients with side effects. The side effects of cancer treatments include recurrence of cancer, resistance to drugs (Rayan, Raiyn, & Falah, 2017) and a cardiac problem (Healey Bird & Swain, 2008) which can restrict the use of anticancer drugs and lowering quality of life (Rayan et al., 2017). Moreover, Trogdon et al. (2017) and Blumen et al. (2016) study demonstrated the burden of cost related to cancer treatment is increased for a different stage of breast cancer. Hence, new effective alternatives should be searched to counter the bad consequences of cancer treatment.

Nature is present with numerous diversity of natural products. They have been used as a natural medicine in treating various diseases, including cancer since ancient times (Graham, Quinn, Fabricant, & Farnsworth, 2000). About 114,000 extracts from 35,000 plant samples collected from 20 different countries by the National Cancer Institute have screened for anticancer activity (Shoeb, 2006). Some anticancer agents have been used in current cancer treatment are derived from plants. They are vinblastine (Velban), vincristine (Oncovin), etoposide, teniposide, taxol (paclitaxel), navelbine (Vinorelbine), taxotere (Docetaxel), camptothecin (Camptosar, Campto), topotecan (Hycamtin) and irinotecan (Demain & Vaishnav, 2011). Thus, natural plants have shown a potential role as an anticancer agent in combating cancers.

Natural products contain great molecules with pharmacological active potential. These molecules can influence more than one biological pathway when existing together (Surh, 2003). Thus, the influence of natural product on the mechanism of

cancer cell death, such as apoptosis and cell cycle is one of the approaches for cancer therapy (Galluzzi, Vitale, Vacchelli, & Kroemer, 2011; Pfeffer & Singh, 2018).

Apoptosis is one of the programmed cell death that plays a vital role in development and homeostasis in normal tissue. Defects in apoptosis pathway will lead to the formation of tumour (Fernald & Kurokawa, 2013). Both intracellular and extracellular signals lead to activation of apoptosis at different pathway which are the intrinsic and extrinsic pathways (Mcilwain, Berger, & Mak, 2013). Therefore, a better understanding of cell death by the naturally-derived anticancer agent will provide new strategies in targeting cell death pathway.

The cell cycle is driven by a controlled event for DNA replication and division of cells. It comprises of G1, S, G2 and M phase. The consecutive event of the cell cycle from one phase to the next phase is tightly regulated by regulatory proteins includes cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors (CDKIs). The disturbance and alteration of this event and its regulatory proteins may cause the development of cancer (Caldon, Daly, Sutherland, & Musgrove, 2006). Hence, targeting cell cycle signalling pathways by the naturally-derived anticancer agent is one of the approaches for anticancer treatment.

Neolamarckia cadamba is one of the underexplored plant. This plant has been reported to possess various pharmacological activities such as antioxidant, wound healing, antimicrobial (Sanjay et al., 2007), antidiabetic (Acharyya, Dash, & Abdullah, 2013), antihelmintic (Islam et al., 2015), analgesic, anti-inflammatory, and antipyretic (Mondal, Dash, & Acharyya, 2009). It has been used by Indian traditional formulation in treating illnesses among different Indian tribes using different parts of the plants including leaf (Pandey & Singh, 2016). Additionally, *N. cadamba* leaf has demonstrated anticancer activity on cancers (Singh, Ishar, Saxena, & Kaur, 2013). But, there is

inadequate of scientific research on this part of the plant. To our best knowledge, no studies have been conducted on *N. cadamba* leaf to investigate its effect and underlying mechanism against breast cancer.

1.2 PROBLEM STATEMENT

For many years, effective treatment of breast cancer still not found. The rising of breast cancer incidence year by year is burdening. Although the advancement of treatment increase the survival rate of the patients, yet, drug resistance and recurrent of cancer have responsible for treatment failure. Moreover, the challenge of finding new strategies by researchers is due to the different morphological and genetic changes in breast cancer (Weigelt, Geyer, & Reis-Filho, 2010).

The discoveries of new cancer treatments become mandatory. It may be suggested by exploration of natural plant-based anticancer agents that are effective with minimal side effects and cost as well as a multi-target mechanism of action on cancer. Therefore, this study is intended to evaluate *N. cadamba* leaf extract effect on breast cancer in *in vitro* and its mechanisms towards apoptosis cell death and cell cycle arrest.

1.3 RESEARCH OBJECTIVES

1.3.1 General Objectives

This research is aim to obtain a better understanding of the anticancer effect of *Neolamarckia cadamba* (*N. cadamba*) leaves on human breast cancer cell line (MCF-7) at the molecular level.

1.3.2 Specific Objectives

i. To determine the concentration of *N. cadamba* leaves (NCL) extract that inhibits 50% of cell growth [IC_{50}] and its antiproliferative effects of NCL extract on human breast cancer cell line, MCF-7.

ii. To investigate the effects of the NCL extract on induction of apoptosis and cell cycle arrest on breast cancer cell line, MCF-7 using flow cytometer.

iii. To determine the molecular signalling pathways with regards to apoptosis and cell cycle arrest of related genes expression of the NCL 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 NCL extract on MCF-7 cells that cause 50% inhibition of cell growth and its inhibition on proliferation of cells?

ii. Is NCL extract can trigger apoptosis on MCF-7 cells upon treatment at [IC_{50}]?

iii. Is NCL extract can induce cell cycle arrest on MCF-7 cells upon treatment at [IC_{50}]?

iv. What is the possible mechanism of signalling pathways of high expression of genes related to apoptosis and cell cycle arrest in response to NCL extract treatment on MCF-7?

1.5 RESEARCH HYPOTHESES

i. The extract of NCL induce 50% inhibition of cell growth at low concentration by decreasing cell viability.

ii. The extract of NCL caused morphological changes related to apoptosis in MCF-7 cells.
iii. The extract of NCL trigger apoptosis on MCF-7 cells upon treatment.
iv. The extract of NCL promote cell cycle arrest on MCF-7 cells upon treatment.
v. The extract of NCL execute apoptosis and cell cycle arrest by up-regulation and down-regulation of related genes expression at the molecular level.

1.6 SIGNIFICANCE OF THE STUDY

This study will provide the underlying effect of the NCL extract on breast cancer cells, MCF-7. The NCL effects on cell death and cell cycle induced are important as a part of the anticancer candidate development and research. Furthermore, the expression of apoptosis and cell cycle-related genes at the molecular level will present extensive information in understanding the underlying mechanism of how NCL extracts react towards MCF-7 cells. Overall, this study will provide a better insight into NCL. Proper strategies can be strategized to forthright the study further as potential anticancer agents in breast cancer treatment in the future.

1.7 RESEARCH FRAMEWORK