



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

BY

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## 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<sub>50</sub>] 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<sub>50</sub> 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<sub>0</sub>/G<sub>1</sub> 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 (*Neolamarckia cadamba* (NCL) 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<sub>50</sub>] ضد خلايا MCF-7 بعد 72 ساعة من العلاج، وتم تقييم تأثيره المضاد للتكاثر على العلاج لمدة 24 ، 48 ، 72 و 96 ساعة باستخدام TBEA. أظهر مستخلص NCL نشاطاً مثبطاً في نمو الخلايا بقيمة IC<sub>50</sub> 0.206 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 G<sub>0</sub> / G<sub>1</sub> 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 cytochrome c* و *caspase-7* و *caspase-9* 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 تأثيراً مضاداً 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 هاماً كعامل مضاد للسرطان في علاج سرطان الثدي.

## APPROVAL PAGE

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## 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 <sub>2</sub>  | Carbon dioxide   |
| DNA              | Deoxyribonucleic acid  |
| DMEM             | Dulbecco's Modified Eagle Medium   |
| DMSO             | Dimethyl sulphoxide  |
| E                | Efficiency   |
| FBS              | Fetal bovine serum   |
| FITC             | Fluorescein isothiocyanate   |
| IC <sub>50</sub> | 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              | <i>Neolamarckia cadamba</i> 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

|                    |                                     |
|--------------------|-------------------------------------|
| $\alpha$           | Alpha                               |
| $\beta$            | Beta                                |
| $\Delta$           | Delta                               |
| Cq                 | Quantification cycle                |
| Ct                 | Threshold cycle                     |
| g                  | Gram                                |
| g                  | gravity                             |
| G                  | Gap                                 |
| M                  | Mitosis phase                       |
| S                  | Synthesis phase                     |
| $\mu\text{L}$      | Microliter                          |
| $\mu\text{g/mL}$   | Microgram per millilitre            |
| $^{\circ}\text{C}$ | Degree Celsius                      |
| %                  | Percent                             |
| -                  | To                                  |
| >                  | More than                           |
| <                  | Less than                           |
| $\pm$              | 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, Trogon 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<sub>50</sub>] 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<sub>50</sub>]?
- iii. Is NCL extract can induce cell cycle arrest on MCF-7 cells upon treatment at [IC<sub>50</sub>]?
- 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

