

ANTI-PROLIFERATIVE ACTIVITY OF CRUDE AND FRACTIONATED
EXTRACT OF *Labisia pumila* ON HUMAN PROSTATE CANCER CELL LINES

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EXTRACTS OF *Labisia pumila* ON HUMAN PROSTATE CANCER CELL LINES

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To Ayah Jamaludin bin Che Matt and Ma Jamilah binti Mahmud who love me unconditionally. Both of you are the most beautiful gift that has ever happened to me.

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ABSTRACT

Labisia pumila (*L. pumila*) or locally known as Kacip Fatimah has been widely used by women in Malaysia to address post-partum health conditions. This herbaceous undershrub of the Myrsinaceae family has been made into beverages, cosmetics health and supplements to promote overall wellness. The objective of the present study was to determine the anti-proliferative property of *L. pumila* crude extract against prostate cancer cell lines. In this study, the crude extract was prepared by using ultrasound assisted extraction method. The result revealed that *L. pumila* crude extract inhibited the proliferation of the prostate cancer cell lines. The crude extract was further fractionated using column chromatography and the most active fraction was determined as 40% methanol (MeOH) fraction. The results showed that both crude extract and 40% MeOH fraction contained phytochemicals that caused the characteristic apoptosis. However, higher percentage of early and late apoptosis were observed in crude extract as compared with 40% MeOH fraction. As for the apoptotic body, this characteristic was only found in the DU 145 prostate cancer cell morphology that was treated with 40% MeOH fraction. The result from the scavenging activity test confirmed that *L. pumila* crude extract and 40% MeOH fraction displayed a strong antioxidant activity with the value of 70%. Total phenolic contents assay was carried out to determine the phenolic content of the extract and its active fraction. Identification on polyphenolic compound was carried out using Ultra-performance liquid chromatography tandem mass-spectrometry and the result revealed that rutin and 3 organic acids including gallic, p-coumaric and salicylic acids were present in both crude extract and 40% MeOH fraction. High performance liquid chromatography analysis confirmed that gallic acid as one of the phytochemical constituents found in *L. pumila*. In conclusion, these findings suggested that crude extract and 40% MeOH fraction of *L. pumila* contains phenolic compound that act as an anti-proliferative agent against proliferation of prostate cancer cell lines *in vitro*.

ABSTRAK

Labisia pumila (*L. pumila*) atau lebih dikenali sebagai Kacip Fatimah telah digunakan secara meluas oleh wanita di Malaysia sebagai perawatan selepas bersalin. Herba renek berasal daripada keluarga Myrsinaceae ini telah diproses dalam bentuk minuman, kosmetik dan makanan tambahan kesihatan untuk menggalakkan kesejahteraan. Kajian ini adalah untuk menentukan aktiviti anti-proliferatif daripada ekstrak akueus *L. pumila* dan sebatian aktif daripadanya terhadap sel kanser prostat. Hasil kajian mendedahkan bahawa ekstrak *L. pumila* yang diekstrak menggunakan kaedah pengestrakan ultrabunyi bertindak menghalang pertumbuhan sel-sel kanser prostat. Oleh itu, ekstrak ini diperingkat lagi dengan menggunakan alat kromatografi turus bagi menuliskan beberapa bahagian yang aktif dan pecahan yang paling aktif telah dikenalpasti sebagai pecahan 40% metanol (MeOH). Hasil yang dipaparkan menunjukkan bahawa ekstrak mentah dan pecahan 40% MeOH mempunyai fitokimia yang menghasilkan ciri-ciri dalam apoptosis. Walaupun peratusan apoptosis awal dan akhir jelas terdapat dalam semua rawatan tetapi morfologi badan apoptotik hanya dapat ditemui pada DU 145 prostat sel kanser selepas dirawat dengan pecahan 40% MeOH. Keputusan yang diperolehi daripada aktiviti pemerangkapan mengesahkan bahawa ekstrak mentah dan pecahan 40% MeOH *L. pumila* menunjukkan aktiviti antioksidan yang banyak dengan nilai adalah lebih 70%. Assai jumlah kandungan fenolik telah dijalankan untuk menentukan sebatian fenolik di dalam pecahan tersebut. Identifikasi kepada sebatian polifenolik telah dilakukan menggunakan spektroskopi jisim tandem kromatografi cecair prestasi ultra dan hasilnya mendapati bahawa rutin dan 3 asid organik termasuk asid galik, p-kumarik dan salisilik telah dikesan dalam kedua-dua ekstrak mentah dan pecahan 40% MeOH. Keputusan analisis daripada kromatografi cecair prestasi tinggi mengesahkan bahawa asid galik adalah sebahagian daripada komponen fitokimia dalam *L. pumila*. Kesimpulannya, hasil kajian ini menunjukkan bahawa ekstrak mentah dan pecahan 40% MeOH *L. pumila* mengandungi kumpulan fenolik yang boleh bertindak sebagai agent anti proliferatif ke atas garisan sel kanser prostat secara *in vitro*.

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LIST OF ABBREVIATION

ATCC	-	American Type Culture Collection
AO/EB	-	Acridine orange/ Ethidium Bromide
AO/PI	-	Acridine orange/ Propidium iodide
cm	-	centimeter
CO ₂	-	Carbon dioxide
DCM	-	Dichloromethane
EMEM	-	Eagle Minimum Essential Medium
EDTA	-	Ethylene Diamine Tetra Acidicacid
FBS	-	Foetal Bovine Serum
g	-	Gram
HSF	-	Human Skin Fibroblast
HPLC	-	High-performance liquid chromatography
kHz	-	Kilohertz
L	-	Litre
MARDI	-	Malaysian Agricultural Research and Development Institute
MeOH	-	Methanol
mg	-	Milligram
mL	-	Millilitre
MTT	-	3-(4,5 Dimethylthiozol-2-yl)-2,5- diphenylettrazolium
PBS	-	Phosphate Buffer Saline
UAE	-	Ultrasound Assisted extraction
UPLC-MS/MS	-	Ultra Performance Liquid Chromatography- Mass Spectrometry/ Mass Spectrometry
USA	-	United State America
WHO	-	World Health organization

LIST OF SYMBOLS

%	-	Percentage of extraction yield
$M_c\%$	-	Percentage of moisture content
m_0	-	Mass in grams of dish
m_1	-	Mass in grams of dish and sample before drying
m_2	-	Mass in grams of dish and sample after drying
mm	-	Millimetre
μm	-	Micrometre
g	-	Gram
mL	-	Millimetre
MPa	-	Megapascal
K	-	Kelvin
$^{\circ}\text{C}$	-	Degree celcius
P_c	-	Critical pressure
T_c	-	Critical temperature
X_i	-	Variables
Y_i	-	Responses
A	-	Alpha
Nm	-	Nanometer
Ppm	-	Part per million
Psi	-	Pound per square inch
P	-	Density

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

In these few years, cancer has become the topmost killer disease worldwide. The term cancer is very inclusive. Cancer arises when within a single cell grows with a time into a mass of cells, some of which can spread to other locations in the body (metastasize), where they will aggressively grow and upset normal bodily functions (Pardee and Gary, 2009). During metastasize, cancer cells enter blood stream and lymphatic system and relocate themselves to other part of the body leading to the growth of secondary tumours. At this point, secondary tumours may leads to fatality due to the loss and interruption of body function

Several cancers have been reported to be prominent among male. Lung cancer tops the list followed by stomach, liver colorectal and oesophagus worldwide (Steward and Wild, 2014). On the other hand, prostate cancer is the most common cancer in male in all races in United States and the second leading cancers that causes of death among white, African American, American Indian and Alaska native. In Asia region, prostate cancer is the fourth most common cancer among male (U.S. Cancer Statistics Working Group, 2015). Prostate cancer occurs when there is uncontrolled growth of prostate cells leading to progression of cancerous cells.

Various kinds of cancer treatments are available for patients as options depending on their age, stages of the cancer and other health conditions. In for the early stages, surgery might be the best option followed by external beam radiation or brachytherapy (radioactive seeds implant). Hormonal therapy also included as the option that may be used along with the surgery or radiation therapy provided with some exceptional cases (Adsul *et al.*, 2015 and Ray *et al.*, 2015) . Unfortunately, these treatment had been known to have side effects. Therefore, alternative solution for the treatment is sight after. Combination of natural sources or phytotherapy and synthetic drug may produce significant synergetic effect in treatment of prostate cancer (Ulrich-Merzenich, 2014).

Throughout the history, plants have been used as food as well as medicinal purposes. Every nation in this world has their own medicinal plants. Plants have been investigated extensively in order to disclose the potent role of plant in treating and averting serious diseases and illnesses such as cancer (Khazir *et al.*, 2014), diabetes (Raafat and Samy, 2014), epilepsy (Sahranavard *et al.*, 2014) and even fever. Certain plants have been reported to possess anti-inflammatory (Zakaria *et al.*, 2011), anti-analgesic and anti-angiogenesis properties due to the active compounds and secondary metabolites derived from the plants.

There has been growing interest in the use of naturally occurring compounds with chemopreventive and chemotherapeutic properties in the treatment of cancers. For instance, herbs have been considered natural and valuable sources for anticancer drug discovery (Cragg & Newman, 2013). Herbal medicine has been prescribed in many countries over centuries for treating various diseases, including infectious and malignant diseases. Plants have played an important role as a source of effective anticancer agents. It is significant to note that 60% of the currently used anticancer agents are derived from natural sources including plants, marine organisms and microorganisms (Cragg & Newman, 2005).

Plants also have been widely used in Malay traditional medicine. For example, *Labisia pumila* or Malaysia commonly called Kacip Fatimah, akar selusuh by old folk in Malaysia is a popular herb and related to women. It is believed that this plant has

been used for centuries in inducing and facilitating the child birth as well as post-partum medicine. It also helped to improve menstrual problem by boiling the water from leaves, root or whole plant of *L. pumila*. Numerous numbers of researches have been done to identify the phytochemicals of this plant. Chua and colleagues (2011), Karimi *et al.* (2011) and Norhaiza *et al.* (2009) have reported that there were abundant of phenolic compounds including phenolic acids and flavonoids in *L. pumila* plant extract. The presence of phytochemicals in the plant could leads to the anticancer activity of *L. pumila* against prostate cancer.

In order to extract the phytochemicals from the plants, several types of extraction like soxhlet, maceration, boiling as well as new technologies such supercritical fluid and ultrasound assisted extraction can be employed using several organic solvents and water. The selective method and solvents that are going to be used must be corresponded with the desired compounds from the plants (Sasidharan *et al.*, 2011). Heat and temperature play an important role to isolate the targeted compound without reducing the quality of the extracts.

Ultrasound-assisted extraction (UAE) has been demonstrated to reduce the temperature during extraction. This method was reported to display better heat reducing compared to soxhlet and heat reflux which the latters require high temperature during the extraction process.(Trendafilova *et al.*, 2010). These methods were performed under lower temperature that was favourable for all thermally unstable compounds without any loss of desired active compounds (Khoddami *et al.*, 2013). In term of solvent dependence, UAE extraction has minimized the usage of solvent. Moreover, UAE is more practical, simple, and convenient for recovery and purification of the active ingredient of plant material such as phenolic, saponins and others (Vázquez *et al.*, 2008).

Toxicity of plant can be assessed through *in vitro* and *in vivo* studies. The potential plant extract can be tested on cell lines to determine their cytotoxicity against particular cancer cells. In corroboration with this fact, normal, cancerous and even wounded cell lines might be used to establish the effectiveness of plant extract. Some plant-derived compound has been extensively studied and considered as potential to

be natural anticancer agent in the future (Cragg and Newman, 2005). The claimed anticancer extract should not be harmful to normal cell and to the human body system. Furthermore, it also has the lowest of inhibitory concentration (IC_{50}) that kill half of cell tested population (Ekwall *et al.*, 1990).

Cell based assay like cell proliferation assay will be carried out in order to investigate the cytotoxicity of extract tested on the cell. Methods such as MTT assay by Mosmann (1983), XTT assay improved from MTT assay, MTS and Water Soluble Tetrazolium Salts (WSTs) assay have been used widely. Counting cell assay is used to determine the number of dead and viable cells using trypan blue in cell staining to differentiate between dead and viable cells. Moreover, cell viability can be seen to determine the viability of cells for certain period of time using trypan blue exclusion method where the dye would enter the membrane of dead cells.

Cell death population can be investigated through many ways such by observation under light or florescence microscope, using prepared kit and analysing using equipment such as flow cytometer (Henry *et al.*, 2013). Microscopic examination could be performed with appropriate staining method. The morphology changes in cells due to the treatment with particular extracts or drugs can be examined using this approach. In flow cytometry, cells are suspended in the stream of fluids and they will pass through the electrons. This technique is used in cell counting, cell sorting, microscopic identification like determination of biomarker and protein (Hossain *et al.*, 2011).

1.2 Problem Statement

Numerous researches was conducted using *L. pumila* on the phytochemical along with potential biological activities derived from this plant. Some studies reported that *L. pumila* strong anti-carcinogenic (Lope Pihie *et al.*, 2012), anti-microbial (Karimi *et al.*, 2011) and antifungal activities (Karimi *et al.*, 2013). Anticancer studies also was determined and proved that *L. pumila* inhibit the activity of several cancer

(Al-Mekhlafi *et al.*, 2012). However, there were no report on phytochemicals of *L. pumila* tested on the prostate cancer cell lines particularly on DU 145 cell lines.

Higher concentration of antioxidant may contribute to the inhibition of the cancer cells. Antioxidants are chemicals that neutralize free radicals, thus preventing them from causing damage. Antioxidants are also known as “free radical scavengers”. If the free radical generated intracellular maybe the major factor behind the growth inhibition and apoptosis observed in the cancer cell. *Labisia pumila var alata* was reported to have higher antioxidant activity compare to other varieties (Norhaiza *et al.*, 2009). Therefore, there are potentially strong relationship between antioxidant activity and mechanisms such as proliferation and apoptosis of prostate cancer cells.

Phenolic compound such as gallic acid is an abundant phytochemical of *L. pumila*. Gallic acid from other plant have displayed the anticancer properties by demonstrating some of hallmarks of cancer such as inducing the apoptosis and blocking the proliferation signalling of cancer cells. Gallic acid from *Toona sinensis* leaf extract showed cytotoxicity towards DU 145 prostate cancer cell through generation of reactive oxygen species and was able to block the growth of DU 145 at early stage of cell cycle by activating certain protein that involved in mitosis mechanism (Chen *et al.*, 2009). Other phytochemicals with similar in *L. pumila* that have the anticancer properties in inhibiting the prostate cancer are apigallocatechin and phytoestrogen. Thus, this will leads to unleash the potential of *L. pumila in* inhibiting the progression of prostate cancer.

In order to isolate the potential compound from the plant, the extraction method should be taken into consideration. Conventional method like soxhlet reflux, maceration and microwave-assisted extraction has been carried out to extract the phenolic compound for many years. It is important to note that these various extraction methods could lead to degradation of active compounds of the plant due to oxidation, hydrolysis and thermal disintegration due to high temperature. New technologies have been invented to improve the functional and ameliorate the disadvantages of conventional method.

1.3 Objectives

The objectives of this study was to investigate the potential of crude and fractions of *L. pumila* extract in inhibiting the cell proliferation of prostate cancer lines (DU 145).

1.4 Scope of Study

In order to fulfil and achieve the objective, the experimental works were divided into three major scopes. The scopes of the research are:

- i) To extract *L. pumila* using ultrasound assisted extraction.
- ii) To fractionate the crude extract of *L. pumila* via column chromatography.
- iii) To study the anti-proliferative activity of crude and fractions *L. pumila* extract on DU 145 prostate cancer cell lines.

1.5 Organization of Thesis

This thesis was organized into five chapters. It starts with introduction chapter, followed by literature review, research methodology, result and discussion, and conclusions and recommendations chapter.

Background of the research and problem statement were stated in the chapter 1. The objectives and scopes of the study were stated in the first chapter.

The statistics, symptoms, stages and treatment for prostate cancer were further described in the first part of the Chapter 2. The taxonomy, medicinal used, potential activities and phytochemical compound of *L. pumila* were reviewed together with the plant chemical compounds, which previously studied to have anticancer properties. In

addition, the cell growth, death and assessment of cell death were explained in the last part of this chapter.

Chapter 3 mainly focused on the methodology of this research. Method for extraction, fractionation, cell proliferation analysis and phytochemical screenings such as antioxidant and total phenolic compound were elucidated in this chapter. The statistical analysis also was described at the end of this chapter.

The results and discussion are discussed in the Chapter 4. Section 4.2 discusses the extraction yield and the fractionation part. The results of proliferation activity of crude and fractions from *L. pumila* tested on human prostate cancer cell lines and human skin fibroblast were discussed in the following section. Then, the morphological study and flow cytometry analysis were analyzed in this chapter. Chapter 4 ends with phytochemical screening results from the total phenolic content, HPLC and UPLC mass spectrophotometry analyses.

Finally, the findings are concluded in the Chapter 5. Recommendations were also made in this chapter for future guidance which hopefully may help to improve further experimental works on *L. pumila* and prostate cancer cell lines.

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