SUPERCRITICAL FLUID EXTRACTION OF BIOACTIVE COMPOUNDS FROM *LYCIUM CHINENSE* AND *LYCIUM BARBARUM*

BY

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ABSTRACT

Cancer is one of the most dangerous diseases, and breast cancer contributes to 22.9 percent of all cancers excluding non-melanoma skin cancer. There are several plant extracts which were reported for their potential to be used in cancer treatment. This includes *L. chinense* and *L. barbarum* which have been reported to contain abundant bioactive compounds with a lot of medicinal properties such as anti-cancer and powerful anti-oxidant. One of the advanced technologies that can be used to extract these bioactive compound is supercritical fluid extraction (SFE), replacing the conventional extraction methods that have several disadvantages. However, no report is available on supercritical fluid extraction for *L. chinense* and *L. barbarum* fruits. Therefore, in this study, supercritical fluid extraction was used to extract bioactive compounds from *L. chinense* and *L. barbarum* fruit. Optimization of the extraction yield were conducted using Response Surface Methodology for three important parameters namely temperature, co-solvent and time. Under different extraction conditions 15 experiments were designed and studied. The optimum yields from *L. chinense* and *L. barbarum* were 0.0656g/g and 0.0332g/g, respectively, which was attained within 60 minutes extraction times at 45 °C using 20% co-solvent. Co-solvent and extraction time had the most significant effect on the yield ($p<0.05$) while temperature showed minor influence on the yield of crude extraction. Furthermore, the assay for bioactive compound such as antioxidant and cytotoxicty were also cunducted for both *L. chinense* and *L. barbarum* crude extract. Anti-oxidant activity of 20mg/ml of *L. chinense* crude extract shows higher DPPH free radical scavenging capacity with 82.79% compared to 20mg/ml of *L. barbarum* crude extract with 81.75% of DPPH scavenging capacity. However, 20mg/ml of *L. chinense* and *L. barbarum* crude extracts on DPPH free radical scavenging were higher to that of 0.1mg/ml of BHA (81.71%) and 0.115mg/ml of BHT (73.87%). In addition, 20mg/ml of *L. barbarum* crude extract *on ABTS+* radical indicated higher anti-oxidant capacity with 93.95% compared to 20mg/ml of *L. chinense* crude extract with 87.93% of scavenging capacity. However, the results for 20mg/ml of *L. chinense* crude extract ABTS+ free radical scavenging was lower to 0.2mg/ml of BHA (93.90 %) and 0.4mg/ml of Vitamin C (93.61%). On the other hand, the 20mg/ml of *L. barbarum* crude extract on ABTS+ free radical scavenging showed higher anti-oxidant than 0.2mg/ml of BHA and 0.4mg/ml of Vitamin C with 93.90% and 93.61%, respectively. Furthermore, *L. chinense* crude extract shows higher cytotoxic activity with IC_{50} value of 1126.4530µg/ml compared to *L. barbarum* crude with IC_{50} value of 1919.304µg/ml. However, when compared to Taxol, *L. chinense* crude extracts showed much lower cytotoxic activity as Taxol`s IC_{50} value was 0.122µg/ml. This result was expected as the *L. chinense* and *L. barbarum* crude extract were not purified while Taxol used was highly purified. However, the cytotoxic activity in *L. chinense* and *L. barbarum* crude extract indicated their potential as cytotoxic agent.

خلاصة البحث

يعتبر السرطان واحد من أخطر الأمراض في العالم، ويشكل سرطان الثدي تحديدا٢٢,٩٪ من مجموع السرطانات عدا سرطان الجلد اللاميلانومي. هنالك العديد من مستخلصات النباتات التي تم تأكيد فاعليتها في علاج السرطان. هذا يتضمن *L. chinense* و *L. barbarum* وذلك لاحتوائهما مركبات حيوية لها العديد من الخواص الطبية كمضادات السرطان ومضادات الأكسدة. واحدة من التقنيات المتطورة لاستخلاص المركبات الحيوية بنجاح هي تقنية إستخلاص الموائع فوق الحرجة، مستبدلة بذلك الطرق التقليدية التي تتميز بكثير من المصاعب. ولكن لم يتم تجربة هذه التقنية على النبتتين المذكورتين اعلاه. لهذا السبب، استخدمت تقنية استخلاص الموائع فوق الحرجة في هذا البحث. لإيجاد المردود الأمثل لعملية الاستخلاص تم استخدام طريقة الاستجابة السطحية على ثلاثة عوامل رئيسية وهي الحرارة، المذيب المشارك والوقت تحت مختلف عوامل الإستخلاص في ١٥ تجربة. المردود الأمثل لمستخلصات *L. chinense* و *L. barbarum* كان ٠,٠٦٥٦غ/غ و ٠,٠٣٣٢غ/غ الذين تمت التحصل عليهم في ٦٠ دقيقة من عملية الإستخلاص في ٤٥ درجة مئوية ل ٢٠٪ من المذيب المشارك. للمذيب المشارك ووقت الإستخلاص تأثير هام على المردود ($p<0,05$) في حين أن الحرارة كان لها تأثير ثابت على مردود إستخلاص الخام. ولهذا تجربة المضادات الحيوية كمضادات الأكسدة والتسمم الخلوي تم على كلى النوعين. أظهر مضاد الأكسدة ل٢٠ مغ/مل من مستخلص *L.chinense* نسبة ٨٢,٧٩% من DPPH لقوة الكسح الجذري الحرة وهي نسبة أعلى من نسبة ٨١,٧٥ للنوع الثاني. ومع ذلك ٢٠مغ/مل من مستخلص كلى النوعين من DPPH لقوة الكسح الجذري الحرة أعلى من ٠,١مغ/مل من BHA(٨١,٧١%) و٠,١١٥ مغ/مل من BHT (73.87%). بالاضافة الى ذلك، ٢٠ مغ/مل من مستخلص *L. barbarum* في +ABTS يظهر قدرة مضادة للاكسدة أعلى تقدر بـ ٩٣,٩٥% مقارنة بـ ٢٠ مغ/مل من مستخلص *L.chinense* مع ٨٧,٩٣% من قوة الكسح الجذري. ومع هذا، نتائج الـ٢٠ مغ/مل من مستخلص *L.chinense* ل +ABTS ادنى من ٠,٢مغ/مل من BHA (٩٣,٩٠ %) و ٠,٤ مغ/مل من الفيتامين سي (٩٣,٦١%). ومن ناحية أخرى، ٢٠ مغ/مل من مستخلص *L. barbarum* في +ABTS يظهر نسبة مضاد أكسدة أعلى بـ ٠,٢ مغ/مل BHA و٠,٤مغ/مل من الفيتامين سي بنسبة ٩٣,٩٠ % و٩٣,٦١% على التوالي. أظهر مستخلص الخام *L.chinense* قدرة تسمم خلوي ب IC_{50} بقيمة١١٢٦,٤٥٣٠ ميكروغرام/مل مقارنة مع *L. chinense* التي اعطت ١٩١٩,٣٠٤ ميكروغرام/مل. تمت مقارنة مضادات السرطان ومضادات الأكسدة المستخلصة مع التاكسول أظهر مستخلص الخام ل *L.chinense* الخلايا أضعف بكثير مقارنةً بالتاكسول ذو ال IC_{50} بقيمة٠,١٢٢ ميكروغرام/مل. هذه النتيجة كانت متوقعة بما أن مستخلص *L. barbarum* و*L.chinense* لم يكن مصفى في حين مستخلص التاكسول كان مصفى جيدا. فبذلك قدرة المستخلص الخام لـ *L.chinense* و *L. barbarum* على تسميم الخلايا يدل على أهليتها لتستخدم كعامل سام للخلايا.

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 Science (Biotechnology Engineering).

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DECLARATION

I hereby declare that this dissertation 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.

Zulipiye Aihemaiti

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In the Name of Allah, the Beneficent, the Merciful and Salam upon his Messenger Prophet, Mohammed (SAW).

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CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND

In medical sciences, cancer is defined as an unfettered cell growth. One of the malignant tumors attacks on the near parts of the body because one of the cancer cells grows in an uncontainable way. Cancerous cell is called metastasis, because it can spread to other parts of the body through blood or lymphatic stream. Some of tumours are benign, thus they do not spread to other parts of the body, and do not attack the nearby tissue; all of them do not malignant in nature. The genetic role in cancer was recognized to be from 5-10%. In general, the disease can be treated using several methods such as chemotherapy, surgery or through radiation therapy. However, these treatment processes tend to cause unwanted side effect, which could be harmful. Therefore, researchers have currently developed the usage of medicinal plants as alternative methods to cure cancer, which are deemed to be safer (Rahmat et al., 2013).

Medicinal plants have been used for the treatment of various diseases such as cancer, diabetes, and obesity. Antioxidants property from different medicinal plants could inhibit cancer by balancing the free radicals inside the body that can damage the DNA, which cause cancer, aging, etc. Several studies have shown that plants have been used for cancer treatments because of its components or constituents that modulate oxidative stress and antioxidants scavenge (Powell et al., 2005) .

Alternative medicine such as traditional Chinese medicine (TCM) is a regular system of diagnostics and therapies, which was trained worldwide, and as a result, it

may help as a beneficial model for scientific inquiry. Among the constituents of TCM, botanical or herbal agents have complex biological activities that might affect many sides of carcinogenesis such as cell growth and proliferation, host-tumor interactions, apoptosis, and immune function and differentiation (Cohen et al., 2002).

Goji berry (*Lycium chinense* and *Lycium barbarum),* an important Chinese herbal medicine is one of the Solanacea family, which has been usually used as health food due to its pharmacological and biological active compound (Luo et al., 2006). Goji is the new name given to two close species which are *L. chinense* and *L. barbarum* that are used as the food and medicinal plants in East Asia, in particularly in China for a long time (Wang et al., 2010). It is rich in bioactive components, such as polysaccharides, polyphenolic compounds and flavonoids. It also exhibit high antioxidant properties that terminate free-radical mediated reactions by contributing hydrogen atom or an electron to the radicals (Ke et al., 2011).

Polysaccharides are important components from *L. barbarum* fruit, and are established to be responsible for multiform biological functions of the fruit (Zhou et al., 2007). Polysaccharides have immunogenicity and anticancer properties. Flavonoids constitute another major group of polyphenol compound, and are usually distributed in plants, vegetables as well as fruits. They are said to have anti-cancer, anti-atherosclerosis, and anti- inflammation functions (Wang et al., 2010). Carotenoids are another important group of bioactive compound. However, flavonoids and carotenoids have been less investigated especially for their antioxidant. In addition, antimicrobial compounds were also found in the *L. chinense* and *L. barbarum* roots. Specific α-galactosidase inhibitors, N-methylcalystegines structure/ activity relationship of calystegines from *L. chinense* and *L. barbarum* have been

reported (Chung et al., 2014). *L. chinense* and *L. barbarum* has a variety of important biological function, such as anti- aging, anti-tumor, enhancing immunity, anti-oxidation and many other pharmacological effects (Zheng et al., 2010).

Therefore, in order to verify the presence of these significant bioactive compounds, it is necessary to isolate and determine the extracts method through which its extraction process can be optimized. One of the latest approaches of phytochemical extraction is Supercritical Fluid CO_2 Extraction (SFE), a promising tool with better performance over the conventional extraction methods.

L. chinense and L. barbarum have been reported to show antipyretic, hypotensive, and hypoglycemic activities in animal models (Huang et al., 2014).In addition, few researches have analyzed anti-oxidant and anticancer properties of *L. chinense* and *L. barbarum* extracts (Huang et al., 2014).

1.2 PROBLEM STATEMENT AND ITS SIGNIFICANCE

Current statistics is pointing to the fact that cancer might be the world's most dangerous disease. Stewart and Wild; (2014) reported that cancer is the cause of death of around 8.2 million people.

Chemotherapy, radiation and surgery are breast cancer treatments; however, there are claims that they are unsafe, unguaranteed and costly with various side effects such as hair loss and weariness along with psychologic loads. Therefore, people are opting for better natural alternatives as many medical plants served as bases of drugs substitution with few side effects.

Finding the best extraction method of bioactive compounds from the medical plants are significant in order to obtain great yield of extracts. Various extraction parameters, such as temperature, pressure and extraction time also play role in attaining high extraction yield. However, the conventional extractions methods that are commonly used have various disadvantages, for instance low yield due to long extraction time, ease of contamination, destructive environmental effects as a result of the usage of huge volume of solvent, as well as less efficiency. A new advanced extraction technology that involves supercritical fluid extraction (SFE) is proposed to give the impression to be a better extraction option because of its ability to achieve higher extraction yield at shorter extraction time. Therefore, in this study, in order to avoid the risks posed by the use of the conventional extraction methods of bioactive compounds from *L. barbarum and L. chinense* will be extracted using the SFE. In addition, alaysis antioxidant and cytotoxic activity of *L. chinense* fruit extract for breast cancer was new research position in cancer research study.

1.3 RESEARCH OBJECTIVES

1. To optimize the extraction of bioactive compounds from *L. chinense* and *L. barbarum* using Supercritical Fluid Extraction (SFE).
2. To determine the antioxidant activity of *L. chinense* and *L. barbarum* extracts using antioxidant assays.
3. To determine cytotoxic effects *L. chinense* and *L. barbarum* against MCF-7 cells.

1.4 SCOPE OF RESEARCH

In this study, *L. chinense* and *L. barbarum* were used. SFE method was chosen to extract crude compounds from *L. chinense* and *L. barbarum* due to its technological advances and environmental friendly compared to other solvent extraction methods. The conditions of SFE process to extract crude compounds were optimized using Response Surface Methodology (RSM). The extraction parameters that were examined comprised of temperature, extraction time and precentage of co-solvent used. The range of temperature selected was 45 - 65 °C, with extraction time extending from 50 - 120 minutes, and co-solvent amount of 10 - 20%.

Furthermore, two antioxidant assay were conducted, which include DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging assay and ABTS [2, 2`- azinobis-(3-3 ethylbenzothiazoline-6-sulfonic acid)] radical scavenging assay. In addition, analysis on cytotoxic effect was tested on two types of mammalian cell cultures, there were normal cells, VERO and MCF-7, breast cancer cells using MTT assay.

1.5 THESIS ORGANIZATION

Chapter one includes, a brief introduction to the research, the problem statement and its significance, research objectives, and scope of study. Chapter Two discussed about the literature review on traditional Chinese medical plant, bioactive compounds and their function, various methods of *L. chinense* and *L. barbarum* crude extraction, including SFE a new method for *L. chinense* and *L. barbarum* crude extraction, optimization studies, antioxidant and cytotoxic activities of *L. chinense* and *L. barbarum*. Chapter Three illustrates all the materials and methods applied to complete the full objectives of the research. Chapter Four highlights the experimental results along with thorough discussion of the research findings. The thesis was

concluded in Chapter Five with required recommendation to improve for future associated research.

CHAPTER TWO

LITERATURE REVIEW

2.1 INTRODUCTION

This chapter delivers evidence related to this research such as traditional Chinese medical plant, *Lycium chinense* and *Lycium barbarum*, bioactive compounds, extraction technology, anti-oxidants, about breast cancer and the role of drug detection in-vivo and in-vitro studies. Furthermore, cytotoxicity assay are also discussed in this chapter.

2.2 TRADITIONAL CHINESE MEDICINE (TCM)

Minerals, herbs, and animals were commonly used to treat various diseases as drugs and healthy food in Asia, which have been recorded and collected in Traditional Chinese Medicine (TCM) as traditional cure and effective in China (Wang et al., 2014). Recently, in the United States and Europe, the use of herbs, dietary plants, and herbal combination formulas as substitute medicine has gained considerable attention because of its good therapeutic efficacy and affordability (Wang et al., 2014).

Traditional Chinese medicine (TCM) is generally taken as an alternative or complementary medicine in the Western world and is presently well accepted as a typical medical care throughout East Asia. TCM is one of the common name of Chinese Material Medicines which have therapeutic properties for healing and medical treatment (Wang et al., 2012). TCM has been widely used to treat the common cold, placenta previa, prevent spontaneous abortion and preterm labour, fetal growth restriction as well as other obstetric problems (Lixia et al., 2013). In addition,

TCM has also been used to treat cancer patients. According to Abdullah et al. (2003), it is reported that about 28%–98% of Chinese cancer patients use TCM in Asia, while in the North America, approximately 25%–47% of people used TCM for their cancer treatment (Ferro et al., 2007; Maskarinec et al., 2000).

Cancer is one of the leading diseases with high mortality rate therefore, finding the best cure for the disease is of utmost necessity. Previous studies have shown that some natural compounds sourced from specific herb were able to effectively interfere with tumor headway, constrain angiogenesis and block metastasis (Aravindaram and Yang, 2010; Lee et al., 2006; Yiyi Sun et al., 2009; Wang et al., 2012). Therefore, with the development of technology and science, mechanisms and pharmacological activities of herbs and natural compounds will be identified and understood in great depthand and more TCMs will be used to treat and protect against cancer as an effective therapy (Hwang et al., 2012; Wang et al., 2012; Xutian et al., 2012).

L .chinense and *L. barbarum* have long been recognized as one of traditional Chinese medicine. The species are deciduous woody perennial plants (Song, 2013). In nutraceuticals they have been widely used as functional ingredient since several studies have demonstrated that *L. chinense* and *L. barbarum* act as a key role in improving vision, age-related diseases, prevention of aging, inhibiting cancer development and boosting immune system because of bioactive components (Gan et al., 2004; Yoo et al., 2005; Wang et al., 2014).

2.3 *L. chinense* AND *L. barbarum*

L. barbarum and *L. chinense*, also known as wolfberry (goji berry), belong to the Solanaceae family, and can be used as valuable tonic, health food and juice

supplement for keeping good health and improving vision (Chung et al., 2014). In addition, *L. barbarum* and *L. chinense* plays an important role in TCM, which has a long history of use as a traditional remedy (Zhou, 2007). They are mainly related with the ability to enhance the immune system, tonifying liver, nourishes kidneys, moistening lungs, reduces blood glucose and serum lipids, and has anti-aging, anticancer and anti-fatigue, and also can improve blood circulation (Chang et al.,2008; Gan and Zhang, 2003; Peng and Huang, 2001; Peng et al., 2001; Peng et al., 2005; Potterat, 2010). Two closely related species are *L. chinense* and *L. barbarum*, both have been used as medical plants and food in China and other Asian countries for a long time (Chung et al., 2014; Potterat, 2010). Their fruits have similar tissue structure and anatomical features (Park et al., 2012).

According to Chung et al. (2014), *L. barbarum* and *L. chinense* contains several bioactive compounds such as Lyciumamide and cerebrosides which are known to display several bioactivities containing health promoting effects. Based on the previous findings, the common metabolites found in the fruits are carotenoids, polysaccharides, glycoconjugate, and flavonoids (Park et al., 2012). *L. barbarum* fruits have been reported to provide immunomodulatory, and neuroprotective effects (Gan et al., 2003; Yumanshan et al., 2005). Besides, antioxidant properties, other bioactivities of *L. chinense* and *L. barbarum* have also been reported (Chung et al., 2014; Ming et al., 2009). The *L. chinense* and *L. barbarum* are shown in Figure 2.1.

Figure 2. 1. *L .chinense* and *L. barbarum*

2.4 BIOACTIVE COMPOUND OF *L. CHINENSE AND L. BARBARUM*

According to Bonilla et al. (2015) , bioactive compounds are natural components that have some nutritional values and biological activity which play a key role in maintaining the health by reducing the risk of disease. Bioactive compounds might be connected to health benefits such as antioxidant compounds (chlorogenic acids, keratin, phenolic compounds and lactones).

L. chinense and L. barbarum contain rich nutrients and active substances, including mineral, amino acids, several vitamins, and antioxidants (Carnés et al., 2013). Furthermore, *L. chinense* and *L. barbarum* have been closely associated with the health and attractive effect because they have many functional compounds, including polysaccharides, flavonoids, and carotenoids (Wang et al., 2010).

2.4.1 Carotenoids

Carotenoids are one of the bioactive compounds in *L. chinense* and *L. barbarum*, they have a group of lipid-soluble compounds and rich important pigments (Wang et al.,

2015). Carotenoids have been confirmed to be effective in averting chronic disease such as skin cancer and cardiovascular disease (Fraser and Bramley, 2004).

In *L. barbarum* fruits, Inbaraj et al. (2008) used a thin-layer chromatographic (TLC) method to determine several carotenoids, such as zeaxanthin, β-carotene, and β-cryptoxanthin. In addition, the composition of carotenoids in *L. barbarum*, zeaxanthin constitute the biggest percentage (0.1%) (Inbaraj et al., 2008; Wang et al., 2010). Zeaxanthin consumption rises plasma zeaxanthin concentration, which consequently increases preretinal pigment concentration, i.e., zeaxanthin and lutein, and finally lowers AMD risk (Bucheli et al., 2011).

2.4.2 Flavonoids

Flavonoids are one of the important secondary metabolites, a class of polyphenol compounds that are commonly distributed in plant kingdom (Erlund, 2004). Flavonoids are the a major functional components of wolfberry, with physiological function and pharmacological action such as antioxidant, anti- aging, eliminating free radical, lowers blood pressure, lowers blood sugar, anti-inflammation, and anti-cancer (Wang et al., 2010; Wang and Hong-li, 2014). Consequently, flavonoids have been widely applied as additives of functional food and natural care food.

2.4.3 Polysaccharides

Polysaccharides, compose more than 100 monosaccharides, are often extant in large amounts in Chinese herbal medicine. They are composed of 100 or more monosaccharides (Wang et al., 2010). Yuanlin et al. (2005), states that polysaccharides can be water-insoluble or water-soluble, with the former kinds being