ULTRASONIC ENZYMATIC MEDIATED EXTRACTION OF EURYCOMA LONGIFOLIA (TONGKAT ALI) FOR COSMECEUTICAL AND NUTRACEUTICAL APPLICATIONS

ELFIRA BINTI ANUAR

Thesis submitted in partial fulfilment of the requirements
for the award of the degree of
Bachelor of Chemical Engineering

Faculty of Chemical & Natural Resources Engineering
UNIVERSITI MALAYSIA PAHANG

JULY 2014

ABSTRACT

This thesis reported ultrasonic enzymatic mediated extraction of *Eurycoma longifolia* (Tongkat Ali) for cosmeceutical and nutraceutical applications. *E. longifolia* has been used for quite a long time by practitioners for medical use in Malaysia. Interest in its commercial value began when it is noted to have an aphrodisiac property for men. The use of active compounds in *E. longifolia* for various industries signifies the need of the most appropriate and standard method to extract it. Previously, several researchers have studied new extraction technique of *E. longifolia*. However, it has not been reported yet the ideal technique to optimize the extraction of active compounds in *E. longifolia*. This study is focused on hydrodistillation, enzymatic extraction, and ultrasonic enzymatic mediated extraction techniques in order to investigate the performance of each extraction technique. Comparison of HPLC chromatogram was made between the standard and sample of *E. longifolia* extract to determine the concentration for eurycomanone in each sample. The findings of the results show that the yield for hydrodistillation technique increased gradually until the 6 hours and declined afterward due to loss of some heat-sensitive bioactive compounds. This technique reported a total of 12.56% of yield for 12 hours of extraction process. Result obtained for the enzymatic extraction shows that the enzyme treatment enhanced the yield by nearly 2.1-fold relative to hydrodistillation technique. By applying the ultrasonic of 10% and 20% duty cycle on the enzyme-treated samples, the overall yield improved by nearly 2.4-fold and 2.6-fold respectively compared to hydrodistillation technique and 1.2-fold and 1.1-fold respectively compared to enzymatic extraction technique. Thus, as a conclusion, apart from being effective, the ultrasonic enzymatic mediated extraction of *E. longifolia* is the best extraction technique to improve the extracted productivity and shorten the extraction time which in turn may be beneficial to commercialization of *Eurycoma longifolia* for cosmeceutical and nutraceutical application in the future.

ABSTRAK

Tesis ini melaporkan pengekstrakan *Eurycoma longifolia* (Tongkat Ali) secara ultrasonik dengan pengantaraan enzim untuk aplikasi kosmeseutikal dan nutraseutikal. *E. longifolia* telah digunakan untuk tempoh yang agak lama oleh pengamal perubatan di Malaysia. Kepentingan dalam nilai komersialnya bermula apabila ia dikatakan mempunyai sifat afrodisiak untuk lelaki. Kegunaan sebatian aktif dalam *E. longifolia* untuk pelbagai industri menandakan kaedah yang paling sesuai untuk penegekstrakan diperlukan. Sebelum ini, beberapa penyelidik telah mengkaji teknik pengekstrakan baru *E. longifolia.* Walau bagaimanapun, masih tiada laporan mengenai teknik yang sesuai untuk mengoptimumkan pengekstrakan sebatian aktif dalam *E. longifolia*. Kajian ini memberi tumpuan kepada teknik *hydrodistillation*, pengekstrakan menggunakan enzim, dan pengekstrakan menggunakan ultrasonik dengan pengantaraan enzim untuk menyiasat prestasi setiap teknik pengekstrakan. Perbandingan HPLC kromatogram telah dibuat di antara standard dan sampel ekstrak *E. longifolia* untuk menentukan kepekatan eurycomanone dalam setiap sampel. Hasil keputusan menunjukkan bahawa produktiviti bagi teknik *hydrodistilation* meningkat secara beransur-ansur sehingga jam keenam dan selepas itu menurun disebabkan oleh kehilangan beberapa sebatian aktif yang sensitif kepada haba. Teknik ini mencatatkan sejumlah 12.56 % hasil untuk 12 jam proses pengekstrakan. Keputusan yang diperolehi untuk pengekstrakan enzim menunjukkan hasil bagi rawatan enzim meningkat hampir 2.1 kali ganda daripada teknik *hydrodistillation*. Dengan menggunakan kitar tugas ultrasonik sebanyak 10% dan 20% ke atas sampel yang dirawat dengan enzim, hasil keseluruhan telah meningkat sebanyak hampir 2.4 kali ganda dan 2.6 kali ganda berbanding dengan teknik *hydrodistillation* dan 1.2 kali ganda dan 1.1 kali ganda berbanding dengan teknik pengekstrakan menggunakan enzim. Oleh itu, sebagai kesimpulan, selain daripada efektif, pengekstrakan menggunakan ultrasonik dengan pengantaraan enzim adalah teknik pengekstrakan yang terbaik untuk *E. longifolia* bagi meningkatkan produktiviti ekstrak dan memendekkan masa pengekstrakan yang seterusnya boleh memberi manfaat kepada pengkomersilan *Eurycoma longifolia* untuk aplikasi kosmeseutikal dan nutraseutikal pada masa hadapan.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ppm	part per million
rpm	revolution per minute

LIST OF ABBREVIATIONS

BW	Body weight
GAE	Gallic acid equivalent
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
LC-MS	Liquid chromatography and mass spectrometer
NaOH	Sodium hydroxide
UV	Ultraviolet

1 INTRODUCTION

1.1 Motivation and statement of problem

The herbal-based phytochemical industry is a new and upcoming sector in Malaysia. One of the important phytochemical products in the Malaysia market is *Eurycoma longifolia*. *E. longifolia* is belongs to family Simaroubaceae which a family of tropical plant that have a bitter taste. According to Kuan *et al.* (2007), roots of *E. longifolia* have its special benefits include enhancing blood flow, functioning after child birth and restoring energy and vitality. It has been used for quite a long time by practitioners for medical use in Malaysia. Interest in its commercial value began when it is noted to have an aphrodisiac property for men.

Previous research had proven that *E. longifolia* contain of more than eighty-five compounds that have been reported till today and mostly from the roots. According to Chua *et al.* (2011), these active compounds are majority from the classes of quassinoids, canthin-6-one-alkaloids, beta-carbolines alkaloids, tirucallane-type triterpenes, squalene derivatives, and biphenylneolignans. In his study by using liquid chromatography and mass spectrometer (LC-MS) metabolites identification showed that the eurycomanone from the quassinoids class of compound, represent the highest amount among the detected quassinoids. This claim is supported by the study done by Chan *et al.* (1998) where the highest peak detected by LC-MS is confirmed to be eurycomanone.

The *E. longifolia* is covalently bonded with lignin and other carbohydrates, thus restricting it from undergoing any chemical changes and enzymatic degradation. Degradation of the cell wall is a basic step to release the active compounds. Numerous studies have been conducted to extract active compounds in *E. longifolia* by using conventional extraction techniques e.g. Low *et al.* (2013), Ahmad *et al.* (2012), Chua *et al.* (2011), Zubairi (2010), and Kuan *et al.* (2007). The major challenges of conventional extraction are low extraction selectivity, degradation of the targeted compounds due to high temperature, high cost required, and longer extraction time (Luque de Castro and Garcia-Ayuso, 1998). To overcome these limitations, nowadays, various new techniques have been developed including microwave-mediated extraction, ultrasound-mediated extraction and enzymatic-mediated extraction.

Figure 1-1: Roots of *Eurycoma longifolia*

Plant cell walls consist of pectin, cellulose, and hemi-cellulose that act as the barrier for the release of bioactive compounds. The *E. longifolia* is covalently bonded with lignin and other carbohydrates, thus restricting it from undergoing any chemical changes and enzymatic degradation. Extraction is the most important step in isolating different types of bioactive compounds from plants. There are numerous methods that have recently been reported for the extraction of bioactive compounds. Up to now, several conventional extraction techniques have been reported for the extraction of plant like solvent extraction (Athimulam *et al*., 2006; Chua *et al.*, 2011; Kuan *et al.*, 2007;), enzyme-assisted extraction (Nagendra chari *et al.*, 2013; Puri *et al.*, 2012) and ultrasound-assisted extraction (Chen *et al.*, 2012; Xia *et al.*, 2006). All these techniques have some common objectives which is to extract targeted bioactive compounds from complex plant sample, to increase selectivity of analytical methods, to increase sensitivity of bioassay by increasing the concentration of targeted compounds, to convert the bioactive compounds into a more suitable form for detection and separation, and to provide a strong and reproducible method that is independent of variations in the sample matrix (Smith, 2003). There is a need to develop optimized and comprehensive protocols for enhanced recovery of bioactive compounds, particularly from plants where the cell wall can inhibit extraction efficiency.

The term nutraceutical is the combination of the word nutrition and pharmaceutical. Bernal *et al*. (2011) defined nutraceutical as dietary supplements that contain significant amount of bioactive components with the purpose of improving health. Nutraceutical field in herbal industry is growing rapidly due to the growing preference among

consumers to consume their nutritional needs in food form rather than in medicinal form. Bioactive compounds are obtained selectively from plants as specialty chemicals and can be used as nutraceuticals, processed foods to complement a balanced diet or as drug leads. *E. longifolia* has been well documented to exert aphrodisiac property as natural testosterone and rejuvenation booster for men to support male hormonal balance including testosterone availability, libido and physical performance. It is also has the anabolic effect on man which functioned for muscle and body building improvement. Therefore, this led to high market demand of *E. longifolia* due to its tremendous health benefits.

Meanwhile, cosmeceutical is the combination of the word cosmetic and pharmaceutical that represents multifunctional products that rely on science and technology to deliver clinically proven active ingredients to the skin. Golubovic-Liakopoulos, Simon, and Shah (2011) stated that cosmeceuticals are often formulated with pharmaceutical-type active compounds and demonstrated to achieve multiple cell-protective effects for rebuilding healthy skin on a cellular level. It is important to identify each of the active compounds unique functional characteristics, which is often a challenge that requires new formulation strategies. *E. longifolia* has been well documented to exert antioxidative properties due to its high concentrations of flavonoids compounds such as quercetin, kaemferol, luteolin, diosmetin, catechin, epicatechin (isomer) and epigallocatechin. These active compounds contribute to the antioxidants properties that can reduce the skin damage caused by oxidation such as the harm caused by free radicals. However, due to a very limited data has been published on determination of *E. longifolia* antioxidant properties to neutralize the production of free radical (free radical scavenging), this claimed are not supported by other research.

In recent years, *E. longifolia* has become popular the market demand has increased due to public awareness and increasing interest among consumers and scientific community. The use of its bioactive compounds in different commercial sectors such as food, pharmaceutical and chemical industries signifies the need of the most appropriate and standard method to extract these active compounds to meet the high market demand. The main objective of this study was to provide the most ideal extraction techniques of *E. longifolia* by implementing the ultrasound technique and combination of enzymes for better improvements of the extracted productivity for the cosmeceutical and nutraceutical application.

1.2 Objectives

This work aims to provide the most ideal extraction techniques of *Eurycoma longifolia* by implementing the ultrasound technique and combination of enzymes for better improvements of the extracted productivity for the cosmeceutical and nutraceutical application.

1.3 Scope of this research

The following are the scope of this research:

i) To determine the bioactive compound of *E. longifolia* for the cosmeceutical and nutraceutical applications.

ii) To optimize the sonication regimens for the extraction of *E. longifolia.*

iii) To compare the results between ultrasonic enzymatic mediated extraction technique with hydrodistillation and enzymatic extraction technique.

1.4 Main contribution of this work

The following are the contributions of this study:

i) Provide the most ideal extraction techniques of *E. longifolia.*

ii) Improve the extracted productivity for the cosmeceutical and nutraceutical applications in the future.

iii) For commercialization of *E. longifolia*.

1.5 Organisation of this thesis

The structure of the reminder of the thesis is outlined as follow:

Chapter 2 provides a description of the bioactive compounds contain in the *E. longifolia* that contribute to its cosmeceutical and nutraceutical properties. This chapter also provides a general discussion of the advanced experimental techniques available that improve the extracted productivity. A summary of the previous experimental work on extraction of *E. longifolia* is also presented. A brief discussion on extraction techniques of *E. longifolia* by implementing the ultrasound technique and combination of enzymes is also provided.

Chapter 3 gives a review on the materials description that is used in this study and the extraction techniques applied to the *E. longifolia*. The justification on the method selected also provided.

Chapter 4 provides the preliminary results and the discussion on the result trend. The explanation on the results is compared with theory and the previous work.

Chapter 5 draws together a summary of the thesis and outlines the future work which might be derived from the model developed in this work.

2 LITERATURE REVIEW

2.1 Overview

This paper presents the experimental studies of ideal extraction techniques of *Eurycoma longifolia* by implementing the ultrasound technique and combination of enzymes for better improvements of the extracted productivity for the cosmeceutical and nutraceutical applications. In this era of science and technology, *Eurycoma longifolia*-based product has become popular in phytochemical industry and the market demand has increased due to public awareness and increasing interest among consumers and scientific community.

Conventionally, several extraction techniques have been used to extract active compounds in *E. longifolia* such as boiling, heating or refluxing. The major challenges of conventional extraction are low extraction selectivity, degradation of the targeted compounds due to high temperature, high cost required, and longer extraction time. To overcome these limitations, nowadays, various new techniques have been developed including ultrasound-mediated extraction and enzymatic-mediated extraction.

2.2 Hydrodistillation

Hydrodistillation is a type of conventional solvent extraction that using water as a medium to separates soluble solutes by diffusion from a solid matrix in the presence of high temperature. The heat of the water weakens the plant cells, thus releasing the constituents of the plant into the water. According Azmir e*t al.* (2013), there are three types of hydrodistillation: water distillation, steam distillation and combination of water and steam distillation. In hydrodistillation, the plant materials are immersed into the water in the flask and then brought to boil until the steam come out and condensed before the distillate is collected. The advantages of hydrodistillation technique include; this method is simple and cheap compared. However, there are some drawbacks of this extraction technique. At high temperature, some of the volatile components may be lost and this limits the use of hydrodistillation for thermolabile compound extraction. Besides, the process requires high energy and time consumption which also resulting in high cost consumption. The utilization of hydrodistillation method for extraction of targeted bioactive compounds has been widely studied. The previous studies on

hydrodistillation method for different plant materials in comparison with other conventional solvent extraction method are shown in Table 2-1.

Table 2-1: Hydrodistillation extraction for different plant materials in comparison with other conventional solvent extraction method

Plant material	Extraction method	Yield (%)	Duration	Reference
Thymus vulgaris L.	Conventional hydrodistillation	1.34	33.74 min	Gavahian *et al.* (2012)
	Ohmic-asissted hydrodistillation	1.32	18.92 min	
Eugenia caryophyllata Thunb.	Hydrodistillation	11.5	6 h	Guan *et al.* (2007)
	Supercritical fluid extraction	19.6	2 h	
	Soxhlet extraction	41.8	6 h	
Lavandula spp.	Hydrodistillation	0.53	3 h	Costa *et al.* (2012)
	Supercritical fluid extraction	3.45	3 h	

2.3 Enzymatic extraction

The enzymes use the common key and lock theory, where the cell wall is the substrate and binds into the active site of the enzyme as illustrates in Figure 2-1. The enzyme acts as catalyst to speed up the reaction by binding sterically to the specific substrate and breaks it down to gives of the products. The reduction of substrate particle provides better accessibility of the enzyme to the cell. Various commercial enzymes have been employed for the extraction of bioactive compounds from plant such as pectinase, cellulase, protease and viscozyme analysis.

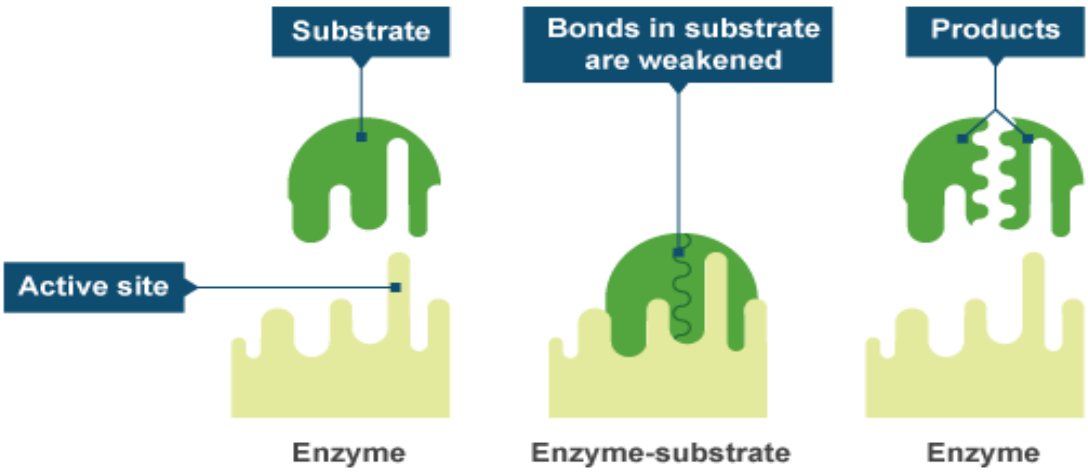

Figure 2-1: The lock and key theory

The enzymes work primarily by macerating the tissues of the herbs plant, breaking down the plant cell walls to release bioactive compounds. Enzymes have the ability to degrade or disrupt cell walls and membranes, thus enabling better release and more efficient extraction of bioactives compounds. According to Puri *et al.* (2012), to obtain optimum process conditions, factors such as pH, concentration and temperature should be considered. The addition of enzymes to the extraction process improves the yield significantly as been reported by the study of Nagendra chari *et al.* (2013) and Li *et al.* (2012). In their study, they proved that enzyme treatment on plant materials had significantly increased the yield of bioactive compounds. The past studies on enzymatic extraction for different plant materials in comparison to control are shown in Table 2-2.

Table 2-2: Enzymatic extraction for different plant materials in comparison to control (without treatment of enzyme)

Plant material	**Treatment**	**Concentration (%, v/w)**	**Yield (%)**	**Duration (h)**	**Reference**
Zingiber officinale R.	Control	-	15	4	Nagendra chari *et al.* (2013)
	Cellulase	0.5	21	4	
	Pectinase	0.5	20	4	
Cardamom	Control	-	1.9	2	Chandran *et al.* (2012)
	Lumicellulase	0.4	2.5	2	
Momordica cochinchinen-sis Spreng.	Control	-	5.9	2	Mai *et al.* (2013)
	Protease	0.5	20.7	2	

Enzymatic extraction has the benefits of being environmentally friendly, highly efficient, short extraction time, higher recovery, low solvent and energy consumption, and easily operated. However, it has some limitations for this type of extraction method such as the cost of enzymes is relatively high for processing large volumes of raw material and enzyme-assisted extraction can be difficult to scale up to industrial scale as the enzymes behave differently as environmental.

2.4 Ultrasonic enzymatic mediated extraction

In recent years, the emerging of ultrasonic extraction technology to replace the conventional extraction techniques for cosmeceutical and nutraceutical applications has received increasing attention.

Figure 2-2: Ultrasound apparatus

Ultrasound is a type of sound wave beyond human hearing which produce wave at frequency in range of 20 kHz to 100 MHz. During the sonication process, cavitation phenomena occur which means the production, growth and implodes of bubbles dissolved in liquid (see Fig. 2-3)

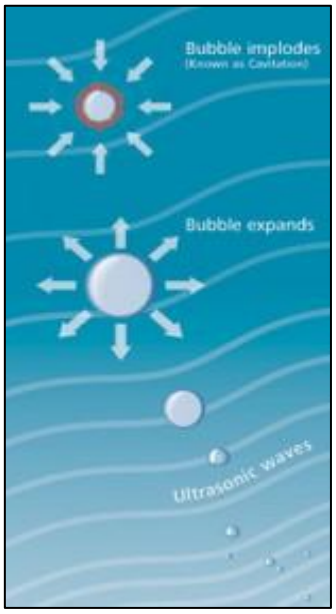

Figure 2-3: Cavitation phenomena

According to Suslick and Doktycz (1990), the bubbles have pressure of 1000 atm, temperature about 5000 K, and cooling rate above 1010 K/s. This cavitation effect only

happen in liquid and liquid containing solid material. As big amplitude of waves pass through a mass media, the bubble implodes at the surface of membranes or in close vicinity causing cell fractures (Vilkhu *et al*., 2008). Ultrasound can damage the plant cells, decrease plant tissue between each component in the close degree of integration, and enhanced the release of cytoplasm, while the effect of ultrasonic vibrations produce extracts in shorten period and increase the extraction productivity (Chen *et al*., 2011). At mild condition of ultrasound, ultrasound can helps the process of enzymatic extraction by increasing the enzymatic activity during the process. The higher ultrasound power caused the stronger vibrations and an increase in the number of cavitation bubbles formed that could increase the extraction efficiency. Therefore, an increase in the power output would bring about an increase in the extraction yield. However, high power and longer sonication time would cause an increase in the bubble numbers in the solvent during cavitation, which may reduce the efficiency of the ultrasonic energy transmitted into the medium. At the same time, high intensity ultrasonic also caused polysaccharide depolymerization, aggregation, and viscosity decrease, which would result in a decrease of the extraction yield. Effects of combination of ultrasound and enzymatic treatment on bioactive compounds from plant material for yield enhancement have been previously reported. The past literatures on the performance of this method are shown in Table 2-3.

Table 2-3: Ultrasonic enzymatic mediated extraction performance on different plant materials in comparison to control (without sonication treatment)

Plant material	**Treatment**	**Ultrasound power (W)**	**Performance (%)**	**Duration (min)**	**Reference**
Acerola mash	Pectinase + sonication	150	Increased 15.5% of yield	100 100	Dang *et al.* (2012)
Cotex phellodendria	Cellulase + sonication	100	Yield increase greatly below 50 °C but declined at 60 °C.	60	Liu *et al.* (2009)
Lycium barbarum	Cellulose + sonication	80	Yield increase by 0.16%	20	Liu *et al.* (2014)

2.5 Bioactive compounds

Various bioactive constituents have been isolated and characterized from *Eurycoma longifolia* and mostly comes from the roots. According to Chua *et al.* (2011), these active compounds are majority from the classes of quassinoids, quassinoidditerpenoids, canthin-6-one-alkaloids, beta-carbolines alkaloids, eurycomaoside, eurycolactone, laurycolactone, eurycomalactone, tirucallane-type triterpenes, squalene derivatives, and biphenylneolignans. Quassinoids form the major bioactive constituents in this plant and are mainly responsible for its bitter taste.

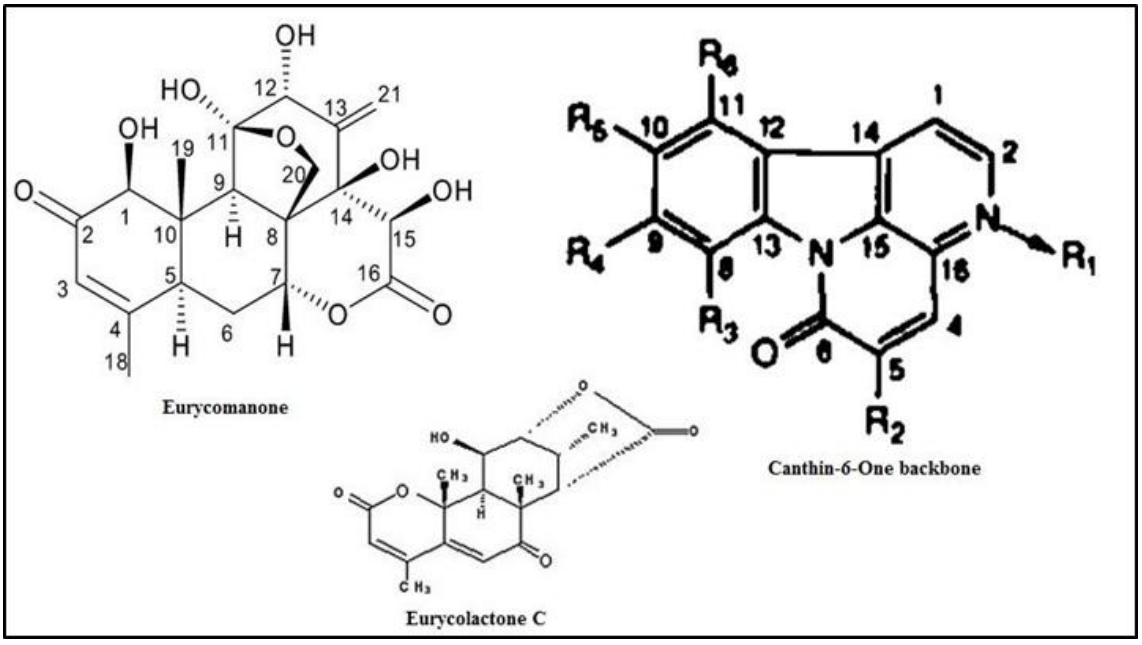

Figure 2-4: Chemical structure of several bioactive compounds in *E. longifolia*

It has been well documented to exert antioxidative properties due to its high concentrations of flavonoids compounds. Flavonoids compounds are secondary metabolites which synthesize in plants which possess biological prosperities like antioxidant and anti-aging. Among all bioactive compounds of *E. longiflia* has the the highest concentration especially in the root part of the plant. Therefore, it usually used as the marker compound for *E. longifolia* extract quantification.

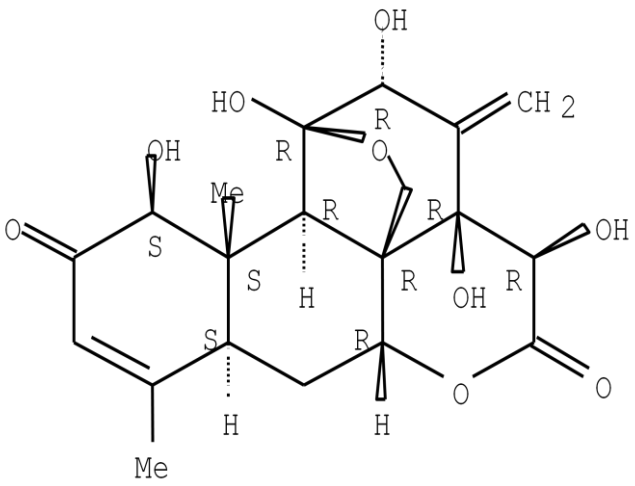

Figure 2-5: Chemical structure of eurycomanone

2.6 Pharmacological properties

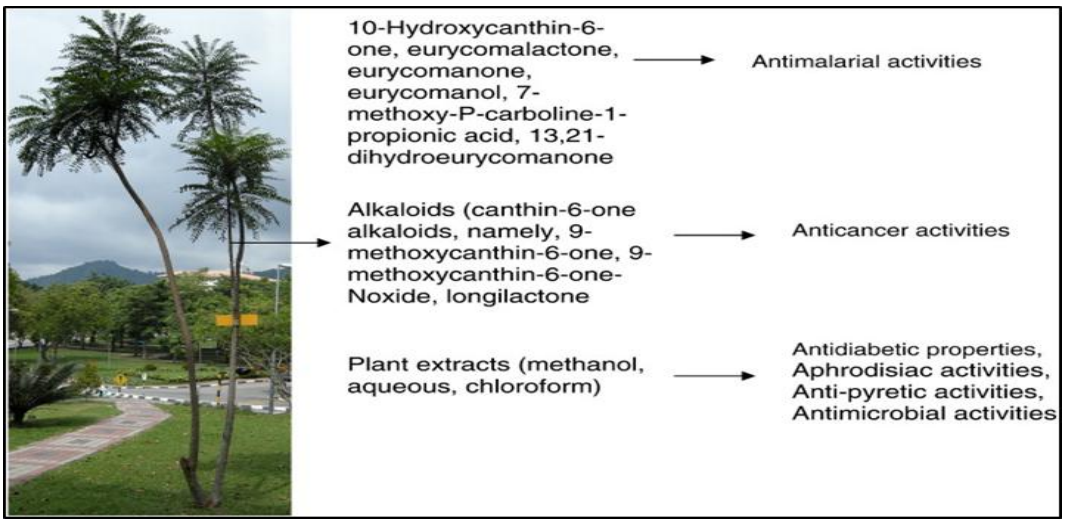

Figure 2-6: Pharmacological properties of *E. longifolia*

Pharmacological properties of *E. longifolia* from plant parts such as roots, stem, bark and leaves have shown many benefit include:

2.6.1 Anti tumor

The effects of quassinoids from *E. longifolia* were tested in vitro on anti-tumor promoting, antischistosomal and plasmodicidal activities (Jiwajinda, 2002) and for antiulcer activities (Mitsunaga, 1994). It was found that the active compound for anti-tumor promotion was 14,15 β-dihydroxyklaineanone.

2.6.2 Anti diabetic

Study of Husen (2004) showed a positive result when using 150 mg/kg BW of the aqueous extract in hyperglyacaemic rats. Decreasing in blood glucose was observed in streptozotocin-induced hyperglycemic adult rats by 38% and 47% for two different extracts.

2.6.3 Antimicrobial activity

Acetone and alcoholic extracts from the stems and leaves of *E. longifolia* were active against gram positive and gram negative bacteria except *E.coli* and *S.typhi* (Farouk, 2007). A standardized root extract was found to defeat the parasitemia of *P. yoelii*-infected mice in dose-dependent manner (Mohd Ridzuan, 2005).

2.7 Previous work on E. longifolia

2.7.1 Increase sexual potential

The quassinoids found in *E. longifolia* are responsible for its aphrodisiac properties. Series of scientific studies confirmed its aphrodisiac properties, thus *E. longifolia* has become popular for its alleged testosterone-enhancing solution. The study of the effects of *E. longifolia* roots extracts (methanol, chloroform, water, and n-butanol) on sexual qualities in middle aged male rats was reported by Ang, Ngai, and Tan (2003) and the study claimed that *E. longifolia* improved the sexual qualities of the middles rats.

The study of Low, Das, and Chan (2013) had provided similar suggestion to the findings of Ang, Ngai, and Tan (2003). The study revealed that quassinoid-rich *E. longifolia* extract improved the rat spermatogenesis. The treatment of quassinoids on the testosterone, play an important role in the spermatozoa production. A research conducted by Tambi (2010) claimed that *E. longifolia* extract improves semen volume, sperm concentration, sperm motility and the percentage of morphologically normal sperm in men with idiopathic infertility. In his study, 350 patients were given 200 mg of the extract daily and follow-up semen analyses were performed every 3 months for 9 months. The analyses showed significant improvement in sperm quality of these patients, allowing for 11 (14.7%) spontaneous pregnancies.

2.7.2 Antioxidant and antiaging

Phenolic compounds are secondary metabolites which synthesize in plants which possess biological prosperities like antioxidant and anti-aging that inhibit cellular aging. The factor that accelerates the aging process is oxidative stress cause by imbalance of body defence system, inhibited stress, and polluted environment. Natural products such as *E. longifolia* contain the antioxidant properties that promote the anti-aging process. According to Purwantiningsih, Hj Hussin, and Kit (2011), the onset of many diseases and aging are caused by the unbalanced mechanism of antioxidant protection and the uncontrolled production of oxygen free radicals. Therefore, phenolic content of substance is usually related with anti-oxidant and anti-aging capacity. The study conducted by Chua *et al.* (2010), showed that the highest phenolic content was produced by methanol extraction (58.26 μg GAE/ mg) and the highest antioxidants activity was produced by water extraction (7684.2 ppm). The phenolic content and free radical scavenging activity in the fractionated extract might due to the presence of flavonol compounds. However, due to a very limited data has been published on determination of *E. longifolia* antioxidant properties to neutralize the production of free radical (free radical scavenging), this claimed are not supported by other research.

3 MATERIALS AND METHODS

3.1 Overview

This paper presents This paper presents the extraction of *E. longifolia* by implementing the hydrodistillation technique, enzymatic assisted extraction technique, and ultrasonic mediated enzymes extraction technique.

3.2 Materials and chemicals

The original roots and the ground roots of *E. longifolia* are obtained from Biotropics Malaysia Berhad (Kuala Lumpur, Malaysia) in dried chip form. Commercial enzyme (pectinase from *Apergillus Aculeatus* with activity of ≥ 3800 unit/ml) used in this study was purchased from Sigma-Aldrich Malaysia. Pure eurycomanone standard was purchased from ChemFaces (China). HPLC grade acetonitrile and 85% ortho-phosphoric acid were purchased from Merck (Germany). Deionized water was prepared using Milipore water purification system.

3.3 Grinding

Before experiment, the *E. longifolia* roots was ground into fine powder (particle diameter: > 0.08 mm) and then stored at 2 - 8 °C until analysis to ensure that the active phytochemicals are maintained before processing.

Figure 3-1: Grinder