World Academy of Science, Engineering and Technology
International Journal of Chemical and Molecular Engineering
Vol:9, No:4, 2015

Effect of Ultrasound and Enzyme on the Extraction of Eurycoma longifolia (Tongkat Ali)

Authors : He Yuhai, Ahmad Ziad Bin Sulaiman

Abstract : Tongkat Ali, or Eurycoma longifolia, is a traditional Malay and Orang Asli herb used as aphrodisiac, general tonic, anti-Malaria, and anti-Pyretic. It has been recognized as a cashcrop by Malaysia due to its high value for the pharmaceutical use. In Tongkat Ali, eurycomanone, a quassinoid is usually chosen as a marker phytochemical as it is the most abundant phytochemical. In this research, ultrasound and enzyme were used to enhance the extraction of Eurycomanone from Tongkat Ali. Ultrasonic assisted extraction (USE) enhances extraction by facilitating the swelling and hydration of the plant material, enlarging the plant pores, breaking the plant cell, reducing the plant particle size and creating cavitation bubbles that enhance mass transfer in both the washing and diffusion phase of extraction. Enzyme hydrolyses the cell wall of the plant, loosening the structure of the cell wall, releasing more phytochemicals from the plant cell, enhancing the productivity of the extraction. Possible effects of ultrasound on the activity of the enzyme during the hydrolysis of the cell wall is under the investigation by this research. The extracts was analysed by high performance liquid chromatography for the yields of Eurycomanone. In this whole process, the conventional water extraction was used as a control of comparing the performance of the ultrasound and enzyme assisted extraction.

Keywords : ultrasound, enzymatic, extraction, Eurycoma longifolia
Conference Title : ICCSE 2015 : 17th International Conference on Chemical Science and Engineering
Conference Location : Istanbul, Turkey
Conference Dates : April 21-22, 2015

1. INTRODUCTION

The global herbal market of nutraceuticals and phytomedicines with an average annual growth rate between 15% to 20% (Gruenwald J, 2002; Sloan, 2002). This market includes herbal products such as herbal supplements and essential oils, and nutraceutical products such as fortified foods and nutritional supplements. In Malaysia, the herbal product market has been estimated to be worth RM 4.55 billion, of which 80% of the products are imported (Puteh, 1999)

A key emerging global market is phytomedicines, which are pharmaceuticals made from plants. Many allopathic medicines, which are produced synthetically, are also originally derived from plants chemicals, i.e. phytochemicals such as quinine for malaria and quinidine for heart arrhythmia from *Cinchina sp*, and digxin for heart failure from *digitalis spp*. About 25% of drugs prescribed worldwide come from plants, 121 such active compounds being in current use (Rates, 2001). Raskin *et al*. (2002) estimated that over 50% of prescription medicines and over the counter herbal remedies contain phytochemicals with market worth of USD 31 billion in 2002.

Unlike allopathic medicines, which are pure chemical compounds, phytomedicines consists of a mixture of phytochemicals. A strength of phytomedicine lies within this mixture, for instance, Tongkat Ali extrcats have been found to more effective against malaria, compared to chloroquinidine (Ang *et al*., 1995a, b), perhaps because the malaria plasmodium cannot build up a resistance to the synergistic mixture of phytochemicals as opposed to a single chemical in a prescription medicine (Raskin *et al*., 2002). As the effectiveness of medicine are measured and prescribed based on dosages, it is essential that a phytomedicine maintain the same potency in every capsule to maintain its dose-response relationship. Therefore, it is important to ensure that the phytochemical concentration and profiles have uniform quality from seed to pill (Raskin *et al*., 2002). This process is known as standardization where the chemical concentration and profile are maintained in every batch of extract.

Tongkat Ali, or *Eurycoma Longifolia*, is a traditional Malay and Orang Asli herb used as aphrodisiac, general tonic, anti-Malaria, and anti-Pyretic. Scientifically, it has also been found to have anti-tumor and anti-oxidant properties. Tongkat Ali root has various benefits and is taken orally. The tap root is processed traditionally, by decocting the root and is drunk for the benefit. Nowadays, Tongkat Ali has become known globally due to its ability to treat erectile dysfunction (ED) and to improve sexual desire. It has been recognized as a cashcrop by Malaysia due to its high value for the pharmaceutical use.

Due to Tongkat Ali's high market demand as health supplement, these phytochemical products have a high commercial value in local and global market. Market demand of this plant has greatly increased as there are almost 200 products from Tongkat Ali available in health-food market specifically for its aphrodisiac properties. Tongkat Ali product are available are either in the form of capsules mixed with other aphrodisiac herbs, in raw crude powder form especially from roots, as additives mixed with coffee and ginseng, or as health products (Bhat and Karim, 2010).

There are various phytochemicals can be found in this plant such as canthin-6-one alkaloids, β-carboline alkaloids, quassinoids, tirucallane-type triterpenes, squalene derivatives, and biphenylneolignans.

Eurycomanon is a very important quassinoids found in Tongkat Ali extract which has a potential to be developed as complementary for anti-cancer therapy due to its ability to inhibit cancer cells such as lung, liver, breast cancer cells, and, decrease tumorigenic and significant activities against Plasmodium falciparum strains (Chan *et al*., 1986; Kardono *et al*., 1991; Zakaria *et al*., 2009). eurycomanone is usually chosen as a marker phytochemical as it is the most abundant phytochemical in Tongkat Ali(Chan *et al*., 1998).

In this part of research, conventional extraction of Tongka Ali by water extraction has been performed. The extracts are analyzed by high performance liquid chromatography (HPLC) for the yields of Eurycomanone. The conventional water extraction will be used as the reference in the future for other extraction method which might improve the productivity of the extraction process.

2. LITERTURE REVIEW

Eurycoma longifolia Jack (*E. longifolia*) is one of the popular medicinal plants in Southeast Asia, including Indonesia and Malaysia. E. longfolia has many local names: in Brunei it is known as tungat ali, langsia siam or pasak bumi; in Cambodia it is known as antoung sar or antong sar; in Thailand it is known as plaalai phuenk, hae phan chan or phiak; in Laos it is known as tho nan; in Vietnam it is known as Cay ba binh; in Indonesia it is known as beseng, bidara laut or pasak bumi; and in Malaysia it is known as bedara merah, bedara putih or tongkat ali (Chan *et al*., 1998).

In Malaysia, Tongkat Ali (*E. longfolia*) has been claimed to improve the stamina of men during sexual activity, increase vitality and restore erection. As such, it is reputed to be an aphrodisiac. The herb is commonly taken as a decoction of roots in water. Nearly 200 Tongkat Ali products are available on the domestic Malaysian market, either in combination with other herbs or as a single preparation (Cyranoski, 2005). Many publications have revealed the aphrodisiac activity of *E. longifolia*, which has been used to enhance male virility during sexual activity (Ang and Lee, 2002; Ang and Ngai, 2001; Ang *et al*., 2003a; Ang and Sim, 1997). Many activity studies have been performed with regard to the many constituents that are contained in E. longifolia. Various bioactive constituents have been isolated and characterized from Eurycoma longifolia, mostly from the roots. Some of the bioactive compounds isolated include: canthin-6-one alkaloids,-β-carboline alkaloids, quassinoids, quassinoid diterpenoids, eurycomaoside, tirucallane-type triterpenes, squalene derivatives, biphenylneolignans, eurycolactone, laurycolactone, and eurycomalactone (Ang and Lee, 2002; Ang *et al*., 2003b; Ang and Sim, 1997). The isolation of nearly sixty-five compounds from the roots of *E. longifolia* was reported by Kuo *et al* (2004) .

Among bioactive constituents of Tongkat Ali, eurycomanone has the highest concentration. Comparatively, the alkaloid content is significantly lower than the content of quassinoids. Thus, eurycomanone is more commonly used as the marker compound for Tongkat Ali extract quantification (Chan *et al*., 1998).

Bioactive compounds in herbal plants are extremely low in concentrations. Therefore, the development of extraction methods to increase the yield of the desired bioactive compounds from the herbal particles while retaining the biological activities is very important (Mardawani Mohamada, 2012).

Typically, these bioactive constituents are isolated using a series of processes beginning with extraction, which involves the transfer of solutes from a solid medium to a solvent. The ideal extraction system should be quantitative, non-destructive and time saving (Zhang, 2007) and is dependent on several factors. The choice of process settings are subjected to the limits set by the chemical nature of the compounds to avoid degradation of their functionality and bioactivity.

Solvent extraction is a process that separates soluble solutes by diffusion from a solid matrix using solvent. The advantages of conventional solvent extraction include using fresh solvent into contact with the solid matrix and no filtration procedure after leaching. Also, this method is simple and cheap compared to other extraction methods(Wang and Weller, 2006). The efficiency of the solvent extraction process is influenced by the type of solvent used, pH, extraction temperature, number of extraction steps, solvent to solid ratio and particle size of the solid matrix(Chirinos R, 2007). These parameters, when optimally selected, provide maximum process efficiency while preserving the desired components functionality which refers to the pharmacological properties of the plant.

Conventional methods of extraction from plant materials, especially of flavonoids involved chemical and mechanical processes such as heating, boiling and refluxing. However, some disadvantages regarding to these methods are the loss of flavonoids owing to hydrolysis, oxidation and ionization during extraction as well as long extraction time (Zhang *et al*., 2011). Shirsath *et al*. (Shirsath *et al*., 2012) stated that there are some limitations that always been related to these conventional methods including time consuming, high energy consumption, damage of extraction quality and limitations of mass transfer resistances due to the involvement of more than one phase in the system. The production of Tongkat Ali water extract is mainly carried out through various conventional methods, such as water extraction and soxhlet extraction which involves boiling or soaking that often lead to high losses and low product yield (Athimulam, 2006). Hence more efforts are needed to develop the various stages of phytochemical processing, be it in planting and harvesting, raw material preparation, processing or as value added production .

In recent years, various novel extraction techniques have been utilized for the extraction of natural products from plants. Latest approaches in extracting herbs or plant material such as ultrasound-assisted extraction (Sharma and Gupta, 2006; Vale *et al*., 2008; Zhang *et al*., 2011; Zhang *et al*., 2008) enzymatic mediated extraction(Vale *et al*., 2008), microwave assisted extraction (Ballard *et al*., 2010; Hayat *et al*., 2009; Jambrak, 2013) and accelerated solvent extraction (Wang and Weller, 2006) were established and furtherly discovered.

Among these methods, ultrasound-assisted extraction is a simple, efficient and inexpensive alternative to conventional extraction techniques (Huang *et al*., 2009). Shear forces created from ultrasonic cavitation can break the cell wall

mechanically and improve the mass transfer tremendously. Moreover, there is no chemical involvement in the ultrasound-assisted extraction, which can prevent possible chemical degradation of targeted compounds (Zhang *et al*., 2011).

In order to overcome the low efficiency of aqueous extraction process, the use of hydrolytic enzymes as eco-friendly alternatives (Sharma and Gupta, 2006) are introduced which help to release the active compounds in the plant cell wall and increase the extraction yield. Rosenthal *et al*. (1996) has listed some advantages regarding the use of enzymatic extraction on terms of lowering the investment cost and energy requirement. The development of this method is also to fulfill the needs towards a clean environment especially the increasing awareness about volatile organic compounds (VOCs) caused by solvent emissions and safety concerns.

2.1 Ultrasound-Assisted Extraction

Ultrasound can be defined as the sound waves having frequency that exceeds the hearing limit of the human ear (20KHz) (Jambrak, 2013). High power (high intensity, high energy) ultrasound operates at frequencies between 20KHz and 500KHz. Intensities higher than $1w/cm^2$ are disruptive and can induce effects on physical, mechanical or chemical properties of samples. Ultrasound can be divided into three frequency ranges which are power ultrasound (16-100kHz), high frequency ultrasound (100kHz-1MHz) and diagnostic ultrasound (1-10MHz) (Patist and Bates, 2008).

Acoustic cavitation and some mechanical effects resulted from the ultrasound-assisted extraction can enhance the extraction efficiency. Zhang *et al*. (2008) highlighted the disruption of cell wall by acoustic cavitation and facilitating the solvent to penetrate into plant material and allowing the intracellular product release. Agitation of solvent used for extraction is one of the mechanical effects of ultrasound. This effect can increase the contact surface area between the solvent and targeted compounds by permitting greater penetration of solvent into sample matrix. It is undeniable that the main advantages of using ultrasound include the reduced extraction time and reduced solvent consumption .

Chemat *et al*. (2011) shows the mechanism of cavitation bubbles that can be (a) generated close to the plant material surface. During the compression cycle, (b) this bubbles collapse and (c) a microjet directed towards the plant matrix is created. The high pressure and temperature involved in this process will (d) destroy the cell walls of the plant matrix and release its content to the medium (Fig. 1).

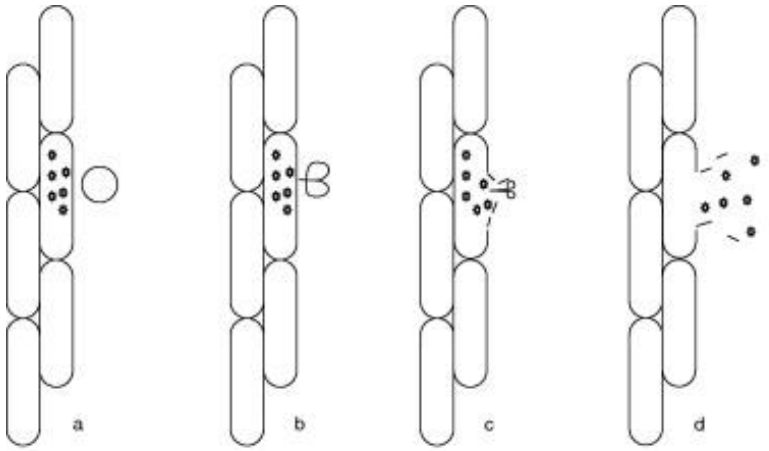

Fig. 1 Cavitation bubble collapse and plant material releasing

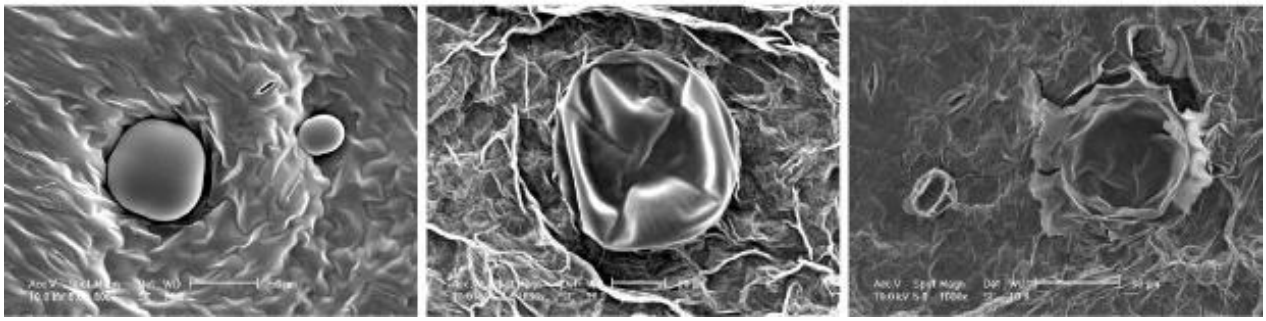

Fig. 2 SEM of Cavitation bubble collapse and plant material releasing in the extraction of essential oil from basil (Chemat *et al*., 2011).

The rupture of cell membrane and mechanical breakdown of cell walls caused by acoustic cavitation phenomenon resulted in good solvent penetration into herbal tissue matrices and rapid exudation of cytoplasmic and cell-sap material into the solvent (Mzevimba *et al*., 2012). Fig. 2 is a example of Cavitation bubble collapse and plant material releasing in the extraction of essential oil from basil under the observation of SEM.

In the ultrasound-assisted extraction, the experiment will conducted by adjusting the sonotrode amplicate to vary the ultrasound power and adjusting the duty cycle to vary the sonication intensity can be calculated using the equation 1.

$$I = \frac{P}{A} \quad (1)$$

Where:

I (W/cm^3)=intensity of sonication (power/area)

P (W)=power of sonication

A (cm^3)=the area of the sonotrode tip

Ultrasonic probe or horn system is chose to be used in this study because it is better and powerful than ultrasonic bath in term of intensity delivery (Chemat *et al*., 2011). Ultrasonic baths are less used for chemical reactions because the reproducibility of reaction is low, even though they are easy to handle and economically advantageous. In fact, the delivered intensity is low and highly attenuated by the water contained in the bath and the walls of the glassware used for the experiment (Mason and Lorimer, 1989; Vale *et al*., 2008). Compared to ultrasonic bath, the ultrasonic probe is more powerful because the ultrasonic intensity is delivered on a small surface (only the tip of the probe). Less attenuation can be achieved because the probe is directly immersed into the reaction flask. The probe system is widely used for sonication of sample with small volumes but special care has to be taken because of the fast rise of the temperature in the sample.

Ultrasound as a form of energy can be used to influence or change the nature of the medium. Some interactions caused by ultrasound vibration and medium included the thermal effects, mechanical effects and cavitation (Xiao *et al*., 2005). Compared to the conventional extraction methods, ultrasonic technology extraction of alkaloids, flavonoids, polysaccharides, saponins, anthraquinone, coumarin and other biologically active compounds from plants costs less time, promotes high yield and has almost no effect on its physiologically activity. Some studies reported that the flavonoids content and yield detected increases with increasing of ultrasound power from 50-120W (Ming Pan, 2013; Xiao *et al*., 2005; Zhang *et al*., 2008). However, at some point, the flavonoid content is decreased with ultrasound power more than 120W due to the enzyme inactivation (Ming Pan, 2013). In terms of reaction time, the first 30 minutes of sonication is more efficient and gives optimized yield (Zhang *et al*., 2008). While Ming *et al*. (2013) stated that the optimum yield can be achieved at 59 minutes of sonication in continual or intermittent sonication.

2.2 Enzymatic Extraction

In order to overcome the low efficiency of aqueous extraction process, the use of hydrolytic enzymes as eco-friendly alternatives (Sharma and Gupta, 2006) are introduced which help to release the active compunds in the plant cell wall and increase the extraction yield. Rosental *et al*. (1996) had listed some advantages regading the use of enzymatic extaction in terms of lowering the investment cost and energy requirement. The development of this method is also to fulfill the needs towards a clean environment especially the increasing awareness obout volatile organic compounds (VOCs) caused by solvent emissions and safety concerns. In this study, cellulase ezyme will be used to catalyse the hydrolysis of cell wall in Tongkat Ali roots. Cellulase is a linear polymer of anhyriglucose units linked together by β-1， 4-glycosidic bonds, to glucose. Cellulose is enzymatically degraded into glucose by the synergic action of three distinct classes of enzymes:

1. Endo-β-D-glucanase (EC 3.2.1.4), which hydrolyze internal β-1, 4-glucosidic linkages or suger residues randomly in the cellulose chain.

2. Exo-β-D-glucanase (E 3.2.91), which progresses along the cellulose and cleave off cellobiose units from the ends.

3. β-glucosidase (EC 3.2.1.21), which hydrolyze cellobiose to glucose and also cleave off glucose units from cello-ologosaccharides.

Endo- β-D-glucanase is one of the major component enzymes of the cellulase complex. Exo- β-D-glucanase and β-glucosidase can syergically convert cellulose into glucose and hence are used on an industrial scale. The optimum temperature range for cellulase is 40-50 ℃ and optimum pH at 4.0-5.0. The conversion of cellulose into glucose by exo-β-D-glucanase and β-glucsidase is shown in figure (Aliyu and Hepher, 2000).

Trichoderma reesei is one of good sources of these types of enzymes. It produces two exo-β-D-glucanase, five endo-β-D-glucanase and two β-glucosidase. The whole hydrolysis process of cellulose can be divided into primary hydrolysis, that involves depolymerisation step by the action of endo- β-D-glucanase and exo- β-D-glucanase on the solid surface of substrate releasing soluble sugars and secondary hydrolysis,which involved the hydrolysis of cellobiose to glucose by β-glucosidase. The action of cellulase hydrolysis can be further understood by the following mechanism showing in figure 3 (Parameswaran Binod, 2011).

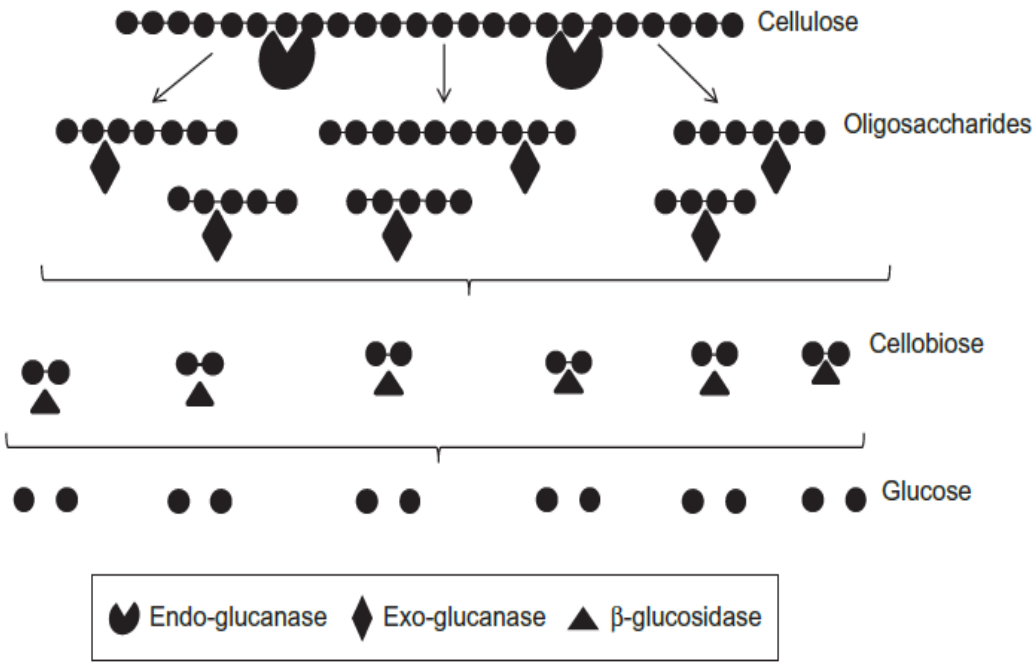

Fig. 3 mechanism of action of cellulose

2.3 Ultrasound-Assisted Enzymatic Extaction

In nature, the enzymatic hydrolysis of lignocellulosic is a low process. The attempts to improve the susceptibility of this material to biodegradation have focused on structural modification, including the use of ultrasound as pretreatment (Imai *et al*., 2004). The pretreatment of ultrasound in cellulose hydrolysis system prior to the enzyme reaction had improved the

reaction rate. A study by Ming *et al.* (2013) showed that the appropriate strength of ultrasonic does not only promote the enzyme inactivation, but also can improve he catalytic activity of enzyme and the enzyme reaction yield.

Sharma and Gupta (2006)stated that the use of the enzyme such as cellulase gives good results in the extraction of cellulosic plant material. On the other hand, the mechanism of caviation bubbles in ultrasound pretreatment result in better contact between the extraction medium and extracted yield. In terms of sample size reduction, Vale *et al*. (2008) mentioned that the reduction of sample size produced by the ultrasound probe is considerably lower than enzyme and ultrasound are jointed. Hence, ultrasonic pretreatment not only enhanced the extract yield, it also reduced the overall precess time signicantly.

3. METHODOLOGY

3.1 Raw Material Preparation

Raw material used for the experiments was purchased from the supplier. The ground Tongkat Ali was stored in a cool and dry environment to prevent fungus growth. Fungus may causes decomposition and changes in the phytochemical in the Tongkat Ali converted to phyto-toxins through bioactivity of bacteria. The raw material was sieved into three size categories: 0.25-0.5 mm, 0.5-1.0 mm and 1.0-2.0 mm. The sieved Tongkat Ali was packed and stored to maintain their quality. Plastic bags were used for sealed packaging and put them in refrigerator. For long time storage, the water content in the raw material should be ensured below than 10 % as recommended by UTM's experience.

3.2 Optimaizaiton of Conventional Water Extraction Using Response Surface Methodology (RSM)

A preliminary study was done to determine the appropriate range of the independent variables on the processing parameters of extraction. Eurycoma longifolia extract using aqueous extraction has been patent and standardized (Sambandan, 2006)(Draft Malaysian Standard, 2010). Therefore, in this study water has been used as the solvent in Tongkat Ali extraction. According to Sim *et al*. (2004) and Kumaresan (2008), the optimum temperature for Eurycoma longifolia roots extraction was found at 100 ℃. Hence, in this work the temperature was constant at boiling point of solvent used which is at 100 ℃. In previous study by Mohamad *et al*. (2010), it was shown that the best agitation rate on the extraction of Tongkat Ali to eurycomanone yield is at 400 rpm. In addition, according to Kumaresan (2008), 400 rpm was chosen as the optimal agitation rate as the agitation rate was sufficient to mix the particles and maintain it suspended while not creating a vortex and overcoming the bulk fluid resistance to mass transfer. Thus, the agitation speed was kept at 400 rpm in this study. As a result, the processing parameters of Tongkat Ali extraction that was investigated in this study are solvent to raw material ratio, duration of extraction and particle size of raw material (Kumaresan, 2003) Effect

of these parameters was observed on eurycomanone yield. Based on the study, the factor level of each processing parameters are as followed:

Table 1: Processing Parameters for the Extraction of Tongkat Ali

Factor Name	Factor levels
Solvent to raw material ratio (g/g)	10:1 to 40:1 (Kumaresan, 2008; Sim, 2004)
Duration of extraction (hour)	1 to 3 (Kumaresan, 2008; Kumaresan, 2003; Sim, 2004)
Particle size of raw material (mm)	0.054-0.154 mm, 0.154-.0.3 mm and 0.3-0.45 mm (modified from Kumaresan, 2003; Sim, 2004)

The experiment was run according to experimental design that developed by Design Expert (V 8.05b). For the optimization of extraction, central composite design was used to evaluate the effects of processing parameters on response variables and 39 experiments were designed for this research. Processing Parameters were used are solvent to raw material ratio, duration of extraction, and particle size of raw material with three levels. The range of extraction process variable are listed as Table 2 and the experimental matrix of the study is shown in Table 3.

Table 2: Processing Parameters of the Tongkat Ali Extraction Process

Factor	Factor name	Factor levels
A	Solvent to raw material ratio (g/g)	10:1, 25:1, 40:1
B	Duration of extraction (hour)	1, 2,3
C	Particle size of raw material (mm)	0.054-0.154, 0.154-0.3, 0.3-0.45

Table 3: Central Composite Design Arrangement

Run	Factor 1	Factor 2	Factor 3
1	25.00	2.00	0.054-0.154
2	40.00	1.00	0.3-0.45
3	25.00	2.00	0.3-0.45
4	40.00	2.00	0.3-0.45
5	25.00	2.00	0.3-0.45
6	25.00	2.00	0.3-0.45
7	10.00	3.00	0.3-0.45
8	40.00	1.00	0.154-0.3
9	40.00	1.00	0.054-0.154
10	40.00	3.00	0.3-0.45
11	10.00	3.00	0.054-0.154

12	25.00	3.00	0.154-0.3
13	25.00	2.00	0.154-0.3
14	40.00	3.00	0.054-0.154
15	25.00	2.00	0.054-0.154
16	10.00	1.00	0.3-0.45
17	10.00	2.00	0.054-0.154
18	10.00	2.00	0.154-0.3
19	25.00	2.00	0.154-0.3
20	40.00	2.00	0.154-0.3
21	25.00	2.00	0.154-0.3
22	25.00	2.00	0.054-0.154
23	25.00	3.00	0.054-0.154
24	10.00	1.00	0.054-0.154
25	10.00	1.00	0.154-0.3
26	25.00	2.00	0.3-0.45
27	40.00	3.00	0.154-0.3
28	25.00	2.00	0.054-0.154
29	25.00	2.00	0.3-0.45
30	10.00	3.00	0.154-0.3
31	25.00	1.00	0.054-0.154
32	25.00	1.00	0.3-0.45
33	25.00	2.00	0.154-0.3
34	10.00	2.00	0.3-0.45
35	25.00	2.00	0.154-0.3
36	40.00	2.00	0.054-0.154

Extraction was carried out in round bottom flask which connected with a condenser on the top to reduce water loss by evaporation as Fig.4 shows. The extraction was heated at boiling temperature and agitated at 400 rpm on a heating mantel. The solvent used for the extraction is water. After extraction, the samples were filtered to remove gross and suspended solids. The filtered extract was analyzed by HPLC to know the amount of eurycomanone. The result from experiments was analyzed using ANOVA to know the optimum condition for extraction process of Tongkat Ali.

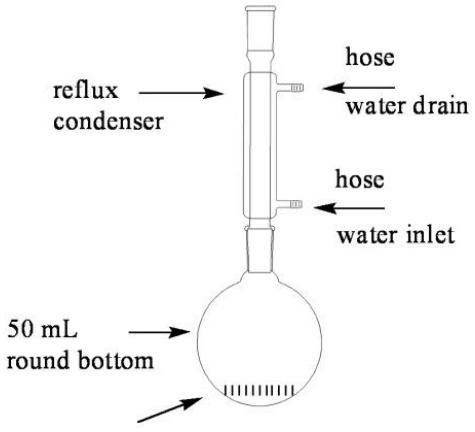

Fig.4 water extraction set

3.3 Amount of Eurycomanone Measurement

To measure the amount of eurycomanone in the Tongkat Ali extract, the High Performance Liquid Chromatography (HPLC) analysis was performed using a Waters 2690 Separation Module auto-sampler and Waters 996 Photodiode-array detector. Separation is achieved using Synerg 4u Fusion-RP80A column with dimension of 150 x 4.60 mm and 4 micron of particle size using a 10 minute water-acetonitrile-ortho-phosphoric acid isocratic system. The mobile phase consisted 85% of 0.05 % phosphoric acid and 15 % of acetonitrile at a flow rate of 1 ml/min (Draft Malaysian Standard, 2010). The low mobile phase flow rate was chosen to allow the peaks to separate more distinctly (Kumaresan, 2003). The UV detector was operated at 254 nm (Draft Malaysian Standard, 2010).

To determine the calibration for eurycomanone, 2 mg standard of eurycomanone was weighed and dissolved into 20 ml deionized water to produce 100 ppm of stock solution. The stock solution was diluted into another four concentrations, 20 ppm, 40 ppm, 60 ppm and 80 ppm to construct a 5 point of calibration curve. The HPLC setting for determination eurycomanone are as following:

Table 4: HPLC Setting

Parameter	Setting
Column	Synerg 4u Fusion-RP80A column with dimension of 150 x 4.60 mm and 4 micron of particle
Detector	Agilent 1100
UV wavelength	254 nm
Flowrate	1 ml/min
Injection volume	20.00 µl
Mobile phase	85% of 0.05 % phosphoric acid and 15 % of acetonitrile

The reference standard solution and sample preparation will be prepared as following: A single injection of an extraction solvent blank was made followed by a single injection of standard preparation. A plot of standard peak areas versus standard concentrations was made with the origin ignored. A single injection of sample preparations. Calculation of the eurycomanone amount in the samples as follow:

$$\text{Amount of eurycomanone } (\%) = \frac{[C][V]}{W} * 100\% \quad (2)$$

where;

C = concentration of eurycomanone (g/ml) from linear regression analysis

V = volume of extract (1 ml = mass of water + mass of solid content)

W = weight of solid content (g)

4. RESULT AND DISCUSSION

This section presents the results of effects of processing parameters on the response variables namely eurycomanone yield in the extraction of Tongkat Ali. The processing parameters considered in the extraction process were solvent to raw material ratio, duration of extraction, and particle size of raw material.

An experimental design with 39 experiments was run on three factorial variables to optimize the extraction of Tongkat Ali process. The three factor variables were solvent to raw material ratio, duration of extraction and particle size of raw material. Design Expert (V 8.05b) was used to carry out the regression analysis and to analyze the points of data. The optimum values for eurycomanone yield in Tongkat Ali extract was simulated from Design Expert using the regression equation and also from the response surface 3D surface graph.

The results for eurycomanone yield from optimization of Tongkat Ali extraction process were gathered in Table 5. The experimental design used was Central Composite Design.

Table 5: Central Composite Design Arrangement and Responses Value for Extraction Process

Run	Factor 1	Factor 2	Factor 3	Response
1	25.00	2.00	0.054-0.154	0.580%
2	40.00	1.00	0.3-0.45	0.069%
3	25.00	2.00	0.3-0.45	0.304%
4	40.00	2.00	0.3-0.45	0.093%
5	25.00	2.00	0.3-0.45	0.270%
6	25.00	2.00	0.3-0.45	0.284%
7	10.00	3.00	0.3-0.45	0.321%
8	40.00	1.00	0.154-0.3	0.151%
9	40.00	1.00	0.054-0.154	0.306%
10	40.00	3.00	0.3-0.45	0.100%
11	10.00	3.00	0.054-0.154	0.309%
12	25.00	3.00	0.154-0.3	0.345%
13	25.00	2.00	0.154-0.3	0.355%
14	40.00	3.00	0.054-0.154	0.528%
15	25.00	2.00	0.054-0.154	0.583%
16	10.00	1.00	0.3-0.45	0.346%
17	10.00	2.00	0.054-0.154	0.300%
18	10.00	2.00	0.154-0.3	0.150%
19	25.00	2.00	0.154-0.3	0.326%
20	40.00	2.00	0.154-0.3	0.165%

21	25.00	2.00	0.154-0.3	0.332%
22	25.00	2.00	0.054-0.154	0.571%
23	25.00	3.00	0.054-0.154	0.513%
24	10.00	1.00	0.054-0.154	0.434%
25	10.00	1.00	0.154-0.3	0.309%
26	25.00	2.00	0.3-0.45	0.274%
27	40.00	3.00	0.154-0.3	0.097%
28	25.00	2.00	0.054-0.154	0.584%
29	25.00	2.00	0.3-0.45	0.269%
30	10.00	3.00	0.154-0.3	0.150%
31	25.00	1.00	0.054-0.154	0.508%
32	25.00	1.00	0.3-0.45	0.254%
33	25.00	2.00	0.154-0.3	0.340%
34	10.00	2.00	0.3-0.45	0.345%
35	25.00	2.00	0.154-0.3	0.330%
36	40.00	2.00	0.054-0.154	0.373%

From Table 5, the best eurycomanone amount of 0.58 % was observed at run 28 which was at a solvent to raw material ratio of 25:1, 2 hour extraction process and 0.054-0.154 mm raw material particle size.

4.1 Analysis of Variance (ANOVA) for Tongkat Ali Extraction Process

Tables 6 and Table 7 display total, regression, residual, sum of squares and mean squares of eurycomanone yield in Tongkat Ali extract. It was observed that F calculated for eurycomanone yield was 25.21. Degree of freedom for regression and residual of eurycomanone yield were 11 and 27, respectively. Hence, the F(11,27,0.05) tabulated was found to be 2.20.

Table 6: The Results fom Analysis of Variance (ANOVA) for Eurycomanone yield from Tongkat Ali Extraction. The Model Value of 22.69 implied that the Model was Significant.

Source	Sum of	df	Mean	F	p-value	
Model	7.36E-05	11	6.69E-06	22.69848	< 0.0001	significant
A-solvent to raw material ratio	3.4E-06	1	3.4E-06	11.52655	0.0021	
B-duration	4.18E-07	1	4.18E-07	1.417844	0.2441	
C-partical size	4.1E-05	2	2.05E-05	69.54715	< 0.0001	
AB	2.14E-06	1	2.14E-06	7.257192	0.0120	
AC	7.06E-06	2	3.53E-06	11.96901	0.0002	
BC	1.44E-06	2	7.22E-07	2.44954	0.1053	
A^2	1.55E-05	1	1.55E-05	52.66224	< 0.0001	
B^2	1.9E-11	1	1.9E-11	6.46E-05	0.9936	
Residual	7.96E-06	27	2.95E-07			
Lack of Fit	7.57E-06	15	5.04E-07	15.29689	< 0.0001	significant
Pure Error	3.96E-07	12	3.3E-08			
Cor Total	8.16E-05	38				

Table 7: Coefficient Estimate of the Quadratic Model for Eurycomanone yield

Term	Coefficient	df	Standard	95% CI	95% CI	VIF
Intercept	3.893E-003	1	1.302E-004	3.626E-003	4.160E-003	
A-solvent to	-4.345E-004	1	1.280E-004	-6.971E-004	-1.719E-004	1.00
B-duration	-1.524E-004	1	1.280E-004	-4.150E-004	1.102E-004	1.00
C[1]	1.440E-003	1	1.230E-004	1.188E-003	1.693E-003	
C[2]	-5.739E-004	1	1.230E-004	-8.262E-004	-3.216E-004	
AB	4.223E-004	1	1.567E-004	1.006E-004	7.439E-004	1.00
AC[1]	7.063E-004	1	1.810E-004	3.350E-004	1.078E-003	
AC[2]	1.094E-004	1	1.810E-004	-2.620E-004	4.808E-004	
BC[1]	3.216E-004	1	1.810E-004	-4.977E-005	6.930E-004	
BC[2]	-3.677E-004	1	1.810E-004	-7.391E-004	3.699E-006	
A^2	-1.369E-003	1	1.886E-004	-1.756E-003	-9.819E-004	1.17

Table 7 shows the coefficients and their confidence interval for quadratic model of eurycomanone yield. The standard error given is the standard deviation associated with coefficient estimates. A full quadratic model was established to express the eurycomanone yield as a function of the chosen variables. The predicted eurycomanone yield model is shown Equation 3.

Eurycomanone yield=+3.893E-003-4.345E-004*A-1.524E-004*B+1.440E-003*C[1]-5.739E-004*C[2]+4.223E-004*AB+7.063E-004*AC[1]+1.094E-004*AC[2]+3.216E-004*BC[1]-3.677E-004*BC[2]-1.369E-003*A^2+1.516E-006*B^2 (3)

From the Equation 3, amount of eurycomanone will be raised when increasing duration to the power of two (B2), interaction between solvent to raw material ratio and duration (AB) and interaction between solvent to patical size (AC[1], AC[2]), interaction between duration and partical size (BC[1]), in conjunction with the decreased of duration (B), solvent to raw material ratio (A),solvent to raw material ratio power two (A^2), and interaction of duration and patical size (BC[2]).

The value of determination coefficient (R^2) for eurycomanone yield was 0.9024 which indicates that only 9.76 % of the total variations were not explained by the model and the value of adj-R^2 was 0.8627. F value for eurycomanone model was 22.7 and value of Prob > F was less than 0.0001 which means that the model was significant. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 17.965 on our model indicates an adequate signal. An adequate signal noise to ratio of 17.965 was achieved thus indicating that the model is significant for the process. The "Pred R-Squared" of 0.7290 is in reasonable agreement with the "Adj R Squared" of 0.8627. A “Pre R-Squared” with the values of 0.7290 implies that the overall mean is a better predictor of experiments response than the current model

4.2 Effect of Solvent to Raw Material Ratio, Duration of Extraction and Particle Size of Raw Material on Eurycomanone Yield and Optimization of Eurycomanone Yield by RSM

Effect of solvent to raw material ratio, duration of extraction and particle size of raw material on the eurycomanone yield from Tongkat Ali extraction was investigated. Fig. 5 shows the interaction between solvent to raw material ratio and

duration of extraction on the eurycomanone amount. Fig.6 shows the interaction between duration of extraction and particle size of raw material on the eurycomanone amount. Fig. 7 shows the interaction between solvent to raw material ratio and particle size on the eurycomanone amount.

From Fig. 5, the eurycomanone yield increases with the increase of extraction duration. The maximum eurycomanone yield obtained was at solvent to raw material ratio of 28.8:1 g/g in 3 hours extraction. Higher amount of extraction solvent will lead to a higher leaching rate where the desired bioactive compound has higher contact with the solvent. Therefore, higher volume of water usage could leach out the bioactive compounds in higher amount (Ahmad et al., 2013). However if solvent to raw material ratio used is higher, the greater quantity of water used requires the extraction process to be longer. As eurycomanone is the marker chemical for standardization, the raw material needs to be processed in the shortest time possible to preserve its quantity and quality.

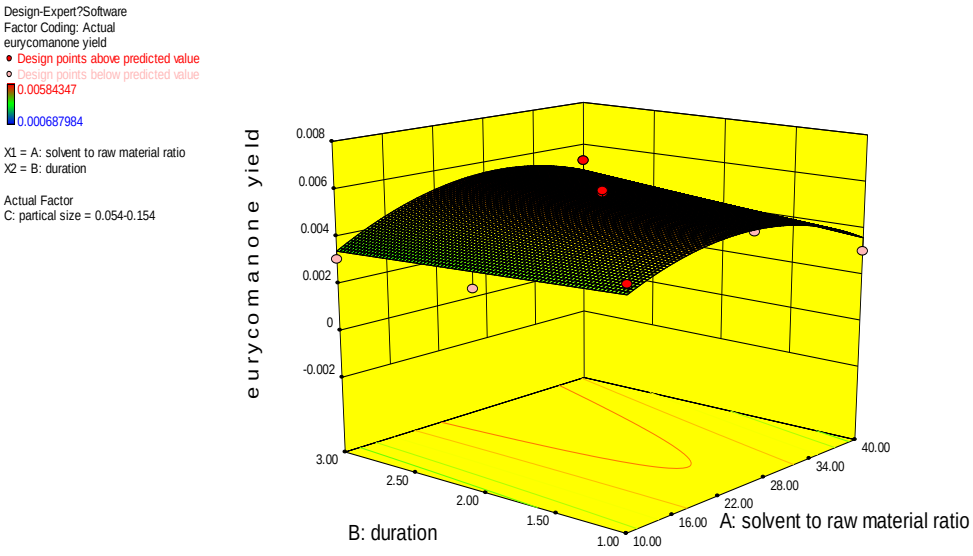

Fig. 5 Tthe interaction between solvent to raw material ratio and duration of extraction on the eurycomanone yield.

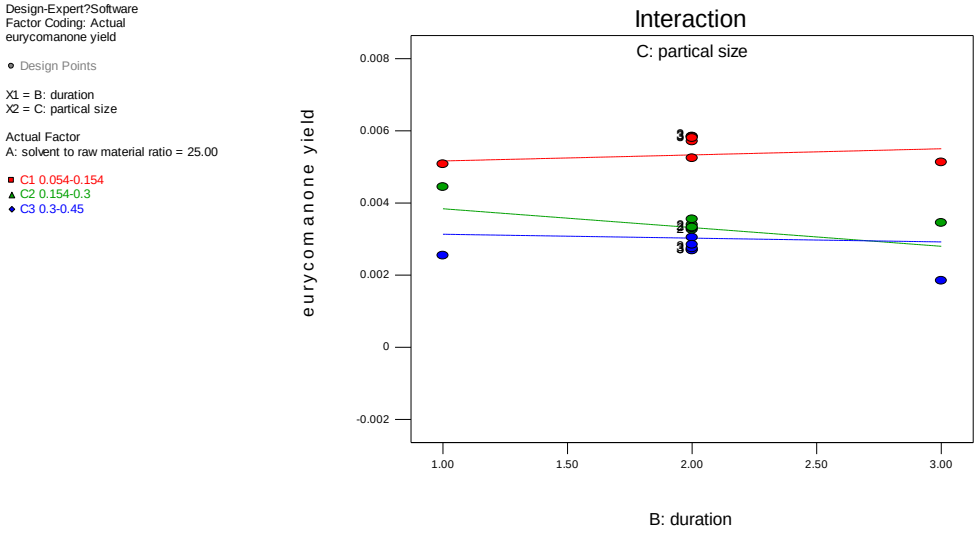

Fig. 6 the interaction between duration of extraction and particle size of raw material on the eurycomanone amount

Fig. 6 illustrates that interaction between duration of extraction and particle size of raw material on eurycomanone yield. The eurycomanone yield highly influenced by partical size. The eurycomanone yield increases with the decrease of raw material particle size. Maximum amount of eurycomanone was observed during 3 hour of extraction when the partical size in the range of 0.054-0.154mm.

For eurycomanone production, the interaction between solvent to raw material ratio and particles size of raw material influenced the responses highly (Fig. 7). Eurycomanone yield increases with the decreasing of particle size of raw material. For example, at 0.3-0.45mm of raw material particle size the eurycomanone yield was 0.364 %. The amount was increased to 0.394 % as particle size decreased to 0.154-0.3mm. When the particle size at 0.054-0.154mm, the yiled of urycomanone increased to 0.559%. Larger particle size has smaller contact surface area which will increase resistance to the water entrance and eurycomanone diffusion towards the water will be lower. Hence compare to the small ones, eurycomanone transferred from inside of the larger particles to the surrounding solution in smaller amount. Therefore, the optimum particle size of raw material to attain the maximum amount of eurycomanone was at 0.054-0.154 mm.

Solvent to raw material ratio affects the eurycomanone yield highly as well. Low solvent to raw material ratio makes lower yield of eurcomanone, and high solvent to raw material ratio dose the same. There is a range of solvent to raw material ratio under different particle size gives the highest eurycomanone yield. For example, When the particle size is 0.3-0.45, the eurycomanone yield could reach 0.364% of its optimum at the solvent to raw material ratio of 15.83:1. And when the particle size is 0.054-0.154mm, the yiled of eurycomanone could reach 0.559% of its optimum at solvent to raw material ratio of 28.8:1.

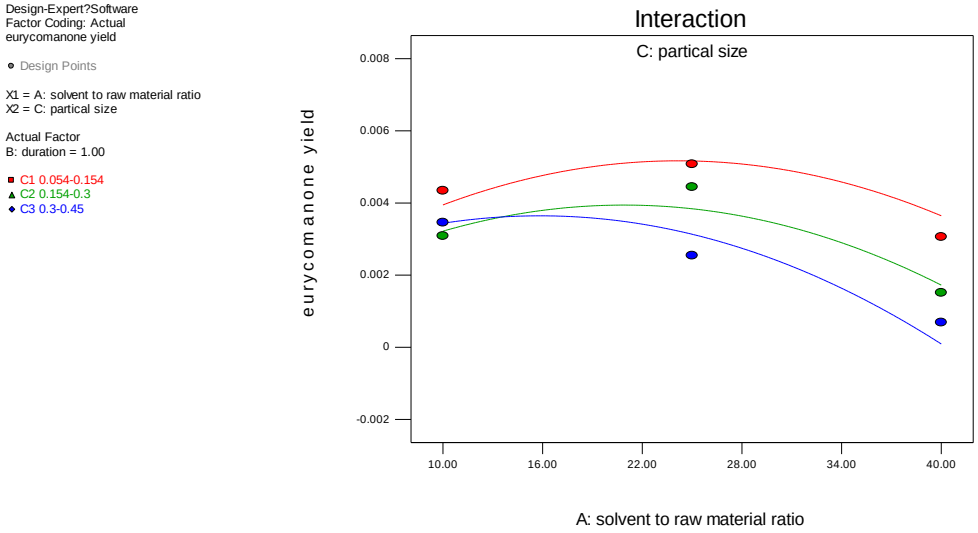

Fig. 7 shows the interaction between solvent to raw material ratio and particle size on the eurycomanone amount

The optimization of solvent to raw material ratio, duration of extraction and particle size of raw material in Tongkat Ali extraction was carried out to identify the optimum condition for the extraction process in order to obtain maximum eurycomanone yield.

The maximum eurycomanone amount which is 0.559% was obtained at solvent to raw material ratio of 28.8:1 g/g, duration of extraction of 3 hour, and 0.054-0.154 mm of raw material.

Table 8: Optimum Condition for Tongkat Ali Extraction Process

Variables			Response
Solvent to raw material ratio (g/g)	Duration of extraction (hour)	Particle size of raw material (mm)	eurycomanone yield (%)
28.8:1	3	0.054-0.154	0.559 %.

5. CONCLUSION

Optimization process on the Tongkat Ali extract was investigated. The optimized operating conditions of extraction was successfully identified using response surface methodology (RSM). In Tongkat Ali extraction process, optimization of processing parameters; solvent to raw material ratio, duration of extraction, and raw material particle size on eurycomanone yield was successfully performed using Central Composite Design (CCD) of Design Expert (V 8.05b) software. The experiment values were analysed using Analysis of Variance (ANOVA), and results showed that the operating parameters have significant effect on Tongkat Ali extraction. The optimum condition obtained was at solvent to raw material ratio of 28.8:1 g/g, 3 hours of extraction duration, and 0.054-0.154 mm of raw material particle size, with the values of eurycomanone yield of 0.559 %.

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