Jurnal Teknologi

Full paper

Effect of Extraction Process Parameters on the Yield of Bioactive Compounds from the Roots of *Eurycoma Longifolia*

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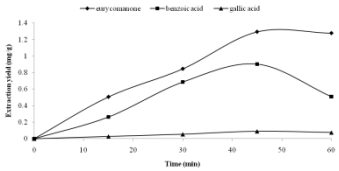
Article history

Received :29 June 2012
Received in revised form :4 Oktober 2012
Accepted :5 December 2012

Graphical abstract



Abstract

The effects of extraction process parameters, i.e., extraction time (15-45 min), temperature (40-110°C) , particle size (Tongkat Ali roots in chip and powder form), agitation speed (200-400 rpm) and solvent to solid ratio (10:1, 20:1 and 30:1) on the extraction yields from *Eurycoma longifolia* were investigated, focusing on eurycomanone, benzoic acid and gallic acid as the product of interests. The concentrations of these bioactive compounds were measured using high-performance liquid chromatography (HPLC). The results obtained showed that the extraction yields increased with reduced particle size and increased of temperature, solvent to solid ratio and agitation speed. The optimum extraction temperature for maximizing yields of eurycomanone, benzoic acid and gallic acid were 100°C, 50°C and 80°C respectively. The highest yields were obtained when the process was run at 45 minutes, with a solvent to solid ratio of 20 to 1, agitation speed of 400 rpm and a smaller particle size (Tongkat Ali roots in powder form) was used. The results provided useful insights for making the process economically feasible and in particular, when the search of other bioactive components is to be continued.

Keywords: *Eurycoma longifolia*; extraction; eurycomanone; benzoic acid; gallic acid; HPLC

Abstrak

Kesan-kesan parameter proses pengekstrakan iaitu masa pengekstrakan (15-45 min), suhu (40-110°C) , saiz partikel (akar Tongkat Ali dalam bentuk serpihan dan serbuk), kelajuan putaran (200-400 rpm) dan nisbah pelarut kepada pepejal (10:1, 20:1 and 30:1) ke atas hasil pengekstrakan daripada *Eurycoma longifolia* telah diselidik yang memfokus kepada eurycomanone, asid benzoik dan asid galik sebagai produk. Kepekatan komponen-komponen bioaktif tersebut diukur dengan menggunakan pemisahan sebatian cecair berprestasi tinggi (HPLC). Keputusan menunjukkan bahawa hasil pengekstrakan meningkat apabila saiz partikel semakin berkurang dan suhu, nisbah pelarut kepada pepejal dan kelajuan putaran semakin meningkat. Suhu pengekstrakan yang optimum untuk memaksimumkan hasil eurycomanone, asid benzoic dan asid galik adalah pada 100°C, 50°C and 80°C. Hasil maksimum pengekstrakan adalah apabila proses pengekstrakan dijalankan selama 45 minit, dengan nisbah pelarut kepada pepejal ialah 20 to 1, kelajuan putaran pada 400 rpm dan saiz partikel yang kecil digunakan (akar Tongkat Ali dalam bentuk serbuk). Keputusan yang didapati memberikan informasi yang berguna bagi memastikan proses tersebut adalah boleh dilaksanakan dari segi ekonomi dan terutamanya apabila pencarian komponen-komponen bioaktif yang lain masih diteruskan.

Kata kunci: *Eurycoma longifolia*; pengekstrakan; eurycomanone; asid benzoik; asid galik; HPLC

1.0 INTRODUCTION

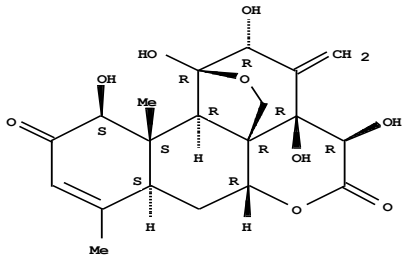
Eurycoma longifolia Jack from the Simaroubaceae family, known locally as Tongkat Ali (Malaysia), PasakBumi in Indonesia, Cay babinh' (Vietnam) and 'Ian-don' (Thailand), is attracting research interests due to its medicinal values. The plant growing wildly in the jungle slopes of Malaysia, is commonly sought after as an essential ingredient in Malay herbal medicine for intermittent fever (malaria).[1-3] Quassinoids form the major bioactive constituents in this plant [3] and are mainly responsible for its bitter taste.[2,4] Traditionally, the roots extract from the plant are used for enhancing testosterone levels in men. It has also been administered as herbal ingredient for women after child birth, restoring energy and vitality and enhancing blood flow. Pharmacological properties of Tongkat Ali from plant parts such as roots, stem, bark and leaves have shown antiplasmodial,

cytotoxic, anti-tumor, antiulcer, antimicrobial andaphrodisiac properties.[5] 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, quassinoidditerpenoids, eurycomaoside, tirucallane-type triterpenes, squalene derivatives, biphenylneolignans, eurycolactone, laurycolactone, and eurycomalactone.[4,6-9] The isolation of nearly sixty-five compounds from the roots of E. longifolia was reported by Kuo *et al.* (2004).[10]

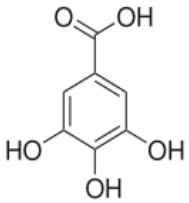
Typically, these bioactive constituents are isolated using a series of processes beginning with extraction, which involves the transfer of solutes from a solid medium to a solvent. The ideal extraction system should be quantitative, non-destructive and time saving [11] and is dependent on several factors. 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. Depending on the extraction methods used, i.e., soxhlet extraction [12], supercritical extraction [13], the available degrees of freedom as to what can be varied would be different. Degree of freedoms for extraction process depends on the range of the specified parameter. Moreover, there are also issues on conditions and duration of storage as well as the presence of interfering substances.

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.[14] 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.[15] These parameters, when optimally selected, provide maximum process efficiency while preserving the desired components functionality which refers to the pharmacological properties of the plant. This is particularly important because 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 is very important.[16]

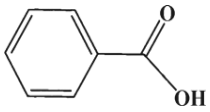
Among bioactive components of Tongkat Ali, eurycomanone has the highest concentration. Comparatively, the alkaloid content is significantly lower than the content of quassinoids. Thus, eurycomanone is more commonly used as the marker compound for Tongkat Ali extract quantification.[17] In this study, in addition to eurycomanone, benzoic acid and gallic acid were also considered. Benzoic acid occurs naturally in different foods such as fruits, vegetables, spices and nuts. It is a colourless aromatic compound and the simplest of the aromatic carboxylic acids, a family of organic compounds containing the carboxyl (-COOH) group. Benzoic acid is extensively used as an important intermediate for preparation of many other organic substances used in the fields of pharmaceuticals, resins, plasticizers, dyes, cosmetics and preservatives.[18] Gallic acid is an intermediate component of plant metabolism and has been associated with a wide variety of biological actions. Various biological activities of gallic acid have been reported, including anti-bacterial [19], anti-viral [20] and anti-inflammatory.[21] Figure 1 shows the chemical structure of the three major compounds from *Eurycoma longifolia* considered in this study, which are eurycomanone, benzoic acid and gallic acid.



(a) eurycomanone



(b) gallic acid



(c) benzoic acid

Figure 1 Chemical structure of (a) eurycomanone ($C_{20}H_{24}O_9$); (b) gallic acid ($C_6H_2(OH)_3COOH$) and (c) benzoic acid (C_6H_5COOH) (Sieber *et al.*, 1995)[24]

This paper focuses on the effect of extraction process parameters which are extraction time, agitation speed, solvent to solid ratio, temperature and raw material particle sizes on the extraction yields. These parameters were varied and optimum values were identified. The results obtained would be useful for further investigations in making the process more efficient to increase the process economy.

2.0 MATERIALS AND METHODS

2.1 Herbal Materials

Tongkat Ali roots were supplied by Depo Herba Enterprise and were originated from a single source in Muar, Johor, Malaysia. The raw materials were supplied as dried roots in chip form (length:2-2.5 cm, width:1 cm and thickness:0.5 mm) and powder forms (particle size: 1 mm).in October 2009 and were harvested when the plant was between 6 to 7 years old.

2.2 Chemicals

Eurycomanone, benzoic acid and gallic acid were purchased from Sigma-Aldrich (St Louis, MO, USA). The standards were stored at -20^{o}C until use. Solvents used for extraction were deionized water, ethanol and iso-propanol, all of analytical reagent grade obtained from Sigma-Aldrich (St Louis, MO, USA). HPLC grade acetonitrile, acetic acid and 85% ortho-phosphoric acid were purchased from Merck (Germany). Ammonium acetate for HPLC analysis was supplied by Fisher Chemicals (USA). Deionized water was prepared using a Millipore water purification system.

2.3 Preparation of Standard Solutions

Standard solutions for eurycomanone, benzoic acid and gallic acid were prepared from concentration ranging from 0.25 mg/ml, 0.5 mg/ml, 0.75 mg/ml and 1 mg/ml for standard calibration. Standard solutions stocks were prepared by dissolving appropriate amounts of eurycomanone, benzoic acid and gallic acid in

deionized water at room temperature. All the standard solutions were stored at -4 °C in a freezer.

2.4 Extraction Procedure

The experimental work of this study was carried out in a 2 liter glass vessel that was soaked in oil bath. The schematic diagram for the extraction system is shown in Figure 2. The extraction system is heated using an oil bath that is insulated by glass wools. The oil is heated using an electrical element using the available control unit. The process parameters, i.e., temperature, time, solvent to solid ratio, raw material particle size and agitation speed were varied within allowable range as prescribed by the process set up and the nature of the raw materials as well as the bioactive ingredients properties.

At the end of the extraction cycle, the extract was decanted from the extraction slurry mixtures and filtered using 0.45 µm Whatman PTFE membrane syringe filter to remove access solids from the liquid extract. This was performed to eliminate rough particles in the extract that could damage the HPLC column that is use in the next process step. The extracts were kept at 4 °C for further analysis. Extractions were performed in duplicate.

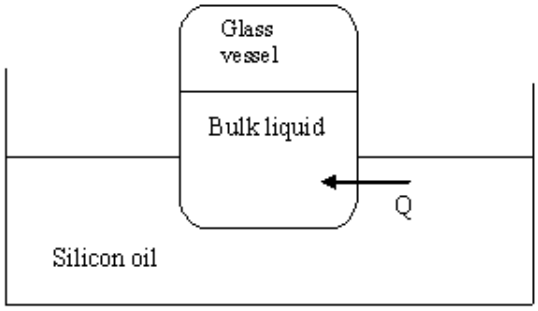


Figure 2 Schematic diagram for the extraction system

Two types of raw materials were used, i.e., Tongkat Ali root chips and Tongkat Ali root powder and the extractions were carried out with deionized water as solvent. The range of extraction process parameters being studied were 40 °C to 110 °C for temperature, 200 rpm to 400 rpm for agitation speed and 15 to 60 minutes for extraction time, and three solvent to solute weight ratios were applied, i.e., 10:1, 20:1 and 30:1. The range of parameters was selected using preliminary works based on previous study conducted by Athimulam *et al.* (2006).[25] The extraction time is defined as the time required to extract at least 90 % of the extractable amount of solute at equilibrium

2.5 HPLC Analysis for Eurycomanone

Pelkin Elmer Series 200 liquid chromatography system comprising of vacuum degasser, pump, auto-sampler and diode array detector was used. The column used was a Phenomenex C_{18} (250 mm × 4.6 mm × 4 µm). Solvents used for separation were acetonitrile and 0.05% orthophosphoric acid (24:76, v/v). The flow rate for the mixed mobile phase was 1.5 ml/min and detection wavelength was 245 nm. Sample injection volume was 10 µl. The total running time for HPLC analysis for eurycomanone was 10 minutes. By comparing the retention times and UV spectra with the reference standard, the chromatographic peaks for the analytes were confirmed and determined

2.6 HPLC Analysis for Benzoic Acid

The column used was a Phenomenex C_{18} (250 mm × 4.6 mm × 4 µm). The mobile phase was consisting of a mixture of acetonitrile-acetate buffer adjusted to pH 4.4 (40:60, v/v). The buffer was prepared by dissolving 3.84 g of ammonium acetate in 1 liter of deionized water and adjusting the pH to 4.4 using acetic acid. The mobile phase was filtered using 0.45 µm membrane filter before use. The flow rate was 1.5 ml/min and the concentration of benzoic acid was monitored using a UV/Vis detector at 254 nm. 10 µl of samples was injected into the chromatographic system. The chromatograph required approximately 6 minutes to reach the equilibrium. The HPLC conditions were similar to those described by Garcia *et al.* (2003) [23] with minor modifications.

2.7 HPLC Analysis for Gallic Acid

The column used was a Kingsorb C_8 (25cm x 4.6mm x 5µm). The column was maintained at 25°C. Solvents used for separation were 0.1% (v/v) orthophosphoric acid and acetonitrile. The flow rate was 1.5 ml/min and detection wavelength was 280 nm. Sample injection volume was 10µl and the chromatograph needs 5 minutes before reaching the equilibrium. Minor modifications were made to the HPLC methods for gallic acid described by Markom *et al.* (2010).[24]

2.8 Data Analysis

All measurements were performed in duplicate and the mean values of the duplicates were presented. All the analytical data from HPLC analysis were determined within significance $P<0.05$ after subjecting to an analysis of variance (ANOVA).

■3.0 RESULTS AND DISCUSSIONS

3.1 Yield Determination

Chromatographic peaks of eurycomanone, benzoic acid and gallic acid were identified by referring the retention times of the external standard. The concentration for each bioactive compound was determined using standard calibration curve for each bioactive compound based on identified from HPLC chromatogram. The yield of eurycomanone was measured in mg eurycomanone per g of raw material used for extraction. The yield determination was also similar for benzoic acid and gallic acid. Standard calibration for eurycomanone is y = 3E+07x + 5E+06; R^2 = 0.971, benzoic acid (y = 5E+06x + 6E+06; R^2 = 0.949) and gallic acid (y = 9E+06x + 3E+07; R^2 = 0.939).

3.2 Effect of Extraction Time

The effect of extraction time was studied by conducting extraction using water as the solvent, carried out at 80°C and agitation of 400 rpm. The extraction time was varied within 15 to 60 minutes. It is important to determine the duration of the extraction process required to extract most of the desired bioactive compounds. Typically, this would be the time at which equilibrium of solvent concentration between inner and outer cells is established.[11] Using this information, suitable duration can be selected and operating cost for the process can be reduced.

Figure 3 illustrates the effect of extraction time on the yield of the three bioactive compounds being studied. The extraction yields of eurycomanone and benzoic acid increased graduallyfor the first 45 minutes, but declined thereafter. For gallic acid, the

yield was only mildly affected by the extraction duration, but a 45 minute extraction was also seen as the point at which the process was reaching a plateau. Since most bioactive compounds are sensitive to elevated temperature, keeping them for a longer period of time would lead to the thermal decomposition of the bioactive compounds similar to those reported by Ma *et al.* (2009).[28] Therefore, based on this result 45 minutes was selected as the optimum extraction time. time.

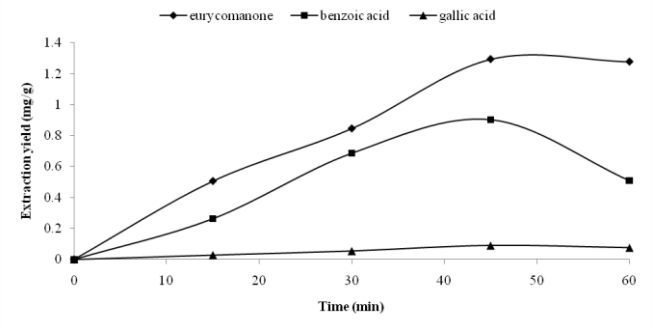


Figure 3 Effect of extraction time on the yield of eurycomanone, benzoic acid and gallic acid using deionized water as extraction solvent at 80 °C and agitation level at 400 rpm using Tongkat Ali roots in chip form

3.3 Effect of Agitation Speed

Figure 4 shows the effect of agitation speed on the extraction yields of eurycomanone, benzoic acid and gallic acid. Three agitation speeds were investigated, i.e., 200, 300 and 400 rpm the results indicated that a higher agitation speed was preferred. This is consistent with the mass transfer theory. In a solid-liquid extraction such as this case, the solute moves from inside the solid to the surface through diffusion or capillary action, and once at the surface, it is limited by convective mass transfer. Higher agitation rate leads to higher mass transfer coefficient, k_L and improves convective mass transfer rate, thus facilitating the extraction process, leading to increase of the extraction yields.[29] Nevertheless, higher agitation rate was not used due to physical limitation of the process such as effect of vortex, splash and geometry effect. The difference on the extraction of gallic acid and benzoic acid as a function of agitation speed is due to gallic acid is simpler tannins and resulted to lower extraction yields due to the long contact with the hot solvent. The effect of long exposure in high temperature for gallic acid leads to the degradation of the compounds. [27]

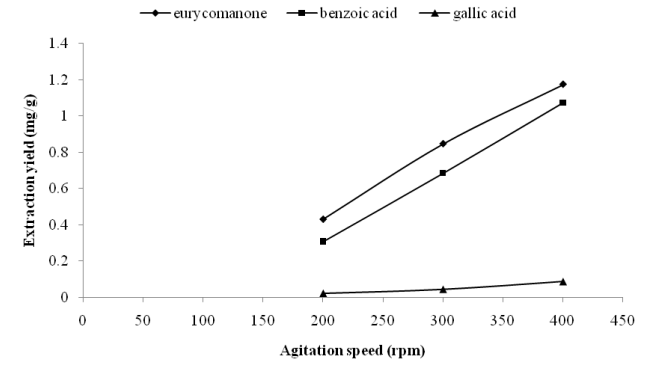


Figure 4 Effect of agitation speed on the extraction yields using deionized water as extraction solvent and solvent to solid ratio is 20:1 for Tongkat Ali roots in powder form

3.4 Effect of Solvent to Solid Ratio

The The effect of solvent to solid ratio on the yield of bioactive compounds under study is shown in Figure 5. Three different ratios between solvent to solid, i.e., 10:1, 20:1 and 30:1 were used. The results show that among the three, 20:1 was most effective. The removal of solute requires a lot of energy using water as solvent. Therefore, if more water usage in extraction may lead to higher energy consumption. This shows that the optimum solvent to solid ratio was found to be similar to the findings by Ma *et al.* (2009).[28]

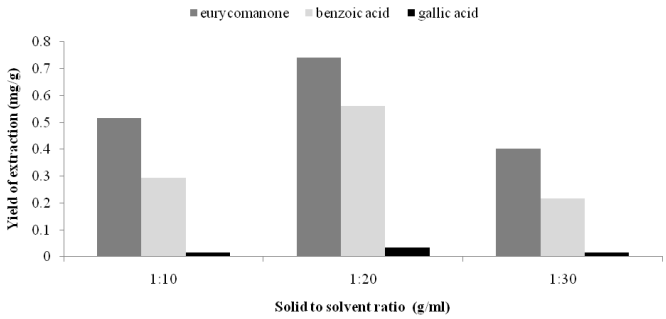


Figure 5 Effect of solid to solvent ratio on the yield of eurycomanone, benzoic acid and gallic acid using deionized water as extraction solvent at agitation level at 400 rpm and 45 minutes extraction time for Tongkat Ali roots in powder form

The dissolving of bioactive components into the solvent is a physical process. When the amount of extraction solvent is increased, the chance of the desired bioactive compounds coming into contact with the solvent leading to higher leaching rates.[11] When the ratio of solvent to solid ratio is higher, it means that the difference of the concentration between the bulk solution and the solutes becomes higher. Thus, more bioactive compounds can leach out if a higher volume of water is used.[30] The solubility of the compounds was affected by the interactions of the compounds with the extraction solvent.[31]

The increase of extraction yields with the increase of solvent to solid ratio is consistent with mass transfer principles. Smaller volumes of solvent can lead to incomplete target extraction while larger volumes can make the extraction procedure becomes complex and wasteful.[28] Therefore, suitable solvent to solid ratio is preferred in order to achieve higher extraction yields. Based on the figure, the optimum solvent to solid ratio is 20:1.

3.5 Effect of Extraction Temperature

Both the equilibrium (solubility) and mass transfer rate (diffusion coefficient) can influence the extraction temperature. In theory, the penetrability and solubility of the solvents increased with the increase of the temperature, thus resulting in an increased of extraction efficiency and speed. On the other hand, the target compounds may decompose if the temperature is higher.[32] A study on the influence of temperature on the overall yield is therefore important.

Figure 6 shows the result on the effect of changing operating temperature on the product yields. The range chosen was between 40 to 110 °C. For eurycomanone, the yield increased with temperature up to 100 °C, beyond which, the yield was reduced. Increase in yield with respect to increasing temperature is attributed to thermal kinetics of mass transfer and the thermodynamic effect of temperature on solubilization. Lower viscosity and lower surface tension of the solvent at higher

temperature improves the diffusion of the solvent inside the solid matrix which together with enhanced diffusivity and solubilization results in higher yield and extraction rate. Thermal-degradation of eurycomanone during the extraction process can be accelerated although increasing the temperature can improve the diffusivity and solubilization of eurycomanone in the solvent.[33]

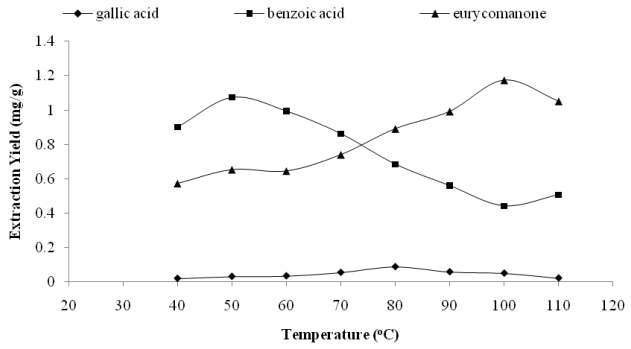


Figure 6 Effect of extraction temperatures on the extraction yields in 45 minutes, agitation level at 400 rpm and solvent to solid ratio is 20:1using water as solvent

Consistent with previous works [30], for eurycomanone, the yield decreases as operating temperature exceeds 100 °C. The main disadvantage of applying higher temperature is the increase of the solvent boil off and reducing effective contact area between solid and liquid phases. As a result, lower yield of the final extracts.[34] Therefore, 100 °C was chosen as optimum extraction temperature for eurycomanone extracts.

For benzoic acid, the yield was increased as the temperature was increased up to 50 °C, beyond which, similarly, the yield for gallic acid was increased with temperature up to 80 °C and decreased thereafter. Since water was used and water boils at 100 °C at atmospheric condition, previous explanation as in the case of eurycomanone is not applicable here. Based on physical understanding of the process, we know that in addition to mass transfer effect which states that higher mass transfer coefficient results in higher rate of solute transfer from solid matrix to bulk solution.[35-36] By applying heat treatment to the extraction process, it can accelerate the mechanism of the diffusion process when extracting bioactive components from plants. However, degradation of bioactive compounds can also be influential to the process, and this phenomenon is affected by temperature. Furthermore, high temperature also can decrease the cell barrier by weakening integrity of the cell wall and membrane. When increasing the temperature, all the reactions are accelerated which contribute to the opening of the lactone ring, such as in the case of andrographolide.[37] The lower obtained yield in second region may be the result of degradation of benzoic acid and gallic acid. In order to prevent the degradation of the components, higher temperature are not recommended for gallic acid.[27]

3.6 Effect of Raw Material Particle Size

Raw material particle size influences the extraction rate by the increase in the total mass transfer area per unit volume when the particle size is reduced.[36] The length of diffusion pathways will also decrease when the particle size decreases, resulting in the increase of mass transfer rate for the process.[38] In this study, two types of raw materials were considered, i.e., Tongkat Ali chips and powder form. The range of particle size for Tongkat Ali in powder form is 1 mm and for chip form, the length is 2-2.5 cm, 1 cm for the width and the thickness is 0.5 mm. Particle size for Tongkat Ali roots in powder form was measured by sieving while for Tongkat Ali roots in chip form was measured manually.

Figure 7 illustrates the effect of raw material particle sizes on the extraction yields of eurycomanone. The extraction yield of eurycomanone was better for smaller particles (powder) compared to larger ones (chips). This is consistent with mass transfer theory.[36] Operating at 100 °C in a 45 minutes extraction, an increase of about 15 % of the eurycomanone yield was obtained when powder was used instead of chips.

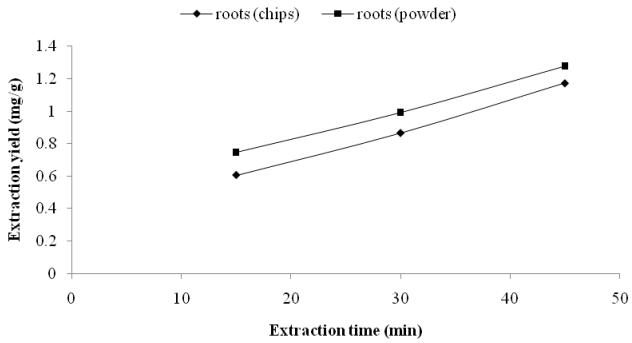


Figure 7 Effect of Tongkat Ali particle sizes on the extraction yields of eurycomanone at 100 °C, agitation level at 400 rpm and solvent to solid ratio is 20:1 using deionized water as extraction solvent

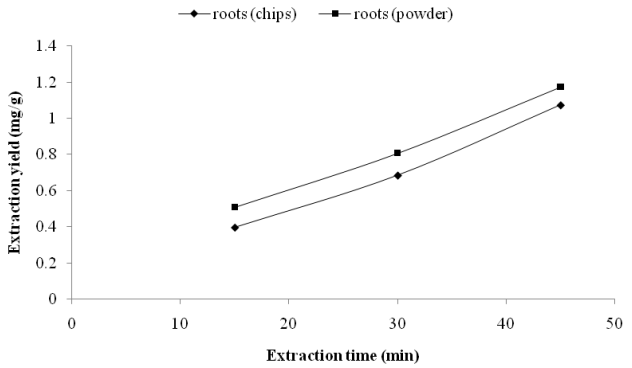


Figure 8 Effect of Tongkat Ali particle sizes on the extraction yields of benzoic acid at 50°C, agitation level at 400 rpm and solvent to solid ratio is 20:1 using water as extraction solvent

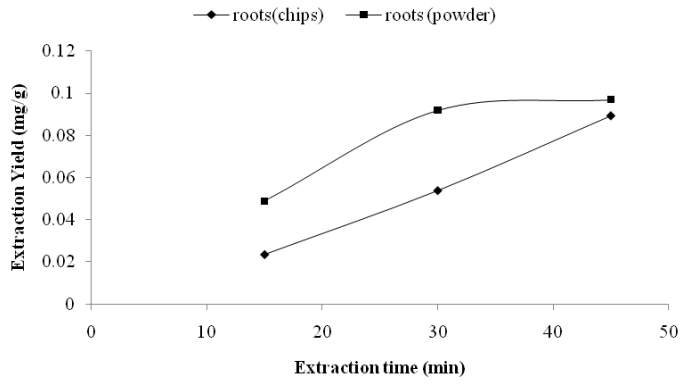


Figure 9 Effect of Tongkat Ali particle sizes on the extraction yields of gallic acid at 80°C, agitation level at 400 rpm and solvent to solid ratio is 20:1 using water as extraction solvent

Similar trend is observed for benzoic acid and gallic acid as illustrated by Figure 8 and Figure 9. For benzoic acid, the operating temperature was 50 ^{o}C, while for gallic acid, a 80 ^{o}C was chosen. These were the temperature which produced the highest yield for the individual components. For benzoic acid, the trends are similar to that of eurycomanone, but the case of gallic acid was somewhat different. In this case, large difference in yield was observed at shorter extraction, but as the extraction time is set at 45 minutes, the difference was small.

The results concluded that smaller particle sizes are better for extraction processes. This is attributed to the larger total surface area presented by smaller particles for extraction.[13] Furthermore, solvent penetration path length decreases when the particle size decreases as the specific surface area of the raw material increases thus influences solubility.[34,39]

■4.0 CONCLUSION

The effect of process parameters, i.e., extraction time, temperature, particle size, agitation speed and solvent to solid ratio, on a batch extraction of *Eurycoma longifolia* has been investigated. Among these, temperature was found to be most influential compared to other parameters, since not only that it has strong influence mass transfer, but also causes decomposition of the bioactive compounds, a phenomenon to be avoided. For eurycomanone, 100 ^{o}C was found to be the optimum temperature. For gallic acid and benzoic acid, the optimum values were 80 ^{o}C and 50 ^{o}C respectively. Consistent to mass transfer theory, smaller particle size was preferred. In this case, powder was found to be better than chips. The optimum agitation speed for the extraction which achieved highest yields was 400 rpm based on the range being studied and a 45 minute was found optimum for the extraction to be carried out. Testing of several solvent to solid ratio pointed preference to the 20:1 proportion.

The results obtained in this study were consistence with mass transfer theory and findings of previous workers which were mostly focused on eurycomanone. In this work, the two additional bioactive components, i.e. gallic acid and benzoic acid were also studied, which proven to be similar in terms of trends, but different in terms of physical limits owing to different level of sensitivity inherently influenced by its individual chemistry. The study has provided useful insights to further development in *Eurycoma longifolia* extraction process in making it more economically feasible and in particular, when the search of other bioactive components was to be continued.

Acknowledgement

This work is supported by the Malaysian Ministry of Science, Technology and Innovation (MOSTI) under National Science Fellowship (NSF).

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