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Effect of number of steps on the quality of *Eurycoma longifolia* extract and cost efficiency of the extraction process

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Abstract

The importance of number of extraction step on cost efficiency of extraction of *Eurycoma longifolia* is undeniable, in addition to main processing factors like temperature, duration, rotation speed, particle size of raw material, and solvent to raw material ratio. In lab-scale, the highest cost efficiency of the process was obtained in two-sequential extraction, i.e. 1.45 gram per RM. This double extraction also seemed appropriate since considerable total yield and amounts of active compounds could be obtained to some level. Multi-step extractions of beyond three steps are not advisable as the severe quality of the extracts. When increasing the number of extraction steps, the amounts of marker compounds such as eurycomanone and polysaccharides decreased, and simultaneously increased the unwanted constituents. Improving the extraction efficiency of *Eurycoma longifolia* particularly in industrial point of view has to be economical at appropriate processing condition.

Introduction

Eurycoma longifolia or Tongkat Ali is exclusively used for enhancing testosterone levels and acting as energy booster in men since ancient times especially in tropical countries such as Malaysia, Indonesia and Thailand. Its phytoandrogenic properties have been proven to improve sexual health and significantly have positive effects as diabetes and stress reducer (George and Henkel, 2014). Apart from the aphrodisiac effect, this native herb is also popular due to its ability to cure a variety of illnesses such as diarrhea, glandular swelling, bleeding, dropsy, persistent cough, hypertension, and relief of pain in the bones.

By scientific evidents, Eurycoma longifolia extracts also possessed cytotoxic, antiulcer, antitumor, anti-pyretic, antischistosomal, and antimalarial activities (Ismail *et al*., 1999; Jagananth & Ng, 2000). There are sixty-five compounds had been isolated from the roots of *Eurycoma longifolia* (Kuo *et al*., 2004). The major form of bioactive compounds in the extracts consists of alkaloids and quassinoids groups, which have been demonstrated to be responsible for its pharmacological activity and therapeutics effect (Kuo *et al*., 2004; Bhat & Karim, 2010). Quassinoid is the characteristic phytochemical of the *Simaroubaceae* family that imparts its bitter taste. The major components of quassinoids in *Eurycoma longifolia* include eurycomanone, 13α(21)-epoxyeurycomanone, eurycomanol, eurycomanol-2-O-β-D-glucopyranoside, and 13,21-dihydroeurycomanone (Teh *et al*., 2011).

Eurycomanone is known as the target marker for *Eurycoma longifolia* extracts due to its high yield and significant phytochemical bioactivities in standardized water extract (MS, 2011; Kumaresan

and Sarmidi, 2003). This active ingredient is well-recognized for its aphrodisiac effects, i.e. increase the testosterone levels and increase the production of sperm in animal models (Ang & Sim, 1998; Low *et al*., 2013). The human study found that it was able to increase the testosterone level up to 440% within a week, which useful to overcome the symptoms of late-onsate hypogonadism (or LOH) in a retrospective analysis (Tambi *et al*., 2012). According to Malaysian Standard (2011), in addition to eurycomanone, other active compounds that should be standardized for commercial purposes include the total protein, total polysaccharides and total saponins.

Due to the various traditional and scientific benefits, the demand for *Eurycoma longifolia* products increases each year, and turns into among the fastest growing products in herbal industry especially in Malaysia. The extract products are available as an additive in coffee or as a replacement for ginseng in health products, such as capsules, tablets, tea bags etc. In order to meet the increasing market demand of this herb, *Eurycoma longifolia* processing needs to be efficient, that is able to provide for the maximum yield and of the highest quality of high concentration of target compounds of the extracts. However, the current yield of *Eurycoma longifolia* extracts in manufacturing industry is still quite low, only about 3% (Athimulam *et al*., 2006). In this study, only the first main step in the recovery and purification of active compounds from plant materials, i.e. extraction process was emphasized.

Solid-liquid extraction (water as a solvent) or specifically water extraction technique is currently preferred for *Eurycoma longifolia* in commercial-scale production due to the simplest, low-cost and safest as compared to other available methods. Studies on the effect of processing parameters on *Eurycoma longifolia* water extraction have been done by a few groups of researchers (Kumaresan & Sarmidi, 2003; Low *et al*., 2005; Mohtar *et al*., 2007; Mohamad *et al*., 2014). In laboratory scale, the yield of *Eurycoma longifolia* extract obtained was in the region of 3 to 11% depending on the different parameters investigated during the extraction process (Wan-Zamri, 2014). Proven by researchers, the efficiency of the water extraction process can be improved by manipulating the temperature, duration, rotation speed, particle size of raw material, and solvent to raw material ratio in order to get the yield the highest possible.

However, in *Eurycoma longifolia* water extraction, the most common technique implemented in a batch boiled-condition is a single-extraction method (Mohamad *et al*., 2013; Sim *et al*., 2004; Kumaresan, 2008; Mohamad *et al*., 2010; Chua *et al*., 2011). There is scarce study had focused on the effect of number of extraction steps in the production of *Eurycoma longifolia* extract. Two-stage of extraction within 2 hours of operating condition each stage had been employed at pilot-scale production, but only described its economic performance using three debottlenecking schemes through simulation (Athimulam *et al*., 2006). Meanwhile, the best extraction condition for *Eurycoma longifolia* suggested by Khari *et al*. (2014) was at three cycles each for 4 hour but they used reflux method, not a batch solvent extraction.

Gonzalez-Montelongo *et al*. (2010) found that the number of extraction steps (three steps for optimum) had the greatest impact on the antioxidant activity for banana peel extract in solvent

extraction. By using methanol as a solvent, extracts rich in antioxidants were obtained from banana by-products. Meanwhile, Xu *et al*. (2008) suggested that 2 times extraction using water as solvent at 100°C for 30 min was proper to extract the considerable amount of minerals and phenolic compounds in citrus peel extract. Although the single extraction could increase mineral contents to some extent, but it couldn't yield higher content of phenolic compounds and stronger antioxidant capacity when to raise the temperature or prolong the time of extraction. Another study (using ethanol as solvent), proposed that the two-step sequential was desirable for extracting bioactive compounds from freeze-dried *Echinacea purpurea* flower (Chen *et al*., 2015).

Generally, it is suggested that a single extraction is not sufficient to remove all the active compounds as compared to multi-steps extractions. Therefore, the objective of this study was to explore further possibilities of varying the number of extraction steps on the yield of *Eurycoma longifolia* extract and the cost efficiency of the process. Effect of number of extraction steps was also emphasized on the quality of the *Eurycoma longifolia* extracts, or precisely the active ingredients, i.e. eurycomanone, total protein, total polysaccharides and total saponins.

Materials and Methods

Herbal Materials and Chemicals

Eurycoma longifolia roots were purchased from Grik, Perak, Malaysia. The plant age was approximately five years and the average growth performance of the cultivated plants was recorded as follows: 6 cm stem diameter at 15 cm above ground and plant height of 470 cm, which met the requirements for production of *Eurycoma longifolia* extract. After subjected to the pre-processing activities of cleaning, drying, grinding and sieving, *Eurycoma longifolia* roots were then stored in a dry environment in order to prevent fungal growth before use.

The aqueous solvent used for the extraction process was deionized water, which prepared using a Barnstead^{TM} water purification system. Eurycomanone standard, acetonitrile, ortho-phosphoric acid, Folin-Ciocalteau reagent, bovine serum albumin, sulfuric acid, glucose anhydrous analytical grade, aescin standard, vanillin etc used for the analytical methods were purchased from Sigma-Aldrich (USA), Merck (Germany), Fischer Scientific (USA) and May Chemical (Qrec, Malaysia).

Water Extraction of Eurycoma longifolia

The lab-scale extractor is a batch solid-liquid of 1-Liter vessel, which was build up as mimicked to the pilot-scale manufacturing extractor (Institute of Bioproduct Development or IBD, University of Technology Malaysia, Johor, Malaysia) to minimize the losses during extraction process. Made of stainless steel, the closed vessel was used to extract *Eurycoma longifolia* roots with heat sources from the hot plate (Thermolyne, USA). Jacketed by palm oil bath, the uniformly non-localized heating was ensuring achieved in this steady state condition (refer to Figure 1). The losses of the extraction system during experiments were approximately 0.01%, which closed to zero losses.

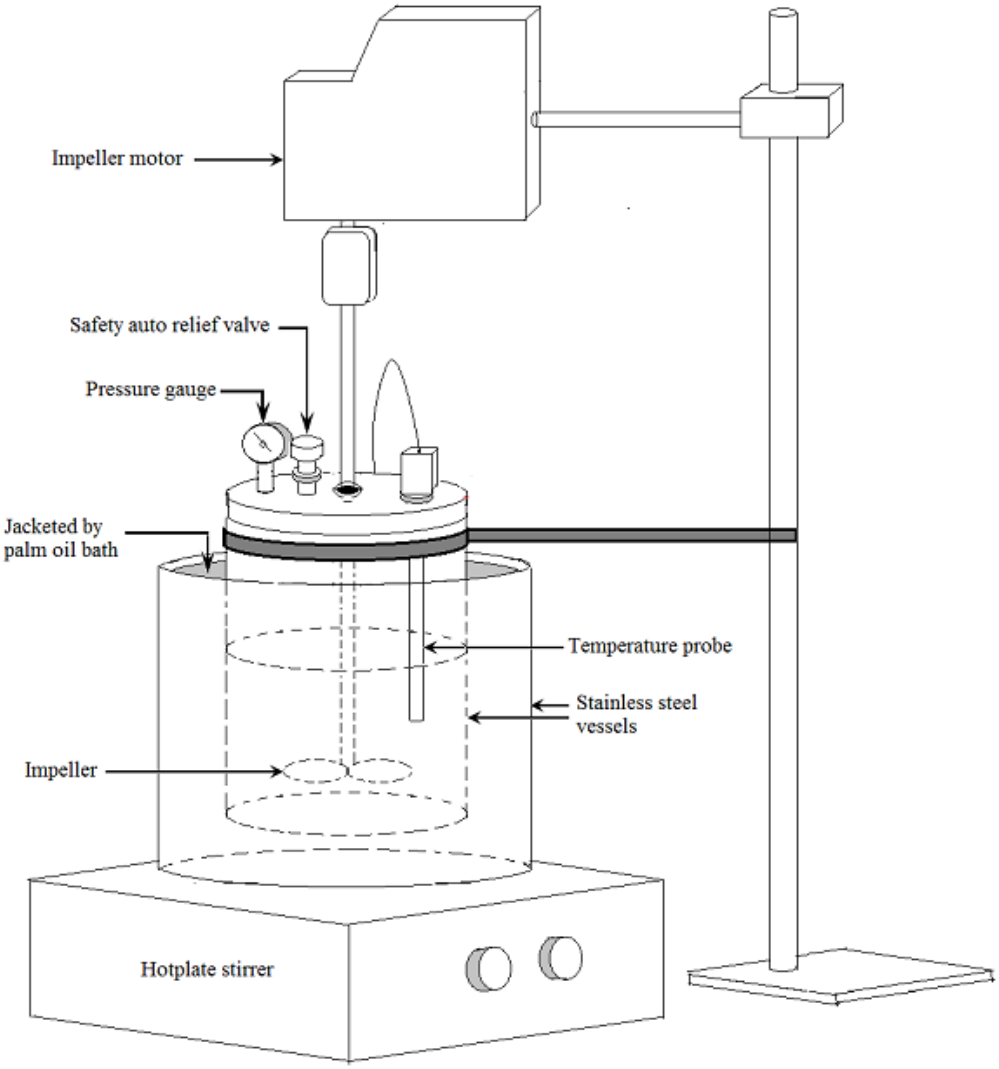

Figure 1. Schematic diagram for a lab-scale water extraction system

Eurycoma longifolia particles with sizes of 0.5 to 1.0 mm were extracted using water at 500 mL working volume in a designed lab-scale extractor. Extraction process was carried out in boiled-condition with pressure up to 2.6 bar, temperature of 129 ± 2^0C, solvent to solid ratio of 10:1 (w/w), agitation speed of 400 rpm, and duration of 1 hour. These operating conditions were based on our recent works with some modifications related to current practice at pilot-scale production (Wan-Zamri, 2014). The extract solutions were then filtered through filter paper using a vacuum filtration system with a Buchner funnel and kept in tight container under refrigeration.

For second-step extraction, the *Eurycoma longifolia* residue from a single extraction was re-extracted with fresh water at the same operating conditions. Then, the residues were subsequently re-extracted for the next steps, i.e. designated as third, fourth and fifth-step extractions. The total volume of each step was fixed at 500 mL. Then, the extracts solutions were mixed together (at that step) for determination of the final volume of the multi-extractions. All experiments were done in triplicate.

Measurement of Extraction Yield and Amount of Active Compounds

Extraction yield (weight percentage) is the most essential criterion to be evaluated in extraction process and become a diagnostic tool for commercial purposes in herbal industry. They represent the total yield or the yield of a certain target compound or compounds. Total yield of extract was measured based on total solid content in the final extract solution. Total solid content is a measure of the amount of material remaining after water has completely evaporated. According to AOAC (1990), determination of total solids was evaluated in terms of the weight percentage of the amount of the water loss on drying that is referred to moisture content of the sample by using equation (1). To measure the water loss on drying, 5 ml of *Eurycoma longifolia* extract solution was placed in a dish and kept in oven at 100^0C until the weight became constant. Then, percentage total yield of the extracts can be generally calculated as in equation (2).

$$Total\ solids(\%) = 100 - Moisture\ (\%) \quad (1)$$

$$Yield\ (\%) = \frac{Mass\ of\ extract\ (g)}{Mass\ of\ raw\ Eurycoma\ longifolia\ (g)} \times 100 \quad (2)$$

Bioactive compounds or known as phytochemicals found in *Eurycoma longifolia* extract ranged from simple to complex structures. The production of an array of phytochemicals from metabolism process can be either primary or secondary. There is a specification prescribed for freeze-dried water extract *Eurycoma longifolia* roots (MS, 2011). Owing to various factors that affect the final profile of the extracts, it is crucial to maintain the *Eurycoma longifolia* extract efficacy level through this standardization. The marker compounds that should be standardized consist of eurycomanone (0.8 to 1.5 %), total polysaccharides (above 30 %), total protein (above 20 %) and total saponins (above 40 %). Thus, in this study, the amount of each marker compounds found in *Eurycoma longifolia* extract was calculated as expressed in equation (3).

$$Amount\ (\%) = \frac{Concentration \times Volume\ extract}{Mass\ of\ total\ solids\ content} \times 100 \quad (3)$$

Measurement of Cost Efficiency

In commercialization, a goal of maximizing the cost efficiency for production of *Eurycoma longifolia* extract is highly desirable. In other words, the highest yield of extract produced with the least amount of financial investment during the production is a target in herbal industry. The calculation for the cost efficiency is expressed in equation (4). Production cost is the amount of added material cost and utility cost of the extraction process in Malaysian Ringgit (RM).

$$Cost\ efficiency = \frac{Total\ solids\ content\ etxract\ (g)}{Production\ cost\ (RM)} \quad (4)$$

Determination of Eurycomanone by HPLC

Eurycomanone in *Eurycoma longifolia* extract was measured by high performance liquid chromatography analysis, which was performed using Waters Alliance 2690 HPLC system with 2487 DAD detector (Milford, MA). Separation was achieved using AscentrisTM RP-Amide 5 µm column

with the dimension of 250 x 4.6 mm. The column was isocratically eluted with water-acetonitrile-ortho-phosphoric acid. The mobile phase was 90 % of 0.05 % phosphoric acid and 10 % of acetonitrile at a flow rate of 1 ml/min with the injection volume of 20 µl. The low mobile phase flow rate was chosen to allow the peaks to separate more distinctly. Eurycomanone was monitored at 254 nm and its concentration was calculated by referring to an external standard curve (MS, 2011).

Determination of Total Protein

Total protein was estimated by Lowry's method, according to Malaysian Standard (2011). It is basically the development of blue colour due to the reduction of the phosphomolybdic components in the Folin-Ciocalteau reagent by the presence of amino acids, tyrosine and tryptophan in the protein. Besides, the colour developed by the Biuret reaction of the protein with the alkaline cupric tartarate was also measured. For protein stock solution of 1000 ppm, it was prepared by weighing 5 mg bovine serum albumin and dissolved in 5 ml distilled water using a volumetric flask. All samples were measured using UV-Vis spectrophotometer at 750 nm of absorbance with bovine serum albumin as a standard.

Determination of Total Polysaccharide

Total polysaccharides such as starch and any storage form of carbohydrate are primary metabolite compounds that were found in *Eurycoma longifolia* extracts. It can be determined using a phenol-sulphuric acid colorimetric assay as proposed by Chen *et al*. (2011). Glucose was used as a standard solution and all samples were measured using a UV-Vis spectrophotometer at 492 nm wavelength.

Determination of Total Saponins

Based on the reaction of saponins with vanillin-sulfuric acid reagent, the total saponins in *Eurycoma longifolia* extract was estimated using the protocol established by Hiai *et al*. (1976) and further modified by Chen *et al*. (2011). The ready samples were measured at wavelength 560 nm using a UV-Vis spectrophotometer with Aescin used as a standard.

Result and Discussion

Effect of Number of Extraction Steps on Total Yield

Under the same conditions in multistep-extractions, the total yield of extract of *Eurycoma longifolia* was determined and compared by its sequential extractions in particular steps, which up to 5 steps. The total yield of each number of extraction steps obtained from 45.45 gram of *Eurycoma longifolia* was demonstrated in Figure 2. The total yield of extracts dramatically increased with increasing the number of extraction steps, namely from 6.83% to 18.83%. The high temperature used in this study made possible by the application of pressure (due to boiling-conditioned), resulted in enhancement in mass transfer properties of the solvent, hence improving extraction efficiency.

In a single batch extraction, the dissolved solids or the solutes in solvent obeyed the first order curve regime or Fick's second law of diffusion that developed for solid-liquid mass transfer (Gertenbach, 2002). The extract of the solute concentrations increased until reached an equilibrium state, after which the extraction of the solutes remain relatively constant at particular operating condition.

However, by prolonged further sequential extractions, the more phytochemical compounds could be released by next fresh water (or solvent) from the plant cell compartments. This is due to adhesive forces or known as underflow, which at the end of each extraction step still a certain amount of solution (consisting of solvent and extracted solutes) was retained in the solid matrix. Gertenbach (2002) described that the plants have the characteristics of very porous, containing a network of passageways and internal pores (or permeable cell walls). Thus, the diffusion process (when solutes were first dissolved) resulted in the formation of pores in the solid matrix which then exposed the fresh (or new) surfaces to subsequent solvent penetration to such surfaces.

Effect of Number of Extraction Steps on Cost Efficiency

Currently, in local market, the *Eurycoma longifolia* water extracts can be sold for up to RM 96 per bottle of 60 capsules (40 mg extract per capsule), that is comparable to RM 2,000 to RM 3,000 per kg extract (Kaur *et al*., 2003). With the current low yield of *Eurycoma longifolia* extract (about 3%), an increase of production of even 0.5 % (in weight) could raise the profits up to 20 % (Mohamad *et al*., 2010). The estimated production cost (summation of utilities, material and manpower costs) for a single extraction process of the pilot-scale at IBD, University of Technology Malaysia (data not shown) was obtained at RM 948.55 for *Eurycoma longifolia* extract of 1253.39 gram. This was equivalent to the cost efficiency of 1.32 gram per RM, which closed to the value obtained in the lab-scale (i.e. 1.34 gram per RM) of a single extraction process (refer Figure 2).

As shown in Figure 2, when the number of extraction steps was two, the cost efficiency of the extract of *Eurycoma longifolia* was the greatest, i.e. 1.45 gram per RM. Even though the total yield of extracts at fifth step of extraction (i.e. 18.83 %) was the highest, but it is not a profitable technique for *Eurycoma longifolia* extraction as the cost efficiency became the lowest, i.e. only 1.28 gram per RM with duration of extraction of 5 hours. It could be seen that beyond the double extraction of *Eurycoma longifolia*, the increased number of extraction steps is time consuming and uneconomical especially in industrial point of view.

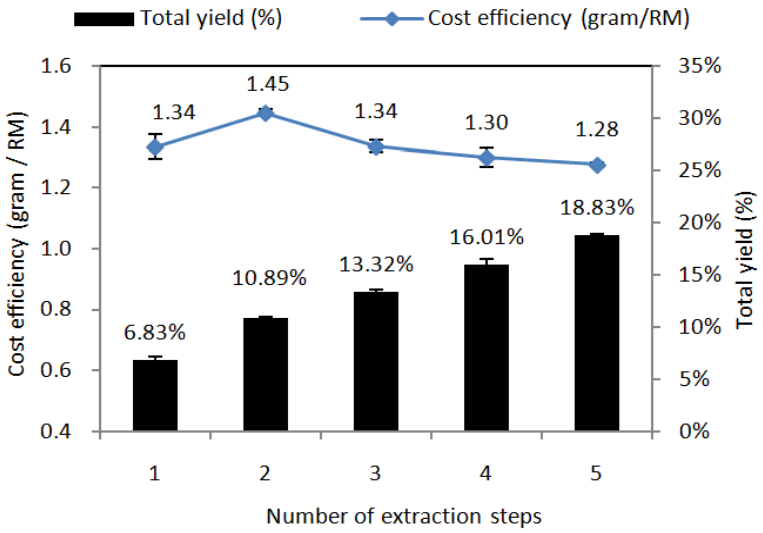

Figure 2: Total yield of extract and cost efficiency for sequential of number of extraction steps

Effect of Number of Extraction Steps on Amount of Eurycomanone

Eurycomanone, the secondary metabolite with a molecular weight of $C_{20}H_{24}O_9$, was proven to be responsible for the aphrodisiac effects in *Eurycoma longifolia* extract (Low *et al*., 2013; Tambi *et al*., 2012). It was also found to exhibit strong antimalarial activity against the resistant *Plasmodium falciparum* (Kuo *et al*., 2004; Yusuf *et al*., 2013). In multi-step extractions, the amount of eurycomanone was significantly decreased with increasing number of extraction steps, namely 2.20%, 1.75 %, 1.45 %, 1.21 % and 1.03 % for first, second, third, fourth and fifth steps respectively, as demonstrated in Figure 3. Nevertheless, those amounts were still in the range which established for eurycomanone specification in Malaysian Standard (2011).

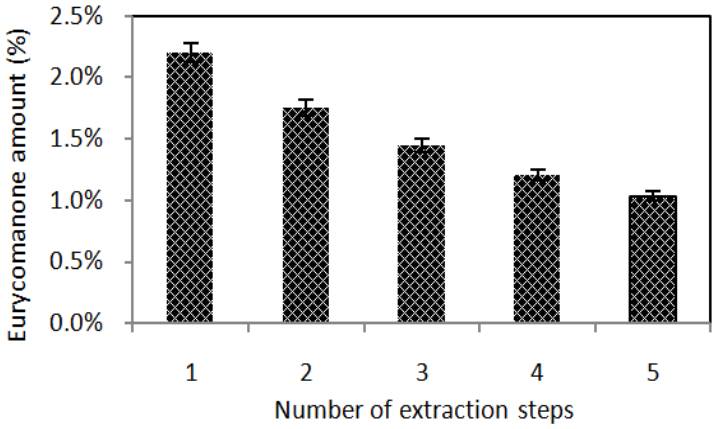

Figure 3: Amount of eurycomanone for multi-steps extractions

In compliance with the first order curve regime, the solid-liquid extraction is a rate limiting step, which internal diffusion of compound itself (i.e. eurycomanone) controlled the process. This phenomenon could be explained by observing the mass of eurycomanone extract in separate extraction steps as indicated in Table 1. It was found that the mass of eurycomanone extract severely

decreased from 68.3 to 0.3 mg from first to fourth steps of extraction. Then, the mass of eurycomanone extract became constant on the next step of extraction. Prolonged the number of extraction steps beyond the first increases the chance of decomposition of eurycomanone due to their long exposure to unfavorable environments like temperature and pressure factors.

Table 1: Total solids and active compounds of *Eurycoma longifolia* extracts in multi-steps extraction

Quality characteristics	Number of extraction steps				
	1st	2nd	3rd	4th	5th
Total solids content (g)	3.1050	1.8455	1.1034	1.2224	1.2833
Eurycomanone (mg)	68.3	18.4	1.0	0.3	0.3
Total polysaccharides (g)	1.2917	0.4776	0.2128	0.1799	0.2543
Total protein (g)	0.6671	0.6085	0.4590	0.4775	0.5225
Total saponins (g)	1.0769	0.7400	0.4206	0.4647	0.4963

Effect of Number of Extraction Steps on Amount of Other Active Compounds

In addition to eurycomanone, other secondary metabolite that should be observed in *Eurycoma longifolia* extraction was total saponins. It has received much attention recently due to their health benefits such as cholesterol lowering and anticancer properties (Gurfinkel and Rao, 2003; Kim *et al*., 2003). Saponins are complex compounds characterized by their structure containing steroidal or triterpernoid aglycone (sapogenin), which linked to one or more oligosaccharide moieties by glycosidic linkage. Saponins are strong surface-active properties due to its amphiphilic nature, i.e. the presence of a lipid-soluble aglycone and water-soluble sugar chain(s) in their structure (Makkar *et al*., 2007).

From Figure 4, amount of total saponins obtained in this study did not significantly different from the first to fifth steps of extraction, that is, ranged from 35 to 37 %. When the extraction steps were compared separately, the mass of total saponins extract decreased from first to third steps, i.e. from 1.0769 to 0.4206 gram. Further extract by sequential extractions (at fourth and fifth) was no longer significant. In multiple-steps of extraction, the degradation may due to the glycosidic bond and the interglycosidic bonds between the sugar residues in saponins, which undergo hydrolyzed by the prolong heat in the presence of water or known as hydrothermolysis (Guclu-Ustundag and Mazza, 2007).

Primary metabolites that important to be observed in *Eurycoma longifolia* extracts include total polysaccharides and total proteins. Primary metabolites are essential for plant life, growth and development, which comprised of substances widely distributed in nature. Most of the dry weight of plants is carbohydrate of one kind or another, which most of them occur in the form of polysaccharides. Total polysaccharides observed in this study include polar substances composed of glycosidically-linked sugar residues as well as molecules that contain polymeric saccharide structures linked via covalent bonds to acid amino, peptides, protein, lipids and other structures. Thus, proteins

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are also derived partly from carbohydrates through the formation of amino acids. As a result, total proteins that desired in this study may occur throughout the plant cells, both in extrinsic and intrinsic, simple and conjugated forms.

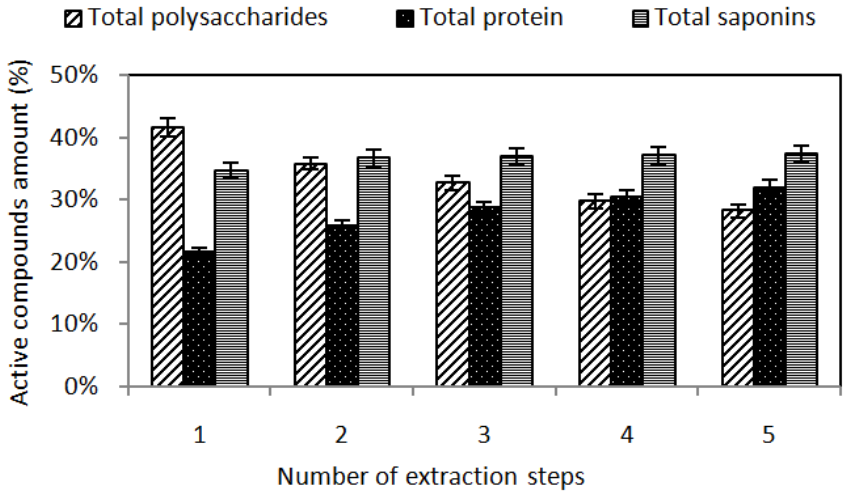

Figure 4: Amount of active ingredients for multi-steps extractions

As also shown in Figure 4, apparently, the amount of total polysaccharides extract at first extraction step was the highest followed by the second, third, fourth and fifth steps in decreasing order, i.e. from 41.6% to 35.7, 32.7, 29.7 and 28.2% respectively. On the contrary, the protein contents demonstrated in a little increasing order, namely from 21.5, 25.8, 28.65, 30.4 and 32.0% respectively for the same sequential extractions. It could be due to the contents of total polysaccharides, protein and saponins were very closely-related when carried out the multi-steps extraction, especially beyond the three steps.

When compared separately, the mass of those compounds have slightly increased in fourth and fifth steps of extractions. Saponins might bind to rich protein and interfere with protein synthesis to form insoluble aggregates, hence increased protein aggregations (Guclu-Ustundag & Mazza, 2007). Under stress condition such as prolong heat, the newly synthesized proteins may not fold correctly, or the proper folded proteins can spontaneously misfold, and consequently contribute to subsequent aggregations. Denatured proteins can exhibit a broad range of characteristics, as of conformational change and loss of solubility to aggregation due to the exposure of hydrophobic portions of the protein, which may toxic if consumed.

Conclusions

To conclude, the extraction process of *Eurycoma longifolia* considerably necessary by increasing to double extraction steps in terms of total yield and cost efficiency. By doing so, adequate amount of active compounds could also be extracted. However, from the results, the greatest quality of the extracts which referring to the highest amounts of standardized active ingredients was only observed in a single or first step of extraction. Although high number of extraction steps can improve the total

yield of extract, the raise of degradation rate of important active compounds such as eurycomanone and total polysaccharides cannot be ignored. As to avoid greater losses of the important compounds in the *Eurycoma longifolia* extracts, the sequential extraction shouldn't beyond the three steps.

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