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Saponins Rich Fractions From *Eurycoma longifolia* Extract

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ABSTRACT
Eurycoma longifolia which is a renowned folk medicinal herb in South East Asia is rich in saponins. This study investigated the different extraction and fractionation techniques of saponins from the roots of the plant. Organic solvents such as acetone and ethyl acetate were used to precipitate and partition saponins from the crude extract, respectively. The organic fractions were then analysed by colorimetric vanillin-sulphuric acid assay for its total saponin content. Solvent fractionation using ethyl acetate was found to produce higher total saponin content than acetone precipitation. Ethyl acetate fraction (676 ± 30 mg AE/g) was showed to have slightly higher saponins than its aqueous counterpart (511.389 ± 82.880 mg AE/g). The technique of acetone precipitation was unable to fully recover saponins as precipitate (95.468 ± 91.621 mg AE/g). The result showed that majority of saponins (881.581 ± 120.676 mg AE/g) still stayed in the aqueous counterpart of acetone precipitation. To conclude, both techniques of acetone precipitation and solvent fractionation had increased the saponin content from the crude extract (99.2 ± 32.1 mg AE/g). The increment was more significant for the technique of solvent fractionation. This study was successfully compared the different fractionation techniques and ethyl acetate could be used to concentrate saponins from the highly complex crude extract of *E. longifolia* roots.

Keywords: *Eurycoma longifolia, saponins, fractionation, precipitation, total saponins*

1. INTRODUCTION

Eurycoma longifolia belongs to the family Simaroubaceae which comprises about 30 genera and more than 200 species. The plant is widely distributed in the primary and secondary, evergreen and mixed deciduous forests of South-East Asian countries like Indonesia, Vietnam, Thailand, Myanmar, Malaysia and the Philippines [1-3]. It is locally recognized as Tongkat Ali Hitam, Tongkat Baginda, Payung Ali, Bedara Pahit, Penawar Pahit, Setunjang Bumi, Pokok Syurga, Pokok Jelas and Jelaih in Malaysia [4], 'Pasakbumi' in Indonesia, 'Ian-don' in Thailand and 'Cay ba binh' in Vietnam. The word 'Tongkat Ali' for this plant literally means "Ali's walking stick" which refers to its long-twisted roots [5]. There are four different species of Tongkat Ali, namely *Polyathia bullata*, *Eurycoma apiculata*, *Eurycoma longifolia* and *Goniothalamus* sp. Among them, *Eurycoma longifolia* is the most frequently used species for the production of root extract in product development [6-7].

Traditionally, the decoction of the plant roots is consumed to treat various ailments such as sexual insufficiency, glandular swelling, dysentery, aches, cough and persistent fever [6]. Scientifically, it has been proven to be male aphrodisiac [8-11], anti-cancer [12-13], and antimalarial [14-15] agents. The pharmacological activities were attributed to the presence of numerous phytochemical groups, mainly quassinoids, β-carboline alkaloids, canthin-6-one alkaloids, tirucallane-type triterpenes, squalene derivatives, and biphenylneolignans [16]. According to the Malaysian Standard MS:2409 [17], glycosaponin is the most abundant phytochemical group in the roots of the plant which could make up more than 40% w/v of the freeze-dried water extract.

The rising of herbal-based phytochemical industry has necessitated this industrial sector to produce high quality medicinal and food supplements [18]. Products that formulated from *E. longifolia* extracts are in high demand in the herbal market. The herbal extract is extensively used as an additive in coffee and as a substitute of ginseng in health products in the forms of tablets, capsules, and tea bags [19-20]. Nowadays, entrepreneurs prefer herbal extract that rich in bioactive compound over the crude extract of plant. This is due the presence of numerous phytochemicals with diverse chemical properties which make the crude extract very complex. Studies have proven fractions enriched with bioactive compounds exhibiting greater bioactivity than its crude extract. The enrichment could be performed by fractionating or partitioning the crude extract into smaller fractions or nearly pure compound [21]. However, there are only few studies on the fractionation of *E. longifolia* crude extract. Hence, this study was aimed to produce the saponins rich extract from the roots of *E. longifolia*.

Glycosaponin is a high molecular weight and structurally complex secondary metabolite that naturally exists as glycosides of triterpenes, steroids, and sometimes alkaloids, but triterpenes are being the prominent group of saponins [22].

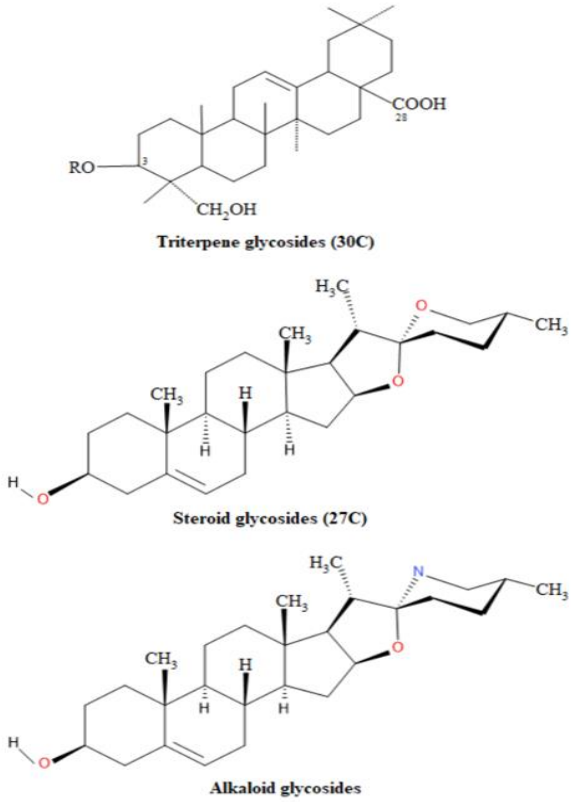

Figure 1 Classifications of sapogenin or aglycone

Glycosaponin or saponin is made up of a polycyclic aglycone coupled to a sugar side chain. The non-sugar component is known as aglycone or sapogenins which is derived from isoprene skeleton that covalently joined to one or more sugar moieties. Depending on the nature of the aglycone, glycosaponins can be classified as triterpenoidal, steroidal, or alkaloidal saponins as shown in Figure 1 [23]. Both triterpenoid and steroid saponins are derived from six isoprene units where the triterpenes have 30 carbon atoms and the steroids have 27 carbon atoms due to the oxidative cleavage of three methyl groups from a C30 intermediate [23-24]. The carbohydrate moiety composes of pentoses, hexoses, or uronic acids. They are also known as monodesmosidic, bidesmosidic and tridesmosidic saponins based on the number of attached sugar moieties. These sugar moieties are oligosaccharide side chains that comprise of 2-11 linear or branched monosaccharide units that usually attached at C3 of saponin through ether or ester linkages [25]. In addition to sugars, there are added substituents such as small aromatic and aliphatic acids, monoterpenoidal derived compounds and acyl groups occasionally attached to sapogenins [26]. The structural diversity of saponins contributes to a vast number of physicochemical and biological activities in *E. longifolia* such as hypocholesterolaemic effect [27-28], anti-cancer [29-30], antiparasitic [31], antibacterial [32-33], antioxidant and antiglycation [34], and adjuvant [35] activities. Apart from that, glycosaponin also exhibits toxicity towards red blood cells [32,35]. Hence, glycosaponins can be exploited for its various applications in food, cosmetics and pharmaceutical sectors. However, glycosaponin usually exists as a multi-component mixture of compounds with close similarity in polarities [36]. This often challenges the extraction and separation process. Therefore, this study attempts to investigate the efficiency of saponin extraction by solvent partition or liquid-liquid extraction and gravimetric assay, as well as to compare the methods for total saponins content.

2. EXPERIMENTAL

2.1. Materials

The spray dried powder of *E. longifolia* crude extract was purchased from the local market (nu-prep, Biotropics Malaysia). HPLC grade of methanol (MeOH) was purchased from Merck (Darmstadt, Germany). Analytical grade of ethanol, ethyl acetate, acetone, and methanol were purchased from QReC (Quality Reagent Chemical, NZ). Ultrapure water (18.2 MΩ-cm) was acquired from arium® pro VF Ultrapure Water System (Sartorius, Goettingen, Germany). Vanillin and silica powder were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Method of Saponin Extraction

Fractionation of crude extract was conducted through liquid-liquid extraction (solvent partition) and gravimetric assay (acetone precipitation). Colorimetric assay was used for the quantification of total saponin content from all fractions obtained from both fractionation methods.

2.2.1. Liquid-liquid Extraction

Saponins were partitioned into organic phase from the aqueous phase using liquid-liquid extraction (LLE). LLE was conducted using two immiscible solvents comprised of water and ethyl acetate. A 5.0 g dried extract of *E. longifolia* was dissolved in 60 mL hot water and thoroughly extracted with 20 mL ethyl acetate in a 500 mL separatory funnel. The organic layer was withdrawn from the separating funnel and replaced with another 20 mL fresh ethyl acetate after extraction again. The procedure of extraction was repeated for six times. The collected organic layers were combined and concentrated by a rotary evaporator, and then dried in an oven at 65 ℃ until dryness. The remaining aqueous solution was also dried and recorded for its weight.

2.2.2. Acetone precipitation

The saponins of *E. longifolia* was also precipitated by gravimetric method [17]. One gram of crude extract was

weighed which is then dissolved in 50 mL methanol. The sample was refluxed at 50 ºC for 30 minutes and then filtered. The filtrate was collected. The residue was re-refluxed using 50 mL fresh methanol. The collected methanolic extract was combined and concentrated to 10 mL under vacuum pressure using a rotary evaporator and the concentrate was slowly dropped into 50 mL acetone in a pre-weighed beaker for saponin precipitation. The precipitate of saponin was dried in an oven at 60 ºC until constant weight. The precipitate was analysed for total saponin content.

2.2.2. Colorimetric Assay

This assay uses the principle of reaction by oxidizing triterpene saponins with vanillin. Sulphuric acid being a strong acid is utilized as an oxidant to break apart aglycone from the complex molecule of saponin [37]. The total saponin in *E. longifolia* samples was estimated according to the method described by Chen et al. [34] based on the reaction of saponins with sulfuric acid-vanillin reagent. A 0.5 mL sample was added into a Falcon tube which contained 0.5 mL vanillin (8 % w/v) and 4.0 mL sulphuric acid (72 % w/v). The mixture of the solution was incubated in a water bath at 60 ˚C for 15 min and cooled in an ice bath for 10 min. The absorbance of sample was read at 560 nm where aescin was used as a reference standard. The total saponin content was estimated from the equation of calibration curve of aescin as shown in Figure 2. The results are expressed as milligram aescin equivalent per gram sample (mg AE/g) as in Equation (1).

$$\text{Total saponin (mg AE/g)} = \frac{(\text{Absorbance of sample} - 0.2315)}{\text{Slope of calibration curve x weight of sample}} \quad (1)$$

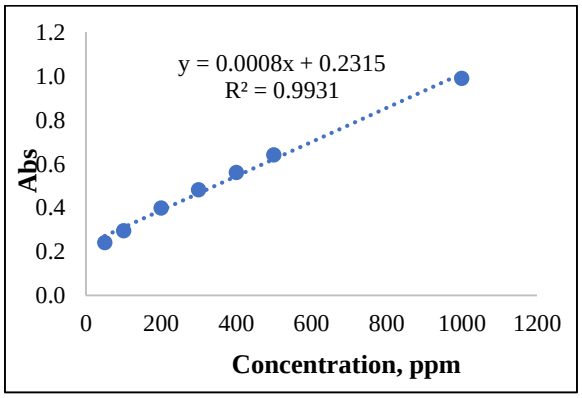

Figure 2 Calibration curve of total saponin content using aescin as the standard chemical

3. RESULTS AND DISCUSSION

Table 1 shows the yield of saponins which is increased in ascending order of precipitate, crude extract, aqueous phase, organic phase (ethyl acetate fraction) and acetone. The highest total saponin, 881.581 ± 120.676 mg AE/g was still remained in the acetone solution of gravimetric assay. The precipitate of *E. longifolia* roots recovered the lowest total saponins (95.468 ± 91.621 mg AE/g). Nevertheless, both fractionation techniques could increase the content of saponins in the fractions from crude extract.

Table 1 Total saponin yield from different samples calculated in aescin equivalent

Sample		Saponins Yield, mg AE/g
Crude Extract		99.20 ± 32.10
Liquid-liquid extraction	Organic Phase	676.00 ± 30.00
	Aqueous Phase	511.39 ± 82.88
Acetone precipitation	Acetone	881.58 ± 120.68
	Precipitate	95.47 ± 91.62

The table also clearly shows that solvent partition could transfer higher saponin content to the ethyl acetate fraction (676.00 ± 30.00 mg AE/g) with some saponins were still remained in the aqueous fraction (511.39 ± 82.88 mg AE/g). The total saponins extracted by ethyl acetate was about 24.35% higher than the aqueous counterpart. The highly polar substances such as proteins, polysaccharides, and organic acid might be remained in the aqueous phase whereas compounds with relatively low polarity such as terpenoids and their derivatives including saponins will be partitioned into the organic phase [21]. Hence, the total saponin became higher after solvent fractionation. This finding was found to be similar to the finding of Chua et al. [21] where ethyl acetate used was produce the highest total saponin content.

From the gravimetric assay, there was still many saponins, 881.58 ± 120.68 mg AE/g remained in acetone after precipitation. The result was significantly higher than its precipitate (95.468 ± 91.621 mg AE/g) formed from the crude extract of *E. longifolia*. Compounds or saponins with limited solubility in acetone would precipitate in acetone. This indicates that most of the saponins of *E. longifolia* roots were soluble in acetone together with other polar phytochemicals. Acetone precipitation was found to have limitation to fully recover saponins as precipitate in this study. Therefore, the total saponins produced by gravimetric assay was poorer than total saponins obtained from the technique of solvent partition using ethyl acetate.

4. CONCLUSION

The fractionation of crude extract was successfully produced high total saponin content in the plant fraction. The performance of solvent partition in LLE was better than gravimetric method to recover saponins from the crude extract of the plant roots. Possibly, saponins in the plant roots have higher affinity towards ethyl acetate or close polarity with the organic solvent.

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