Saponins Rich Fractions From *Eurycoma longifolia* Extract

Abirame Segaran¹,², Lee Suan Chua¹,²*¹

¹Institute of Bioproduct Development, Universiti Teknologi Malaysia, 81310 UTM Johor Bahru, Johor, Malaysia.
²Department of Bioprocess and Polymer Engineering, School of Chemical and Energy Engineering, Faculty of Engineering, Universiti Teknologi Malaysia, 81310 UTM Johor Bahru, Johor, Malaysia.

*Corresponding author: lschua@ibd.utm.my

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, ‘fan-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 *Polypatia 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-caroline alkaloids, canthin-6-one alkaloids, tirucallane-type triterpenes, squalene derivatives, and biphenyleolinolignans [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*. 

Copyright © 2020 The Authors. Published by Atlantis Press B.V.
This is an open access article distributed under the CC BY-NC 4.0 license -http://creativecommons.org/licenses/by-nc/4.0/.
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 sapogenin 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, bidessmosidic and tridesmosidic sapogenins 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 °C 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 °C 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 °C 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 sulphuric 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).

Total saponin (mg AE/g) =
\[ \frac{(\text{Absorbance of sample} - 0.2315) \times \text{weight of sample}}{\text{Slope of calibration curve}} \]  

(1)

![Figure 2](Image)

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, $881.581 \pm 120.676$ mg AE/g was still remained in the acetone solution of gravimetric assay. The precipitate of E. longifolia roots recovered the lowest total saponins ($95.468 \pm 91.601$ 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 mg AE/g | Yield, % |
|----------------------|------------------|---------|
| Crude Extract        | 99.00 ± 32.10    |         |
| Organic Phase        | 676.00 ± 30.00   |         |
| Aqueous Phase        | 511.39 ± 82.88   |         |
| Acetone Precipitate  | 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 ($676.00 \pm 30.00$ mg AE/g) with some saponins were still remained in the aqueous fraction ($511.39 \pm 82.88$ 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, $881.58 \pm 120.68$ mg AE/g remained in acetone after precipitation. The result was significantly higher than its precipitate ($95.468 \pm 91.601$ 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.
ACKNOWLEDGMENT

The authors would like to acknowledge the financial support from Ministry of Higher Education (Malaysia) for giving research fund (HICoE:4J263) to conduct the study.

REFERENCES

[1] H.H. Ang, H.S. Cheang, Studies on the anxiolytic activity of Eurycoma longifolia Jack roots in mice, The Japanese Journal of Pharmacology, 79(4) (1999) 497-500. DOI: https://doi.org/10.1254/jjp.79.497

[2] I.A. Bhat, P.K. Rathor, I.N. Mir, P. Gireesh-Babu, M. Goswami, J. Sundaray, R. Sharma, Toxicological evaluation and effective dose selection of eurycomanone, a quassinoid of Eurycoma longifolia plant in fishes, Aquaculture. 481 (2017) 94-102. DOI: https://doi.org/10.1016/j.aquaculture.2017.08.030

[3] Z. Khanam, C.S. Wen, I.U.H. Bhat, Phytochemical screening and antimicrobial activity of root and stem extracts of wild Eurycoma longifolia Jack (Tongkat Ali), Journal of King Saud University - Science. 27(1) (2015) 23-30. DOI: https://doi.org/10.1016/j.jsus.2014.04.006

[4] A. Athimuthu, S. Kumaresan, D.C.Y. Foo, M.R. Sarmidi, R.A. Aziz, Modelling and Optimisation of Eurycoma longifolia Water Extract Production, Food and Bioprocess Technology. 84(2) (2006) 139-149. DOI: https://doi.org/10.1205/fbp.06004

[5] R. Bhat, A.A. Karim, Tongkat Ali (Eurycoma longifolia Jack): A Review on its Ethnobotany and Pharmacological Importance, Fitoterapia. 81(7) (2010) 669-679. DOI: https://doi.org/10.1016/j.fitote.2010.04.006

[6] B.M. Abubakar, F. Salleh, A. Wagiran, Chemical composition of Eurycoma longifolia (Tongkat Ali) and the quality control of its herbal medicinal products, Journal of Applied Sciences. 17(7) (2017) 324-338. DOI: 10.3923/jas.2017.324.338

[7] A. Norhidayah, J. Vejayan, M.M. Yusoff, Detection and quantification of eurycomanone levels in Tongkat Ali herbal products, Journal of Applied Sciences. 15(7) (2015) 999-1005. DOI: 10.3923/jas.2015.999.1005

[8] S.B. Ismail, W.M.Z. Wan Mohammad, A. George, N.H. Nik Hussain, Z.M. Musthapa Kamal, E. Liske, Randomized clinical trial on the use of PHYSTA freeze-dried water extract of Eurycoma longifolia for the improvement of quality of life and sexual well-being in men, Evidence-Based Complementary and Alternative Medicine. 2012 (2012) 1-10. DOI: https://doi.org/10.1155/2012/429268

[9] S.M. Talbott, J.A. Talbott, A. George, M. Pugh, Effect of Tongkat Ali on stress hormones and psychological mood state in moderately stressed subjects, Journal of the International Society of Sports Nutrition. 10(1) (2013) 1-7. DOI: https://doi.org/10.1186/1550-2783-10-28

[10] M. Tambi, M. Imran, R. Henkel, Standardised water-soluble extract of Eurycoma longifolia, Tongkat ali as testosterone booster for managing men with late-onset hypogonadism?, Andrologia. 44 (2012) 226-230. DOI: https://doi.org/10.1111/j.1439-0272.2011.01168.x

[11] J.K. Udani, A.A. George, M. Musthapa, M.N. Pakdaman, A. Abas, Effects of a proprietary freeze-dried water extract of Eurycoma longifolia (Physta) and Polygonum minus on sexual performance and well-being in men: a randomized, double-blind, placebo-controlled study, Evidence-Based Complementary and Alternative Medicine. 2014 (2014). DOI: https://doi.org/10.1155/2014/179529

[12] C.S. Chuen, A.H.L. Pihie, Eurycomanone exerts antiproliferative activity via apoptosis upon MCF-7 cells, in: The 4th annual seminar of natural science fellowship. Universiti Sains Malaysia, Penang, 2004, pp. 73-77.

[13] P.C. Kuo, A.G. Damu, K.H. Lee, T.S. Wu, Cytotoxic and antimarial constituents from the roots of Eurycoma longifolia, Bioorganic & medicinal chemistry. 12(3) (2004) 537-544. DOI: https://doi.org/10.1016/j.bmc.2003.11.017

[14] Q. Le Tran, Y. Tezuka, J.Y. Ueda, N.T. Nguyen, Y. Maruyama, K. Begum, H.S. Kim, Y. Wataya, Q.K. Tran, S. Kadota, In vitro antiplasmodial activity of antimalarial medicinal plants used in Vietnamese traditional medicine, Journal of Ethnopharmacology. 86(2-3) (2003) 249-252. DOI: https://doi.org/10.1016/S0378-7417(03)00045-X

[15] J. Nguyen-Pouplin, H. Tran, H. Tran, T.A. Phan, C. Dolecek, J. Farrar, T.H. Tran, P. Caron, B. Bodo, P. Grellet, Antimalarial and cytotoxic activities of ethnopharmacologically selected medicinal plants from South Vietnam, Journal of Ethnopharmacology. 109(3) (2007) 417-427. DOI: https://doi.org/10.1016/j.jep.2006.08.011

[16] A.M. Adib, Z. Abdullah, Rapid discrimination of Eurycoma longifolia extracts by Fourier transform infrared spectroscopy and two dimensional correlation infrared spectroscopy, Vibrational Spectroscopy. 96 (2018) 1-9. DOI: https://doi.org/10.1016/j.vibspect.2018.02.003

[17] Phytopharmaceutical Aspect of Freeze Dried Water Extract from Tongkat Ali Roots—Specification; Department of Standards Malaysia: Cyberjaya, SGR, Malaysia, 2011.

[18] M.M.A. El Aziz, A.S.G. Melad, A review on saponins from medicinal plants: chemistry, isolation, and determination, Journal of Nanomedicine Research. 8(1) (2019) 6-12. DOI: 10.15406/jnmmr.2019.08.00199

[19] N. Mohd Effendy, N. Mohamed, N. Muhammad, I.N. Mohamad, A.N. Shuid, Eurycoma longifolia: Medicinal plant in the prevention and treatment of male osteoporosis due to androgen deficiency, Evidence-Based Complementary and Alternative Medicine. 2012 (2012) 1-9. DOI: https://doi.org/10.1155/2012/125761
[20] C.H. Teh, H. Morita, O. Shirotta, K.L. Chan, 2,3-Dehydro-4α-hydroxylongilactone, a novel quassinoid and two known phenyl propanoids from Eurycoma longifolia Jack, Food chemistry. 120(3) (2010) 794-798. DOI: https://doi.org/10.1016/j.foodchem.2009.11.012

[21] L.S. Chua, C.H. Lau, C.Y. Chew, D.A.S. Dawood, Solvent Fractionation and Acetone Precipitation for Crude Saponins from Eurycoma longifolia Extract, Molecules. 24(7) (2019) 1416. DOI: https://doi.org/10.3390/molecules24071416

[22] J.H. Doughari, Phytochemicals: extraction methods, basic structures and mode of action as potential chemotherapeutic agents, in: Phytochemicals-A global perspective of their role in nutrition and health (pp.: InTechOpen, 2012.

[23] E. Madland, Extraction, isolation and structure elucidation of saponins from Herniaria incana, Master Thesis, Norwegian University of Science and Technology, 2013.

[24] J.P. Vincken, L. Heng, A. de Groot, H. Gruppen, Saponins, classification and occurrence in the plant kingdom, Phytochemistry. 68(3) (2007) 275-297. DOI: https://doi.org/10.1016/j.phytochem.2006.10.008

[25] L. Dinan, J. Harmatha, R. Lafont, Chromatographic procedures for the isolation of plant steroids, Journal of Chromatography A. 935(1-2) (2001) 105-123. DOI: https://doi.org/10.1016/S0021-9673(01)00992-X

[26] J.M. Augustin, V. Kuzina, S.B. Andersen, S. Bak, Molecular activities, biosynthesis and evolution of triterpenoid saponins, Phytochemistry. 72(6) (2011) 435-457. DOI: https://doi.org/10.1016/j.phytochem.2011.01.015

[27] S.W. Kim, S.K. Park, S.I. Kang, H.C. Kang, H.J. Oh, C.Y. Baee, D.H. Baee, Hypocholesterolemic property of Yucca schidigera and Quillaja saponaria extracts in human body, Archives of Pharmacal Research. 26(12) (2003) 1042-1046. DOI: https://doi.org/10.1007/BF02994756

[28] J. Milgat, D.C.K. Roberts, The nutritional & biological significance of saponins, Nutrition Research. 15(8) (1995) 1223-1249. DOI: https://doi.org/10.1016/0271-5317(95)00081-S

[29] D.M. Gurinkel, A.V. Rao, Soyasaponins: the relationship between chemical structure and colon anticarcinogenic activity, Nutrition and Cancer. 47(1) (2003) 24-33. DOI: https://doi.org/10.1207/s15327914nc4701_3

[30] S. Man, W. Gao, Y. Zhang, L. Huang, C. Liu, Chemical study and medical application of saponins as anti-cancer agents, Fitoterapia. 81(7) (2010) 703- 714. DOI: https://doi.org/10.1016/j.fitote.2010.06.004

[31] S.G. Sparg, M.E. Light, J. Van Staden, Biological activities and distribution of plant saponins, Journal of Ethnopharmacology. 94(2-3) (2004) 219-243. DOI: https://doi.org/10.1016/j.eph.2004.05.016

[32] S.M. Hassan, A.U. Haq, J.A. Byrd, M.A. Berhow, A.L. Cartwright, C.A. Bailey, Haemolytic and antimicrobial activities of saponin-rich extracts from guar meal, Food Chemistry. 119(2) (2010) 600-605. DOI: https://doi.org/10.1016/j.foodchem.2009.06.066

[33] V.G. Khanna, K. Kannabiran, Antimicrobial activity of saponin fractions of the leaves of Gymnema sylvestre and Eclipta prostrata, World Journal of Microbiology and Biotechnology. 24(11) (2008) 2737. DOI: https://doi.org/10.1007/s11274-008-9758-7

[34] Y.F. Chen, H.Y. Roan, C.K., Lii, Y.C. Huang, T.S. Wang, Relationship between antioxidant and antiglycation ability of saponins, polyphenols, and polysaccharides in Chinese herbal medicines used to treat diabetes, Journal of Medicinal Plants Research. 5(11) (2011) 2322-2331. DOI: https://doi.org/10.5897/JMPR.9001080

[35] H. Sun, L. Chen, J. Wang, K. Wang, J. Zhou, Structure–function relationship of the saponins from the roots of Platycodon grandiflorum for hemolytic and adjuvant activity, International Immunopharmacology. 11(12) (2011) 2047-2056. DOI: https://doi.org/10.1016/j.intimp.2011.08.018

[36] W. Oleszek, Z. Bialy, Chromatographic determination of plant saponins— an update (2002–2005), Journal of Chromatography A. 1112(1-2) (2006) 78-91. DOI: https://doi.org/10.1016/j.chroma.2006.01.037

[37] C.Y. Cheok, H.A.K. Salman, R. Sulaiman, Extraction and Quantification of Saponins: A Review, Food Research International. 59 (2014) 16-40. DOI: https://doi.org/10.1016/j.foodres.2014.01.057