# Sequential Extraction of Saponins from *Eurycoma longifolia* Roots by Water Extraction and Ultrasound-assisted Extraction

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**Abstract**

Central composite designs (CCDs) for UAE and WE were established by seven and five extraction factors, respectively, to determine their optimum conditions that maximized the saponins yields. These optimum conditions were employed successively to establish a sequential extraction (SE) of saponins from *Eurycoma longifolia* roots. Results identified the agitation speed as an influencing factor in altering the interactive effects of the extraction factors in UAE and WE. Optimized conditions illustrated that higher agitation speeds increased the yields of saponins in WE while UAE preferred lower agitation speed ranges. This implied that the high agitation speeds decreased the collision of microjets and particles and hence the decrease of sonication efficiency. The SE extraction was proposed by initial sonication of the plant sample for a duration of 5 minutes and 29 seconds to 10 minutes followed by conventional WE for 20-25 minutes.

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*Keywords: Eurycoma longifolia*; saponin; optimization; CCD; agitation speed; ultrasound; extraction

## 1. Introduction

Saponins are secondary metabolites derived from a 30-carbon precursor named oxidosqualene. They are classified into triterpenoid and steroid saponins [1] that vary in the numbers of their attached sugar moieties at different positions [2]. Triterpenoid saponins represent the majority of saponins in nature [3, 4] and are divided into two groups. The first group is the neutral triterpenoid saponins which contain normal sugar attached to sapogenin. The second group is the acidic triterpenoid saponins in which sugar moieties contain uronic acid or carboxylic groups attached to the sapogenin [2]. Steroid saponins are either a spirostanol or a furostanol with a glycone part that consists of linear or branched

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oligosaccharides [5].

Saponins are known for their bioactivities such as their ability to increase immune responses [6] as immunostimulatory adjuvants [7]. Other effects of saponins include antioxidant, anticancer and antidiabetic activities [8]. They also possess antibacterial effects [9] by causing membranes disintegration which lead to inhibition of mobility or death of the protozoans [2]. Saponins are also known as bio-surfactants that improve the surface properties of food [10]. Saponins are extracted from various plant roots [11, 12] by maceration with the assistance of magnetic stirring [13-15] or by other methods such as ultrasound assisted extraction (UAE) [16] and subsequent extraction which employed using different solvents [17, 18].

This study investigated the total saponins yield in water extracts of *Eurycoma longifolia* roots which is known for its aphrodisiac [19], anticancer [20] and antiulcer [21] effects. Most of the studies have been focused on secondary metabolites such as quassinoids [22, 23]. However very few studies have investigated the saponins concentrations in the root extracts of *E. longifolia* with contradicted results [24, 25]. Sequential extraction (SE) has been defined based on the applied process. It is a series of selective partition extraction methods that identify elements associated with solid phases in the environment [26]. It is also a series of various extraction methods to fractionate distinctive metabolites from a plant material [27] or a repeated extraction process with different solvents to obtain targeted metabolites from the plant tissue [28]. Saponins from *E. longifolia* roots were mainly obtained by maceration or water extraction (WE) [24, 25, 29]. However, the implementation of SE by applying a series of successive extraction methods with the same extraction solvent was not previously studied to extract saponins from *E. longifolia* root.

The aim of this work was the employment of SE method to extract saponins from *E. longifolia* roots. Saponins were extracted by the conventional WE and non-conventional UAE to determine the optimum conditions to maximize the yields. The selected optimum conditions were employed to establish a SE process to increase the saponins yields with less extraction time and avoidance of saponins degradation.

## 2. Materials and methods

### *2.1. Material preparation*

Dried roots of *E. longifolia* were obtained at University Malaysia Pahang (UMP) under the research Grant RDU 161601 and RDU 160801. The roots were pulverized to obtain particle sizes with the radii lengths 0.424±0.025, 0.375 ±0.075, 0.223 ±0.073, 0.071 ±0.017 and 0.022±0.22 mm.

### *2.2. Reagents and equipment*

All reagent were obtained from Merck (Germany), Sigma Aldrich (USA) and Fisher scientific (UK) and all were analytical grade. Standard diosgenin was obtained from Sigma Aldrich (USA). Ultrapure water was provided by Milli-Q ultrapure water system. Water extractions were perform by a IKA® MAG HS7 hot plate magnetic stirrer. UAE was performed by a QSonica ultrasonic processor Q700 (700 watts, 20 kHz) with a replaceable flat tip ultrasonic probe (length: 127 mm, diameter: 12.7 mm) made from titanium alloy to the WE experiment. Concentrations of saponins were investigated using a Hitachi U 1800 UV/VIS spectrophotometer (UK) equipped with a Hitachi U1800 D2 lamp (Deuterium UV lamp).

### *2.3. Experimental design and statistical analysis*

Experimental designs and statistical analyses were established by Response surface methodology (RSM) [30, 31]. in Minitab 17 software [32]. The central composite design (CCD) was employed to permit the fitting of second order model for the yields as in (Eq. 1).

$$Y = b_0 + \sum_{n-1}^{n} b_n x_n + \sum_{n=1}^{n} b_{nn} x_n^2 + \sum_{n \neq m=1}^{n} b_{nm} x_n x_m \qquad (1)$$

Where $Y$ is the predicted response variable of the yield (%) for saponins; $b_0$ is the average response obtained at the replicated center point (0, 0, 0, 0) of the CCD; $b_n$, $b_{nn}$ and $b_{nm}$ are the linear, quadratic and interaction regression coefficients respectively.

Star points, which are axial experiments located on variables axes at an (±α) distance from the centre were provided for estimation of the model curvature. The value (α= ±1.32) was chosen as fixed value from the UAE experiments.

*2.4. UAE and WE parameters*

According to the CCD design of UAE, 5 g of pulverized roots with a certain particle size (*P*) were extracted by different values of Liquid-solid ratios (*S*), extraction temperatures (*T*), agitation speeds ($R_s$), durations of sonication ($t_1$) amplitudes (*A*) and duty cycles (*D*). The WE process was conducted with the same factors at extraction times ($t_2$) without the sonication regiments (*A*) and (*D*). Table 1 illustrated the coded and corresponding real values of the parameters for both UAE and WE. All experiments were triplicated and carried out randomly to minimize effect of extraneous factors on the observed responses. All extracts were filtered through Whatman filter paper No. 1 and preserved in -20°C for further investigations.

Table 1: Levels of variables employed for the construction of Central Composition Design (CCD) in UAE and WE.

| Extraction factors | | Levels | | | | |
|---|---|---|---|---|---|---|
| | | -1.322 | -1 | 0 | 1 | 1.322 |
| Liquid-solid ratio | *S* | 16.8 | 20 | 30 | 40 | 43 |
| Particle size (radius (mm)) | *P* | 0.022 | 0.071 | 0.223 | 0.375 | 0.424 |
| Extraction temperature (°C) | *T* | 45 | 50 | 65 | 80 | 85 |
| Agitation speed (200-800 rpm) | $R_1$ | 103 | 200 | 500 | 800 | 897 |
| Agitation speed (800-1500 rpm) | $R_2$ | 687 | 800 | 1150 | 1500 | 1613 |
| Sonication time (seconds) | $t_1$ | 91 | 120 | 210 | 300 | 329 |
| Extraction time (minutes) | $t_2$ | 41 | 60 | 120 | 180 | 199 |
| Amplitude (W) | *A* | 1 | 2 | 6 | 10 | 11 |
| Duty cycle (%) | *D* | 37 | 40 | 50 | 60 | 63 |

*2.5. Isolation and determination of total saponins*

From each experiment, 1 mL of water extract was taken and the saponins were isolated in *n*-butanol fractions as in references [33]. Concentrations of total saponins (g/mL) were detected by the vanillin-sulfuric acid colorimetry assay at 544 nm against the established calibration curve of the standard diosgenin [34]. Yields were expressed by their percentage (%) according to Eq. 2 and stated by their means and standard deviations.

$$Y(\%) = \frac{C \times V}{W} \times 100\% \qquad (2)$$

Where *Y* is the yield of saponins, *C* is the concentration (g/mL), *V* is the whole volume of extract (mL), *W* is the weight of the raw sample (g).

*2.6. Statistical analysis*

Precisions of the mathematical models were estimated by determining the corresponding values of the standard error of regression (*S*), coefficients of determination ($R^2$), adjusted coefficient of determination (*adj* $R^2$) and predicted coefficient of determination (*pred* $R^2$). Further statistical evaluation was conducted by estimating the error sum of squares (*SSE*) and root mean square error (*RMSE*) in Matlab R2010a by the curve fitting tool. The lack of fit estimated the adequacy of each mathematical model to express the experimental data. The lack of fit was evaluated by the corresponding *p* value that should be larger than 0.05.

## 3. Results and Discussion

*3.1. Experimental design and mathematical models for the extraction of saponins (Y)*

Extraction is an important step for investigating bioactive phytochemicals from plant materials [35] by the selection of a suitable combination of extraction factors for proper standardization of herbal products [36, 37]. The

generated CCD design for UAE was composed of the combinations of 7 factors (Table 1) to establish 54 experiments in replicate. Experimental values of saponins yields from both extraction processes were employed in multiple regression analysis performed by RSM to fit the second order polynomial equation and generate the full quadratic models named predicted models. The enhanced models were obtained by deleting the insignificant terms that possess p values ($p$>0.05) from the predicted models.

*3.2. Saponins yields by UAE* ($Y_{UAE}$)

*3.2.1. Saponins yields with agitation speed $R_1$ (200 to 800 rpm)*

Saponins extraction with UAE illustrated that the highest saponins yield (8.398±0.666%) was obtained with the combination of $S$=40, $P$= 0.071±0.017mm, $T$=80°C, $R_1$=800, $t_1$=5 minutes, $A$=2W and $D$=2%. Generated quadratic and enhanced models by Minitab 17 were expressed in (Eq.3) and (Eq.4) respectively.

$$\begin{aligned} Y_{UAE_{(\,full\ quadratic\,)}} &= 0.9133 + 0.9364S - 0.2710P + 0.799T + 1.0539R_1 + 0.0809t_1 + 0.0274A + 0.0014D \\ &+0.370S^2 + 0.229P^2 + 0.267T^2 + 0.456R_1^2 + 0.229t_1^2 + 0.229A^2 + 0.299D^2 - 0.006S.P \\ &+0.632S.T + 0.342S.R_1 + 0.0275S.t_1 + 0.0487S.A - 0.070S.D + 0.096P.T - 0.135P.R_1 \\ &-0.011P.t_1 + 0.082P.A - 0.007P.D + 0.467T.R_1 - 0.200T.t_1 - 0.253T.A - 0.175T.D \\ &+0.251R_1.t_1 - 0.017R_1.A - 0.004R_1.D + 0.072t_1.A + 0.014t_1.Dd + 0.0964A.D \end{aligned} \quad (3)$$

$$\begin{aligned} Y_{UAE_{(enhanced)}} &= 0.9133 + 0.9364S - 0.271P + 0.799T + 1.054T + 0.081t_1 + 0.370S^2 + 0.229P^2 + 0.267T^2 \\ &+0.456R_1^2 + 0.229t_1^2 + 0.2290.229A^2 + 0.299D^2 + 0.632S.T + 0.342S.R_1 - 0.070S.D \\ &+0.096P.T - 0.135P.R_1 + 0.082P.A + 0.47T.R_1 - 0.200T.t_1 - 0.253T.A - 0.175T.D \\ &+0.251R_1.t_1 + 0.072t_1.A + 0.096A.D \end{aligned} \quad (4)$$

*3.2.2. Saponins yields with agitation speed $R_2$ (800 to 1500 rpm)*

Saponins extraction with UAE illustrated that the highest saponins yield (9.095±1.242%) was obtained with the combination of $S$=20, $P$= 0.071±0.017mm, $T$=50°C, $R$=1500, $t_1$=5 minutes, $A$=10W and $D$=60%. Generated quadratic and enhanced models by Minitab 17 were expressed in (Eq.5) and (Eq.6) respectively.

$$\begin{aligned} Y_{UAE_{(\,full\ quadratic\,)}} &= 0.8058 - 0.0732S - 1.6566P - 0.6362T + 0.2054R_2 + 0.1930t_1 + 0.4462A + 0.5352D \\ &+0.0394S^2 + 1.976P^2 + 0.458T^2 + 0.0586R_2^2 + 0.0389t_1^2 + 0.0454A^2 + 0.0446D^2 \\ &-0.0534S.P + 0.1286S.T + 0.0070S.R_2 - 0.1286S.t_1 + 0.2160S.A - 0.0864S.D \\ &+0.2331P.T - 0.3119P.R_2 - 0.0631P.t_1 - 0.2950P.A - 0.1991P.D - 0.2050T.R_2 \\ &-0.0211T.t_1 - 0.0481T.A - 0.4159T.D - 0.1223R_2.t_1 - 0.1473R_2.A - 0.0147R_2.D \\ &+0.0092t_1.A + 0.0668t_1.D + 0.0073A.D \end{aligned} \quad (5)$$

$$\begin{aligned} Y_{UAE_{(enhanced)}} &= 0.8405 - 0.0732S - 1.6566P - 0.6362T + 0.2054R_2 + 0.1930t_1 + 0.4462A + 0.5352D \\ &+2.0696P^2 + 0.5514T^2 - 0.0534S.P + 0.1286S.T - 0.1286S.t_1 + 0.2160S.A - 0.0864S.D \\ &+0.2331P.T - 0.3119P.R_2 - 0.0631P.t_1 - 0.2950P.A - 0.1991P.D - 0.2050T.R_2 \\ &-0.0481T.A - 0.4159T.D - 0.1223R_2.t_1 - 0.1473R_2.A + 0.0668t_1.D \end{aligned} \quad (6)$$

*3.3. Saponins yields by WE* ($Y_{WE}$)

*3.3.1. Saponins yields with agitation speed $R_1$ (200 to 800 rpm)*

The highest saponins yield by WE with the low range of agitation speeds (10.839±1.515%) was obtained with the combination of extraction factor $S$=40, $P$= 0.071±0.017mm, $T$=80°C, $R$=800 and $t_2$=60 minutes. Quadratic and

enhanced models were respectively represented in (Eq.7) and (Eq.8).

$$Y_{WE_{(\,full\ quadratic\,)}} = 8.316 + 1.904S - 0.3407P + 0.1293T + 0.2034R_1 + 0.723t_2 - 0.364S^2 - 0.030P^2 - 0.056T^2 - 0.034R_1^2 - 0.463t_2^2 - 0.0337S.P + 0.0328S.T + 0.054S.R_1 + 0.207S.t_2 - 0.275P.T - 0.212P.R_1 - 0.164P.t_2 + 0.071T.R_1 - 0.238T.t_2 + 0.105R_1.t_2 \quad (7)$$

$$Y_{WE_{(\,full\ quadratic\,)}} = 8.159 + 1.904S - 0.3407P + 0.203R_1 + 0.7226t_2 - 0.693t_2^2 - 0.207S.t_2 - 0.275P.T - 0.212P.R_1 - 0.238T.t_2 \quad (8)$$

*3.3.2. Saponins yields with agitation speed $R_2$ (800 to 1500 rpm)*

The highest saponins yield by WE with the high range of agitation speeds (24.83±0.474%) was obtained with the combination of extraction factor *S*=40, *P*= 0.071±0.017mm, *T*=80°C, *R*=1500 and $t_2$=60 minutes. Quadratic and enhanced models were respectively represented in (Eq.9) and (Eq.10).

$$Y_{WE_{(\,full\ quadratic\,)}} = 14.536 + 2.625S - 1.241P + 0.664T + 4.288R_2 + 0.232t_2 - 1.410S^2 - 0.268P^2 - 0.808T^2 - 0.785R_2^2 - 0.408t_2^2 - 0.415S.P + 0.413S.T + 1.081S.R_2 + 0.006S.t_2 - 0.146P.T - 0.373P.R_2 - 0.086P.t_2 + 0.969T.R_2 - 0.563T.t_2 + 0.856R_2.t_2 \quad (9)$$

$$Y_{WE_{(\,full\ quadratic\,)}} = 14.424 + 2.625S - 1.241P + 0.664T + 4.288R_2 - 1.575S^2 - 0.973T^2 - 0.950R_2^2 - 0.415S.P + 0.413S.T + 1.081S.R_2 - 0.373P.R_2 + 0.969T.R_2 - 0.563T.t_2 + 0.856R_2.t_2 \quad (10)$$

*3.4. Statistical evaluation and model fitting*

Table 2 represented the results of the statistical analyses of goodness of fit and Lack of fit. The precisions of the predicted and generated models were evaluated by the values of *S*, $R^2$, *adj* $R^2$ and *pred* $R^2$ beside *SSE* and *RMSE*. *S* represented the standard distance data values fall from the fitted regression, and $R^2$ expressed the capability of the regression line in representing the experimental data. *adj* $R^2$ reflected the fitness of the model for the data when adjusted, and *pred* $R^2$ demonstrated the ability of the models to predict responses for new observations. *SSE* measured the variation within the experimental data and *RMSE* represented the standard deviation of the prediction errors that measured the scatter of residuals around the fit line. The values illustrated high precisions of the models suggesting good fit for the experimental data. With the generation of enhanced models a decrease in *S* and $R^2$ was noticed while other coefficients and statistical values increased. The increase in the values of *pred* $R^2$ *of* the enhanced models indicated higher capability to present the new entries of data. Lack of fit values stated the adequacy of the models to represent the experimental data with *p* values ($p$>0.05).

*3.5. Main effect of extraction factors*

*3.5.1. Main effects of UAE factors*

Main effects of UAE factors on the saponins yields (Fig. 1.a) illustrated that the increase of the agitation speed altered the topographic features of the main effects of the other extraction factors. At agitation speed range ($R_1$), the saponin yield increased with the increase of the solvent volume due to the rise of the diffusion rate [38]; this was on the contrary to the application of agitation speed range ($R_2$) as less volumes were slightly efficient than larger ones. This indicated that UAE benefited the extraction process by less consumption of the extraction solvent [39]. Reduction of the particle size increased the main effect by declining of the diffusion pathway lengths [40] and increasing the exchange area between solid and solvent [41]. The increase of the agitation speed also altered the main effect of temperature. At low agitation the effect increased with the increase of temperature, while with higher agitation speeds lower temperatures were favoured. As temperature was actually a combination of heat introduced by the hot plate [42] and from the probe horn [43]; the results suggested that the final main effect of the temperature is actually a combination of stirring and the introduced heat.

Table. 2: Statistical analyses for goodness of fit and Lack of fit for all models

| Extraction method: | Ultrasound assisted extraction | | | | Water extraction | | | |
|---|---|---|---|---|---|---|---|---|
| Agitation | 200-800 rpm | | 800-1500 rpm | | 200-800 rpm | | 800-1500 rpm | |
| Models | Predicted | Enhanced | Predicted | Enhanced | Predicted | Enhanced | Predicted | Enhanced |
| Goodness of fit: | | | | | | | | |
| *S* | 0.517 | 0.515 | 0.449 | 0.447 | 0.895 | 0.903 | 1.298 | 1.311 |
| $R^2$ | 0.936 | 0.9346 | 0.961 | 0.961 | 0.825 | 0.808 | 0.939 | 0.935 |
| *adj* $R^2$ | 0.930 | 0.9308 | 0.958 | 0.958 | 0.797 | 0.794 | 0.929 | 0.928 |
| *pred* $R^2$ | 0.925 | 0.9273 | 0.954 | 0.955 | 0.755 | 0.773 | 0.913 | 0.916 |
| *SSE* | 105.000 | 106.60 | 7.942 | 8.237 | 103.8 | 111.9 | 248.3 | 256 |
| *RMSE* | 0.481 | 0.485 | 0.23 | 0.234 | 0.806 | 0.836 | 1.246 | 1.265 |
| Lack of fit: | | | | | | | | |
| *F* value | 0.86 | 0.84 | 1.16 | 1.1 | 1.12 | 1.17 | 0.69 | 0.84 |
| *p* value | 0.814 | 0.857 | 0.172 | 0.265 | 0.332 | 0.265 | 0.839 | 0.69 |

The increase of agitation speeds facilitated the solubility of saponins [44] that implied the maximization of solid-liquid surface contact [45] in addition to cooling the extraction process [46]. Sonication time ($t_1$) revealed a proportional relationship with the saponins yields with high agitation speeds; even though it was considered short to detect any degradation of saponins. However, the addition of sonication regiments was beneficial for short durations and considered more efficient [42] as the acoustic-induced cavitation in UAE enhanced the mass transfer by developing shockwave damages on the solid-liquid interface [47].

*3.5.2. Main effects of WE factors*

The application of the two different agitation ranges illustrated noticeable differences in the main effects of the extraction factors (Fig.1.b). The highest saponin yields corresponded to the application of high agitation speed range $R_2$ due to the maximization of solid liquid collisions [45]. These results suggesting the higher agitation speeds for the next investigations.

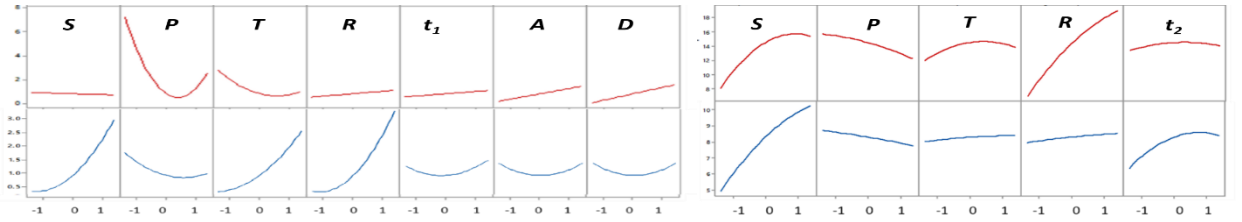
*3.5.3. Interactive effects of conventional WE factors*

Classical techniques for the solvent extraction of nutraceuticals from plant matrices are based on the choice of solvent coupled with the use of heat and agitation [48]. According to the contour plots (Fig. 2) the highest yield was determined with the maximization of solvent volume, temperature and agitation speed. This pointed out the importance of the triple combination of solvent volume, temperature and agitation speed [48]. Large solvent volumes provided higher concentration gradients between the metabolite concentrations at the surface and inside the particles [49]. However, it was noticed that the saponin yields decreased with excess solvent volumes that might be due to dilution. The reduction of particle sizes increased the saponin yield [29] because smaller particles led to enhancement of the diffusion mechanism [50] by maximizing the surface area and declining the diffusion pathway lengths which in turn released larger amounts of phytochemicals and increased the mass transfer [40].

The increase of temperature usually increases mass transfer, efficiency of the solvent and subsequently the solubility of the solutes [38]. In this study saponins yields increased with the increase of temperature due to increasing the diffusivity of solvent into the internal parts of the particles [51]. However a decrease in saponin yields was noticed with application of temperature higher than 84$^o$C that could be due to thermal denature of saponins with exceeding temperature [29].

The experiment illustrated that agitation resulted in maximizing the mass transfer [45] by facilitating the solid solvent contact [45] accompanied with a cooling phenomena and equal distribution of heat in the extraction system [52]. However, it was noticed that the yield decreased when applying high speeds at small volumes that may be due

to the formation of vortexes that cause reduction of solute-solvent collisions [40]. The quadratic effect of extraction time ($t_2$) illustrated possible degradation of saponins associated with extended extraction durations [53].




(a) (b)

Fig.1. Main effects of UAE and WE factors on saponin yield (%); (a) UAE; (b) WE. blue line: main effects at $R_1$ (200 to 800 rpm), red line: main effects at $R_2$ (800 to 1500 rpm). *S*: Liquid-solid ratio; *P*: particle size; *T*: temperature; *R*: agitation speed; $t_1$: sonication time; $t_2$: extraction time; *A*: amplitude: *D*: duty cycle

### *3.6. Selected criteria from UAE and WE for sequential extraction*

#### *3.6.1. Criterion of UAE optimum conditions*

Response optimizer in Minitab 17 had been employed to identify optimum conditions for UAE. Both agitation speed ranges ($R_1$=200 to 800 rpm and $R_2$= 800 to 1500 rpm) were considered. With the application of $R_1$ the prediction of saponins yield was 12.256% by applying the optimum conditions of *S*: 43:1 (g:g); *P*: 0.022 mm; *T*: 85$^o$C; $R_1$: 897 rpm; $t_1$: 329 seconds; *A*: 1W; *D*: 37%. The application of $R_2$ illustrated a predicted yield of 13.306% with the application of optimum conditions *S*: 16.8 (g:g); *P*: 0.022 mm; *T*: 45$^o$C; $R_1$: 1613 rpm; $t_1$: 329 seconds; *A*: 11W; *D*: 63%. Experimental values of the yields were 14.131±1.302% and 13.145±1.498% respectively (N=3), ($p<0.05$). Extension of sonication time for both criteria until 13 minutes illustrated behaviour of the extraction process outside the design boundaries (Fig. 3). Results determined that the optimum conditions (*S*: 43:1 (g:g); *P*: 0.022 mm; *T*: 85$^o$C; $R_1$: 897 rpm; $t_1$: 329 seconds; *A*: 1W; *D*: 37%) were more efficient for saponin extraction that might be due to the suitable combination of heat-agitation speed- sonication combination. The extraction criteria with agitation speed $R_1$= 897 rpm was adopted for initiating the SE process.

#### *3.6.2. Criterion of WE optimum conditions*

Based on the CCD, the response optimizer in Minitab 17 was also employed to identify optimum conditions for WE. The criterion was determined at *S*: 43:1; *P*: 0.022±0.02mm; *T*: 84.8 $^o$C; *R*: 1613 rpm and $t_2$:175 minutes. This criteria was adopted in the SE process.

### *3.7. Sequential extraction of saponins*

Previous studies on the saponins from *E. longifolia* roots did not avoid the drawbacks of conventional extraction. Khanam et al. (2015) used a 24 hour maceration method with various organic solvents at 40$^o$C. The results stated that the absence of saponins in both root and stem extracts were investigated by Froth test. On the other hand Harun et al. (2015) applied a temperature of 129±2$^o$C and agitation speed 400 rpm under 2.6 bar for 1 hour at each extraction step and the yields ranged between 35 and 37%. This contradiction could be explained by the different extraction parameters and extraction solvents. For example, water was stated as a better extraction solvent for glycosaponins from *E. longifolia* roots [29].

Sequential extraction of saponins was encouraged to overcome these drawback by selecting a suitable extraction process and optimizing the various parameters as they are important for upscaling bench and pilot scales [37]. SE was established by applying the optimum conditions of UAE followed by the optimum conditions of WE (Fig.4). The majority of saponin extraction processes were established by maceration [54]. So WE was adopted as the major extraction process while UAE was only employed to initiate the extraction process and facilitate the metabolite extraction [55] by breaking down of the cell walls [56]. This was accomplished to avoid the time consumption in WE [51] and the degradation of saponins by excessive heat in UAE.

Comparing this SE method with previous studies on saponins extraction from *E. longifolia* roots the SE was conducted with less extraction duration with the employment of water as a safe extraction solvent with low cost [57]. WE recorded the lowest saponin yields and the highest obtained yield was 25.436±0.571 (%) in 90 minutes and the

yield was increasing via time that indicated minimum rate of degradation. However WE was time consuming when compared with the other extraction methods.

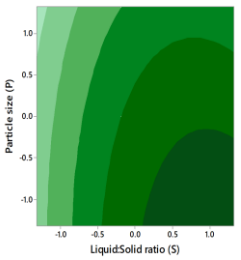



Fig. 2.a. Liquid-solid ratio (S) vs. particle size (P)

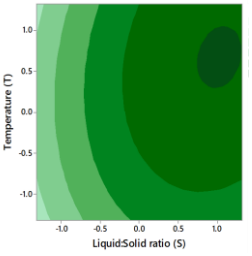



Fig. 2.b. Liquid-solid ratio (S) vs. temperature (T)

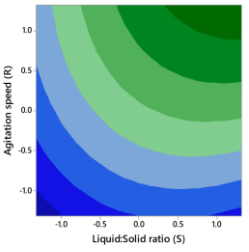



Fig. 2.c. Liquid-solid ratio (S) vs. agitation speed (R)

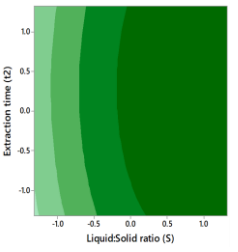



Fig. 2.d. Liquid-solid ratio (S) vs. extraction time ($t_2$)

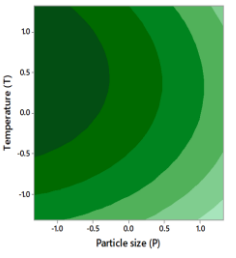



Fig. 2.e. Particle size (P) vs. temperature (T)

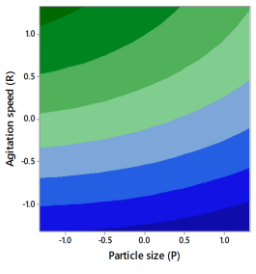



Fig. 2.f. Particle size (P) vs. agitation speed (R)

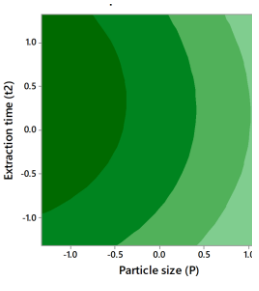



Fig. 2.g. Particle size (P) vs. extraction time ($t_2$)

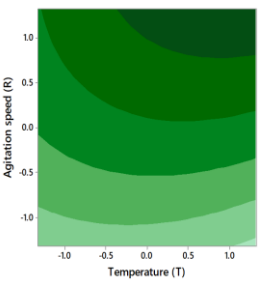



Fig. 2.h. Temperature (T) vs. Agitation speed (R)

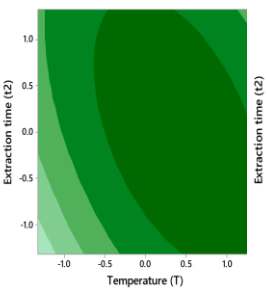



Fig. 2.i. Temperature (T) vs. extraction time ($t_2$)

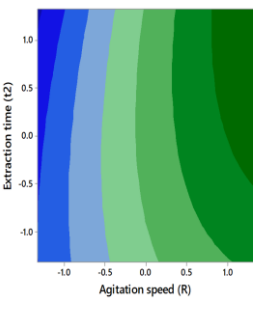
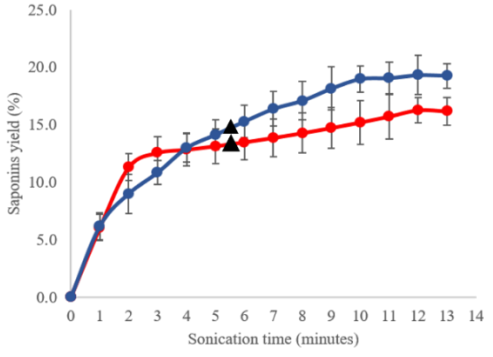



Fig. 2.j Agitation speed (R) vs. extraction time ($t_2$)

Key of saponin yield (%) <6 % yield >18 % yield

*Fig. 2. Contour plot of interactive effects of extraction factors for water extraction (WE) with agitation speed 800 to 1500 rpm*




Key: ▲ Saponin yields (%) at 5 minutes and 29 seconds

Fig.3. Comparison between the saponins yields by UAE. Blue: agitation speed range (200-800 rpm). Red: agitation speed range (800-1500 rpm)

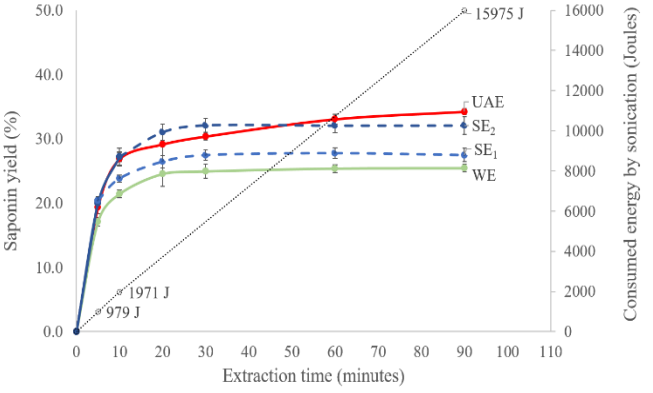



Fig.4. Comparison between saponin yields (%) by water extraction (WE), ultrasound assisted extraction (UAE) and three different criteria of sequential extraction (SE)

The highest saponin yield 34.215±0.483 (%) was obtained by UAE at 90 minutes. However the process consumed 15975 J only by sonication regardless to the energy consumed by the introduction of heat and agitation by the hotplate. To establish SE, two sonication times were done to initiate the process, namely $SE_1$ and $SE_2$ that represented 5 and 10 minutes respectively. After the sonication phase the extraction continued with WE. Both SE processes recorded higher saponins yields than WE. $SE_2$ illustrated higher saponin yields than those obtained by UAE until the 50 minutes. $SE_1$ and $SE_2$ used 979 J and 1971 J respectively by UAE before the switching to WE. The highest saponin yield (32.066±1.112 %) was obtained by $SE_2$ at 30 minutes. Therefore, $SE_2$ was more efficient than $SE_1$, this maybe due to longer sonication time that caused more breakdown of the cell walls.

## 4. Conclusion

A criteria for SE was established for extracting saponins from *Eurycoma longifolia* roots by applying extraction processes of UAE and WE successively. The optimum conditions of both extraction methods were obtained by investigating the factor effects of each extraction method separately. Central Composite Designs (CCDs) were established for WE and UAE. Agitation speed was considered as an important factor that effects other factors and contribute to the extraction rates of saponins. Therefore the experiments were repeated by applying two agitation speed ranges to indicate the effect of agitation speed on the yield. The results identified the best agitation speed for UAE was 897 rpm while for WE was 1613 rpm.

The optimum conditions from both processes were employed to establish the SE process. The process showed efficiency in extracting the saponins by illustrating higher yields than those of WE. The SE processes also illustrated higher yields than UAE in the first 30 minutes accompanied with less heat and energy consumption by UAE.

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