Sequential ultrasound-microwave assisted extraction of water soluble proteins from *Eurycoma longifolia* roots

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Sequential ultrasound-microwave assisted extraction of water soluble proteins from *Eurycoma longifolia* roots

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Abstract. Even though *Eurycoma longifolia* was extensively studied, few researches investigated the protein content in its water extracts with the consideration of establishing the most suitable extraction method to increase their yields with high efficiency and less time consumption. The study applied a sequential extraction method to increase the yields of water soluble proteins (WSPs) in *E. longifolia* root extracts by the application of two nonconventional extraction methods, Microwave assisted extraction (MAE) and ultrasound assisted extraction (UAE). The study was established by circumscribed central composite designs (CCCDs) to indicate the optimum extraction conditions and the corresponding maximum WSPs yields for both the methods by investigating the MAE factors which were temperature (*T*); microwave power (*W*) and irradiation time (*i*) and UAE factors temperature (*T*); ultrasonic intensity (*UI*); and sonication time (*s*). The optimum conditions of MAE (*T*: 54ºC, *W*: 301W and *i*: 15 min) led to the WSPs yield 23.101±1.647% .The optimum conditions of UAE (*T*: 46ºC; *UI*: 1.84 W.cm^{-2} and *s*: 24 min) led to 24.181±0.321%. These predicted optimum conditions were then employed for the two proposed sequential extraction method, sequential ultrasound-microwave assisted extraction (SUMAE) and sequential microwave-ultrasound assisted extraction (SMUAE). Results revealed that the highest WSPs yield (27.172±1.086%) was obtained by initiating the sequential extraction with UAE for 10 minutes followed by MAE treatment for 5 minutes was the best extraction process. Therefore, SUMAE illustrated more efficiency than SMUAE. This concluded that the SUMAE is a more efficient extraction process than the one-step nonconventional extraction methods and was nominated for the upscaling of extracting proteins from *E. longifolia* roots in pilot and industrial scales.

1. Introduction

The roots of the well-known Tongkat Ali *(Eurycoma longifolia)* is identified as an important economical source of phytomedicinal therapy and dietary supplements due to its' aphrodisiac, antiulcer, antimalarial and antitumor activities. The root extracts are usually obtained by decoction, therefore most of the previous studies were based on conventional methods [1]. However, few studies investigated the implementation of nonconventional methods for extracting metabolites from the plant roots [2; 3], even though numerous studies investigated the extraction enhancements and improvement of bioactive compound recoveries by Microwave assisted extraction (MAE) and Ultrasound assisted extraction (UAE) [4] due to their significant efficiencies in the provision of short extraction times accompanied with less energy consumption [5; 6].

The MAE is an optimum choice for obtaining pure plant extracts without any undesirable residues at short duration with limited degradation [7]. The main characteristic of MAE is the direct delivery of energy to the material by molecular interactions with the electromagnetic field and conversions of electromagnetic energy into thermal energy [8]. Here, the heat causes evaporation of the moisture inside cells which leads to a tremendous increase in pressure on the cell walls followed by the rupture in the plant tissue leading to the release of targeted compounds [9].

On the other hand, UAE is highly considered as an economically profitable method in the food industry [10] because it is a reliable non-thermal and inexpensive green extraction technology [11] with minimization of product wastes and environmental impacts [12]. Sonication is based on the generation of microjets that develop shockwave damages on the solid-liquid interface on the plant cell walls and facilitate the release of the inner components [13] leading to the improvement in mass transfer [12].

The combination of UAE and MAE proved to be an attractive extraction technique exhibiting the highest efficiency [9; 14] and introduces advantages and higher yields [15; 16]. These combinations included sequential extraction, such as microwave-ultrasound assisted extraction (MUAE) [17] and ultrasound-microwave assisted extraction (UMAE) [9]. The term sequential extraction was first introduced as a series of extractions for the partitioning of trace metals [18] or a repeated extraction process targeting plant metabolites with different solvents [19]. Sequential extraction was also introduced with two different extraction methods, the nonconventional UAE and the conventional heat assisted extraction (HAE) to extract saponins from *E. longifolia* roots [20].

The sequential extraction based on various extraction methods is rarely studied; therefore, for the first time, this study employed the optimum conditions of UAE and MAE to establish two types of sequential extraction; sequential microwave-ultrasound assisted extraction (SMUAE) and sequential ultrasound-microwave assisted extraction (SUMAE). Both sequential extractions were compared to determine the most suitable method. The first step was to determine the optimized conditions of MAE and UAE to maximize the water soluble proteins (WSPs) yields from *E. longifolia* roots by circumscribed central composite designs (CCCDs). The optimum conditions were then employed to establish sequential extraction processes MUAE and UMAE to determine the most efficient sequence of extraction method.

2. Material and methods

2.1. Plant material

Fresh roots of *E. longifolia* were obtained by a local supplier, air-dried and pulverized by a SZ-1000A-3 grinder. The powdered roots were sieved by a calibrated granulometric sieve GB/T60031-1997 to obtain the desired particle size with radius 0.071±0.017 mm.

2.2. Reagents and equipment

All regents were purchased from Merck (Germany), Sigma Aldrich (USA) and Fisher Scientific (UK) and were analytical grade. Bovine Serum Albumin (BSA) was used to establish the standard curve. The MAE was performed by An ETHOS-Milestone extractor (ATC-FO-300, North America) (Figure 1.a). The UAE was performed by a hot plate IKA MAG HS7 attached to an ultrasonic processor Q700 (700 watts, 20kHz) from Q Sonica, Newtown, U.S.A) (Figure 1.b) with a replaceable flat tip ultrasonic probe [3]. Ultrapure water was provided by Milli-Q ultrapure water system. Concentrations ($g.mL^{-1}$) of WSPs and standard solution of BSA were detected at 750 nm by Hitachi U 1800 UV/VIS spectrophotometer.

2.3. Experimental design and statistical analysis

The CCCDs were generated in Minitab 17 [21] to optimize the processes with three levels (-1, 0, +1) and star points ($\alpha = \pm 1.3$) from the centre base to permit the fitting of the second order model (Eq. 1) for the effect of the independent variables and their interactions on the WSPs yields (%).

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

Where Y is the predicted response variable of the yield (%) for WSPs; b_0 is the average response obtained at the replicated centre point (0, 0, 0) of the CCCD; b_n, b_{nn} and b_{nm} are the linear, quadratic and interaction regression coefficients, respectively.
The lower, middle and higher coded values for all factors were coded according to (Eq. 2).

$$X_i = \frac{x_i - \bar{x}_i}{\Delta x_i} \qquad (2)$$

Where the X_i is the coded value, x_i is the real value of the independent variable, $\bar{x}_i$ is the real value of an independent variable at the centre point and x_i is the step change.

Figure 1. The extraction instruments (a) Microwave apparatus (b) Ultrasound processer with the attached hot plate.

2.4. Extraction of WSPs by MAE
The extraction process was performed by the ETHOS-Milestone extractor (ATC-FO-300, North America) with a round bottomed extraction vessel that was attached to a cooling system for maintaining the balance between the microwave power and the boiling temperature of the solvent (Figure 1). Each experiment was conducted of a preheating process that was carried out for 5 min followed by an irradiation phase and then a cooling phase for 5 min. The application of pulsed heating was used to reduce the bumping phenomenon [22]. The microwave factors, temperature (*T*), microwave power (*W*) and irradiation time (*i*) are illustrated in (Table1). The liquid to feed ratio was constant in all the experiments (20:1) by adding 20 g of the pulverized roots to 400 mL water.

2.5. Extraction of WSPs by UAE

The UAE was performed by the hot plate for heating the constant water volume (400 mL) until the designated temperatures accompanied with the constant agitation speed 1314 rpm [3]; while the ultrasonic processor provided the sonication regiment, amplitudes and duty cycles, that were set up before each extraction experiment to provide the designated ultrasound intensity. The probe tip immersed 3 cm in the water. The sonication process started immediately after the addition of 20 g of pulverized root to the water in each experiment. The independent variables of UAE (Table 1) were temperature (*T*), ultrasound intensity (*UI*) and sonication time (*s*).

Table 1. Levels of variables employed for the construction of circumscribed central composite designs (CCCDS) for microwave assisted extraction (MAE) and ultrasonic assisted extraction (UAE).

Method	Factors		Parameters				
	Variable names	Terms	-1.3	-1	0	+1	+1.3
MAE	Temperature	*T* (°C)	45	50	65	80	85
	Microwave power	*W* (W)	168	200	300	400	432
	Irradiation time	*i* (min)	7	10	20	30	33
UAE	Temperature	*T* (°C)	45	50	65	80	85
	Ultrasound intensity	*UI* (UI)	0.36	0.88	1.70	2.53	3.05
	Sonication time	*s* (min)	2	5	15	25	28

2.6. Sequential extraction

The optimum conditions for MAE and UAE were adopted for the sequential extraction processes. Sequential MUAE (SMUAE) was carried out by applying MAE optimum parameters followed by UAE optimum parameters [17] while sequential UMAE (SUMAE) was done by applying UAE optimum parameters followed by MAE optimum parameters [9].

2.7. Measurement ultrasonic intensity (UI)

The ultrasonic intensity (*UI*) was calculated (Eq. 3) by employing the obtained values of the ultrasonic power (*P*) in (Eq. 4) [23; 24].

$$UI = \frac{4P}{\pi D^2} \qquad (3)$$

$$P = m.C_p.\frac{dT}{dt} \qquad (4)$$

Where *UI* is the ultrasonic power intensity (W.cm^{-2}), *P* is the ultrasonic power (W), *D* is the diameter (cm) of the tip of the probe, *m* is the water mass (g), C_p is the specific heat of the water at constant pressure (4.184 J.g^{-1}.°C^{-1}) and (*dT*/*dt*) is the initial rate of change of temperature over time (°C.s^{-1}) which was determined by fitting temperature change obtained by a thermometer against sonication time.

2.8. Estimation of total dry yields

The yield (g) of the total dry extracts from each experiment was estimated by heating 5 mL of each extract in a pre-weighted glass petri dish at 80°C in an oven for 15 min followed by drying in room temperature for 24 hours. The difference in weights after the drying process and the petri dishes was considered the approximate weight of the dry extract in 5 mL [25]. The total weight of the extract calculated as in (Eq.5).

$$W = \frac{M \times V_L}{V_S} \qquad (5)$$

Where W is the weight of total dry extract (g); M is the mass of the dry extract (g) in 5 mL; V_L is the liquid total volume (400 mL); and V_S is the sample volume (5 mL).

2.9. Determination of WSPs Yields (%)

The recovery of WSPs was established by the acetone precipitation method [26] to separate the WSPs for 1mL extract. Each precipitated WSPs pellet was then dissolved by vortex in 1 mL of ultrapure water and then quantified by Lowry method [27]. The WSPs yields (%) were expressed by their percentages of the extracted WSPs weights (g) to the dry extract weight (g) according to (Eq. 6).

$$Y(\%) = \frac{C \times V}{W} \times 100\% \qquad (6)$$

Where Y is the yield of WSPs yields, C is the concentration ($g.mL^{-1}$), V is the whole volume (mL) which is constant to 400 mL and W is the dry extract weight (g).

2.10. ANOVA analysis and interactive effects of the extraction factors

Statistical analyses determined the goodness of fit by calculating the coefficient of determination (R^2), the adjusted coefficient of determination (R^2_{adj}) and the predicted coefficient of determination (R^2_{pred}). The lack of fit was determined by the p values of the lack of fit tests at significant level of 95% ($p > 0.05$) to determine the adequacy of the models. The 3D Surface plots were generated to visualize the interactive effects between the extraction factors by Design expert (Version 7.1.6; Stat- Ease Inc, Minneapolis, MN, USA).

2.11. Sequential extraction

The optimum conditions for MAE and UAE were adopted for the sequential extraction processes. Sequential MUAE (SMUAE) was carried out by applying MAE optimum parameters followed by UAE optimum parameters [17] while sequential UMAE (SUMAE) was done by applying UAE optimum parameters followed by MAE optimum parameters [9].

3. Results and discussion

3.1. Experiment setup

This study employed MAE and UAE for extracting WSPs from *E. longifolia* roots due their high efficiencies, short extraction durations and reduction of solvent wastes [6; 13]. According to the CCCD of MAE and UAE, each method included 20 experiments that were triplicated and were expressed by their means and standard deviations (M±SD %) (Table.2). Each experiment was established by extracting 20 g of the pulverized root in 400 mL water to fulfil the constant liquid to solid ratio (20:1).

Table 2. The CCCD for MAE and UAE for the WSPs yields (%) and their corresponding predicted yields (%).

Order	Factors			WSPs yields (%) by MAE				WSPs yields (%) by UAE		
	T	*W/UI*	*i/s*	Dry extract(g)	Experimental	Predicted	Dry extract (g)	Experimental	Predicted
1	1.3	0	0	1.888	10.537±0.435	10.725	2.059	19.089±0.048	19.405
2	0	0	0	1.772	22.062±0.428	21.796	2.103	21.579±0.136	21.055
3	0	0	0	1.772	21.934±0.446	21.796	2.103	21.045±0.283	21.055
4	0	0	-1.3	1.677	20.381±0.613	20.654	1.908	18.782±0.295	18.840
5	-1.3	0	0	1.858	21.478±0.874	21.821	2.035	22.912±0.306	22.704
6	1	-1	-1	1.756	10.829±0.467	10.795	1.819	17.280±0.322	17.478
7	0	0	0	1.772	21.775±0.654	21.796	2.103	20.609±0.336	21.055
8	-1	-1	1	1.826	19.697±0.657	19.618	1.872	21.680±0.351	21.547
9	1	1	-1	1.633	12.929±0.435	12.784	1.975	18.926±0.393	18.514
10	1	-1	1	1.702	11.950±0.437	11.793	1.879	18.365±0.422	18.260
11	-1	1	1	1.615	18.098±0.630	17.908	2.019	22.298±0.434	22.583
12	0	-1.3	0	1.716	19.399±0.574	19.690	1.903	19.175±0.447	19.236
13	0	0	0	1.772	22.004±0.706	21.796	2.103	21.162±0.447	21.055
14	0	0	0	1.772	21.805±0.478	21.796	2.103	21.012±0.477	21.055
15	-1	-1	-1	1.837	21.964±0.721	21.752	1.808	19.321±0.479	19.266
16	0	0	1.3	1.741	18.365±0.557	18.623	1.996	20.816±0.498	20.831
17	1	1	1	1.743	11.807±0.450	11.794	2.045	19.379±0.499	19.296
18	-1	1	-1	1.464	22.099±0.691	22.032	1.945	20.122±0.548	20.302
19	0	0	0	1.772	21.889±0.679	21.796	2.103	21.052±0.630	21.055
20	0	1.3	0	1.499	19.633±0.590	19.873	2.099	20.571±0.722	20.583

T: Temperature (°C); W: Microwave power; *UI*: Ultrasound intensity; *i*: irradiation time (min); *s*: sonication time (min).

3.2. Mathematical models and statistical analysis

The full quadratic models were generated in Minitab 17 [21] to represent the mathematical expressions of the WSPs yields and the effects and interactive effects of their corresponding extraction factors. The models for WSPs yields by MAE and UAE were illustrated in (Eqs. 7 and 8) respectively. ANOVA results for the MAE and UAE (Table 3) illustrated the high values of the R^2 and R^2a_{dj} that indicated the suitability of the models while the values of the R^2_{pred} represented the high ability of predictability of the generated models. The accuracy of the models were represented by the p values (> 0.05) of the lack-of-fit tests.

$$Y_{(\text{MAE quadratic})} = 21.796 - 4.2679T + 0.0701W - 0.7812i - 3.268T^2 - 1.192W^2 - 1.276i^2 + 0.428T.W + 0.783T.i - 0.497W.i \quad (7)$$

$$Y_{(\text{UAE quadratic})} = 21.0580 - 1.2689T + 0.5180UI + 0.7659s - 0.0088T^2 - 0.6760UI^2 - 0.7199s^2 + 0.1551T.UI - 0.3748T.s - 0.1019UI.s \quad (8)$$

3.3. MAE extraction factors and their interactive effects on the WSPs yields (%)

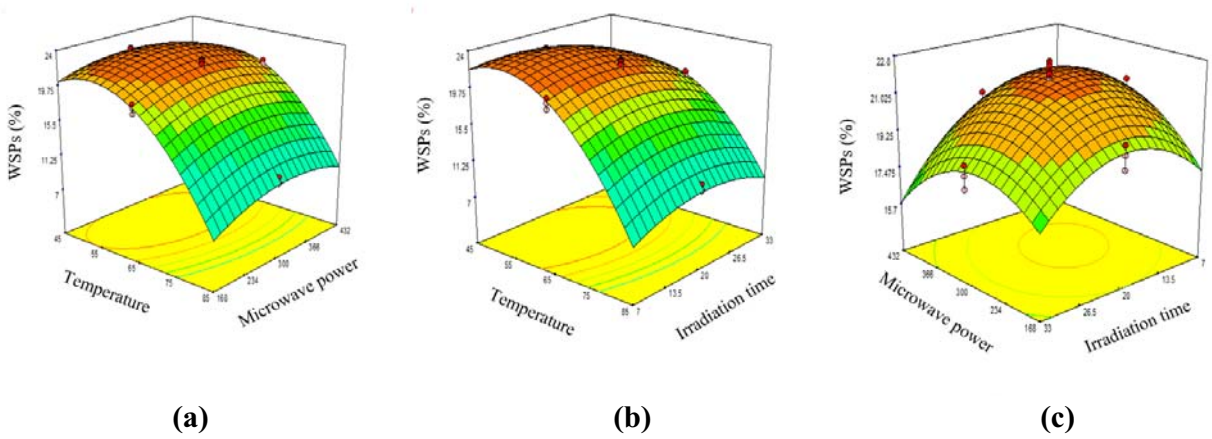
3.3.1. Extraction temperature in MAE. Heat in MAE is known to dissipate volumetrically inside the irradiated medium [8] and is affected by the nature of the reactants and the geometry of the microwave vessel [28]. The study illustrated that the yields (Figures 2.a. and 2.b.) increased with the increase of the temperature until an optimum temperature (54°C) was reached. This might due to the drop in surface tension and viscosity that enhanced the solvent capability to solubilize solutes and improved the matrix wetting and penetration [29]. However, the exceeding temperatures over the optimum caused decreases in the yields due to the overheating effect on denaturation of the thermolabile proteins [7].

3.3.2. Applied microwave power. Microwave power is an indispensable factor that is related to the quantity of sample and the required extraction time [8]. Surface plots (Figures 2.a. and 2.c.) illustrated that the increasing of the power generally improved the extraction rate and increased the yields until the optimum power (301 W) was reached. Further increasing of the power caused insignificant increases or declines in the yields due to thermal degradation due to temperature escalations [29].

3.3.3. Irradiation time of MAE. MAE facilitated the application of short extraction durations [30] when compared to previous studies that adopted conventional methods [25; 31]. The 3D surface plots (Figures 2.b. and 2.c.) illustrated that the short irradiation times possessed a positive effect on increasing the yields [32] until 15 minutes which agreed with Chan et al. (2011) [30] while prolonged durations illustrated decreases in the yields due to the degradation of the targeted compounds by the microwave overheating [33].

Table 3. ANOVA results for MAE and UAE of WSPs yields.

Statistical terms		MAE				UAE			
Source	*Df*	*SS*	*MS*	*p* value	Source	*Df*	*SS*	*MS*	*p* value
Model	9	1053.70	117.08	<0.05	Model	9	114.09	12.68	<0.05
T: Temperature (°C)	1	621.86	621.86	<0.05	*T*: Temperature (°C)	1	54.97	54.97	<0.05
W: Microwave power	1	0.17	0.17	0.473	*UI*: Ultrasound intensity	1	9.16	9.16	<0.05
i: Irradiation time	1	20.83	20.83	<0.05	*s*: Sonication time	1	20.03	20.02	<0.05
T^2	1	214.86	214.86	<0.05	T^2	1	0.02	0.02	0.929
W^2	1	28.58	28.58	<0.05	UI^2	1	9.19	9.193	<0.05
i^2	1	32.78	32.78	<0.05	s^2	1	10.43	10.426	<0.05
T.W	1	4.39	4.39	<0.05	*T.UI*	1	0.58	0.58	0.089
T.i	1	14.73	14.73	<0.05	*T.s*	1	3.37	3.37	<0.05
W.i	1	5.93	5.93	<0.05	*UI.s*	1	0.25	0.25	0.260
Error	50	16.03	0.32		Error	50	9.59	0.19	
Lack-of-Fit	5	1.96	0.39	0.299	Lack-of-Fit	5	0.72	0.14	0.608
Pure Error	45	14.07	0.31		Pure Error	45	8.88	0.19	
Total	59	1069.73			Total	59	123.69		
R^2		0.9850			R^2		0.9224		
R^2_{adj}		0.9823			R^2_{adj}		0.9085		
R^2_{pred}		0.9779			R^2_{pred}		0.8883		



(a) **(b)** **(c)**

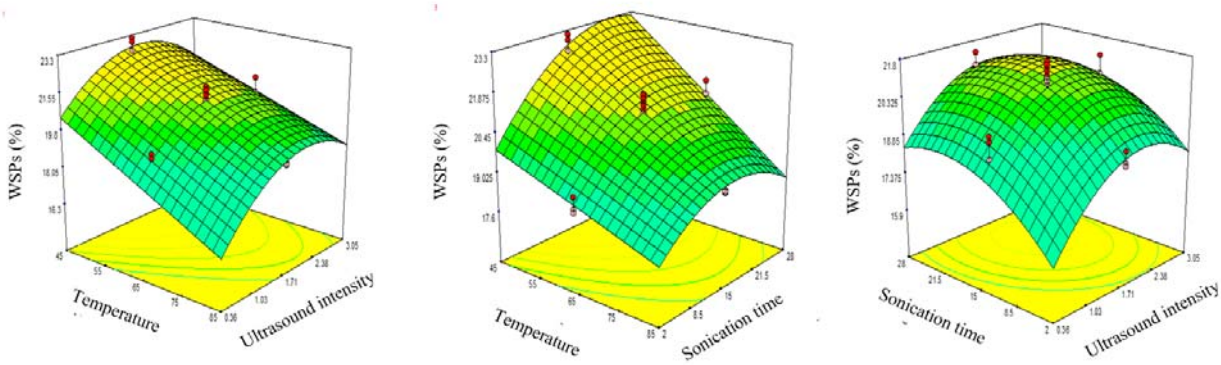
Figure 2. The interactive effects of the MAE factors on the WSPs yields (%). (a) Temperature and Microwave power; (b) Temperature and irradiation time; (c) Microwave power and irradiation time.

3.4. UAE extraction factors and their interactive effects on the WSPs yields (%)

3.4.1. Extraction temperature in UAE. Determination of a suitable temperature is important for a proficient UAE process to avoid degradation of the bioactive compounds [34]. In UAE, the temperature was considered as a complex UAE factor [35] of the dissipated heat from the cavitation phenomenon and the introduced heat from the hot plate [3]. Surface plots (Figures 3.a. and 3.b.) illustrated the quadratic effect of temperature as yields increased with the initial increase of the temperature until optimum temperature (46^{o}C) was reached and then followed decreases with higher temperatures. This was previously explained by the inducement of low vapour pressure at low temperatures that allowed stronger collapses of the cavitation bubbles [36]. On the contrary, high temperatures caused increases in the vapor pressure that might have caused the filling of the voids with water resulting in gentle collapses of the cavitation bubbles [37] that might have weaken the penetration mechanism of UAE on the plant material and eventually decrease the mass transfer rate. Another effect might be related to the effect of heat on the proteins physical properties, as the increasing heat caused thermal treatments can cause structural alterations and hydrolysis of the peptide bonds which subsequently impact the protein functionality [38] and eventually its solubility [39].

3.4.2. Effect of ultrasound intensity. The selection of the sonication regiments should be considered for the efficiency of the cavitation phenomenon [36], therefore, sonication effect was adjusted by the amplitude and duty cycle. To avoid the misleading of evaluating the ultrasound power efficiency only by the amplitude and duty cycle, the ultrasound intensity was estimated as in Eq. (3). The 3D plots (Figures 3.a. and 3.c.) illustrated the increase of the yields with the increase of the ultrasound intensity until it reached 1.84 W.cm^{-2}, followed by decreases of the yields with exceeding intensification. The effect of ultrasound intensity on the yields was previously considered as a complex effect of temperature, amplitudes and duty cycles. It increases proportionally with the decrease of temperature due to the low vapor pressure. On the other hand, it increases with the increase of amplitude and duty cycle but attenuates by high amplitudes and elongated pulse durations due to the gentle collapses of the cavitation bubbles [3; 37].

3.4.3. Sonication time of UAE. One of the efficiencies of UAE is the short extraction durations that consume less time and energy [5] when compared with conventional extraction methods. Surface plots (Figures 3.b. and 3.c.) revealed the quadratic effect of sonication time as the yields increased proportionally with time until 24 minutes, then started to decrease. This was due to the escalation of temperature through the sonication time [24] which could lead to degradation by overexposure to heat [35].



(a) **(b)** **(c)**

Figure 3. The interactive effects of the UAE factors on the WSPs yields (%). (a) Temperature and ultrasound intensity; (b) Temperature and sonication time; (c) ultrasound intensity and sonication time.

3.5. Experimental validation of the extraction optimized conditions of MAE and UAE for the WSPs yields
The response optimizer in Minitab 17 was employed to identify the combination of predictor values that jointly maximize the WSPs yields of MAE and UAE. For MAE, the predicted optimizing conditions (*T*: 54°C; *W*: 301W and; *i*: 15 min) corresponded to a predicted yield of 23.529% while for UAE, the optimizing conditions (*T*: 46°C; *UI*: 1.84W.cm^{-2} and *s*: 24 min) corresponded to the predicted yield 23.257%. Validation tests (*N*=3) for MAE and UAE conditions led to the WSPs yields of 23.101±1.647% and 24.181±0.321% respectively. Both results illustrated no significant differences ($p < 0.05$) which proved the validity of the designed model in this study.

The comparison between the obtained WSPs yields by the MAE and UAE optimized conditions (Figure 4) illustrated that the extraction rate of UAE was higher than that of MAE in the first 10 minutes; this might due to rapid rupture of the cell walls at low temperature (46°C) [13] while in MAE, the extraction process is series of phenomenological steps through the interaction period between the water and the protein-containing particle [40; 41].

This suggested that UAE was a more reliable technique to initiate WSPs extraction. However, the highest yields obtained by MAE was after 15 minutes while the highest yields by UAE was around 25 minutes. Therefore, a serial combination or a sequential technique of both extraction methods was suggested to overcome this two observations and to obtain high yields at shorter time by the higher initiation capability of UAE and the slightly-delayed high mass transfer phenomenon of MAE.

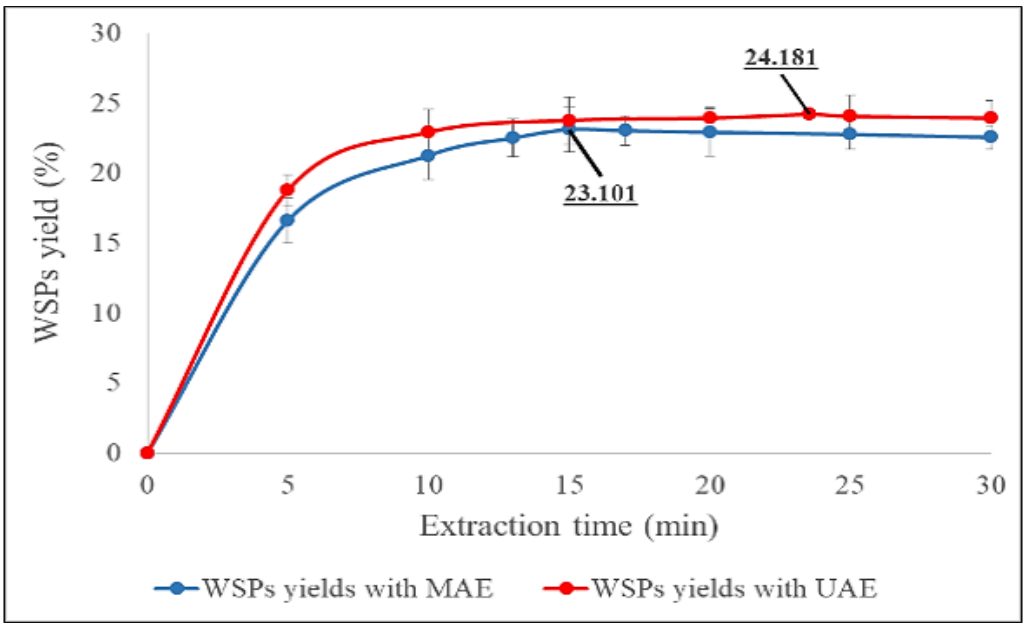


Figure 4. Comparison between the WSPs yields obtained by the optimized conditions of MAE (blue) and UAE (red)

3.6. Sequential ultrasound microwave assisted extraction (SUMAE)

The significance of MAE and UAE efficiencies is the provision of short extraction times accompanied with less energy consumption [5; 6]; this fact invited the investigation of combining UAE and MAE that proved to be an attractive extraction technique exhibiting the highest efficiency [9; 14]. Therefore, the study employed the optimum conditions to establish two types of sequential extraction; sequential microwave-ultrasound assisted extraction (SMUAE) and sequential ultrasound-microwave assisted extraction (SUMAE). The initiation phase of the extraction was considered an important phase for comparing the extraction methods; therefore, two initiating phases that differ by their timing were investigated, the first was 5 minutes and the second was 10 minutes followed by the treatment phase that extended the extraction durations to 30 minutes (Table 4).

Table 4. SMUAE and SUMAE phases and corresponding highest yields of WSPs.

Extraction method	Initiation phase		Treatment phase		Highest yield %	Total time
	Method	Time	Method	Time		
SMUAE	MAE	5 min	UAE	25 min	25.243±0.853%	25 min
	MAE	10 min	UAE	20 min	24.126±0.811%	20 min
SUMAE	UAE	5 min	MAE	25 min	26.481±0.911%	20 min
	UAE	10 min	MAE	20 min	27.172±1.086%	15 min

The experiments indicated the preference of sonication for 10 min as a pre-treatment followed by the rapid heating of the microwave that increased the rupture process and enhanced the release of the targeted compounds into the surrounding solvent [9]. The effect of the MAE was clear with the sudden increase in

the extraction rate after the first 5 minutes of irradiation; which corresponded to the highest yields of all the experiments (27.172±1.086%) while the highest yield by SMUAE was 25.243±0.853% after 5 minutes of irradiation followed by 20 minutes of sonication (Figure 5). Increasing the initiating irradiation phase to 10 minutes led to lower yields due to the exceeding sonication time [35] or by thermal impacts [42]; however, it is preferred to apply sonication in initiating the extraction processes to facilitate the metabolite extraction [43]. The results also illustrated that prolonged irradiation time should be avoided due to the decomposition and degradation [15]. The study concluded that when comparing the outcomes of the various extraction methods in this study, the SUMAE was the most efficient method to the obtained WSPs yields with shorter extraction time; however, further kinetic studies should be conducted due to the lack of reports regarding the optimization of SUMAE [14].

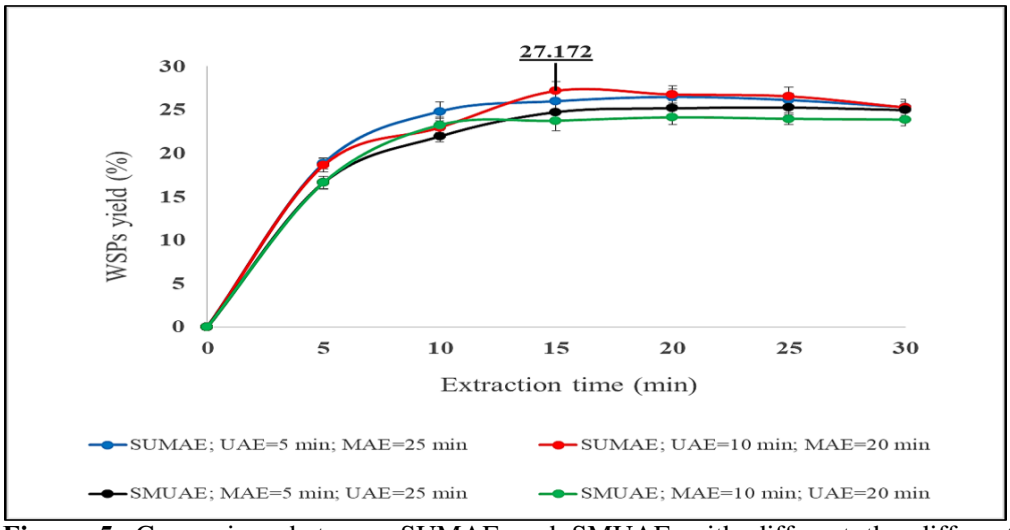


Figure 5. Comparison between SUMAE and SMUAE with different the different initiation and extraction phases.

3.7. Upscaling sequential extraction method

Necessary solutions for improving the herbal–based industry sector are required for the production of high quality nutraceutical and food supplements. As the products of *E. longifolia* are highly demanded. The implementation of green extraction technology accompanied with enhancements of combining the nonconventional techniques is highly demanded for low operating costs at short extraction durations. The sequential method provided a solution to a complexity of lower penetration depth of MAE [44] by initiating the process with sonication and the relatively longer extraction duration of UAE by introducing the MAE with its high mass transfer rate in the second phase. Finally, the introduction of this lab-scale sequential extraction for nonconventional methods reflected a high potentiality in furnishing the upscaling of protein extraction from Tongkat Ali plants, to pilot and industrial scales.

4. Conclusion

This study illustrated for the first time the implementation of nonconventional extraction methods UAE and MAE for extracting water soluble proteins (WSPs) from *E. longifolia* roots, followed by the attempt to combine both methods sequentially to increase the WSPs recovery and overcome possible shortcomings of the extraction methods. The study established the sequential extraction by studying the MAE and UAE individually to predict the optimum condition for maximizing the WSPs. Optimum condition for MAE were

T: 54ºC; *W*: 301W and *i*: 15 min and for UAE were *T*: 46ºC; *UI*: 1.84W.cm^{-2} and *s*: 24 min. The optimum conditions were employed to establish various sets of sequential extraction methods by using MAE or UAE. Results illustrated the favour of sequential ultrasound-microwave assisted extraction (SUMAE) by initiating the process by UAE for 10 minutes then followed by MAE treatment for 5 minutes. The method was nominated for upscaling in pilot and industrial scales to increase the profitability of *E. longifolia* products in the herbal-based industrial sector.

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