

## Probe Ultrasonic-Assisted Extraction of Andrographolide Rich Extract from *Andrographis paniculata*: Processing Parameters Optimization Using Response Surface Methodology

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### Abstract

The conventional extraction method has low extraction efficiency. This study aims to optimize the ultrasonic-assisted extraction conditions of andrographolide from *A. paniculata*. A ½ inch ultrasonic probe sonicator was used to extract *A. paniculata*. The effects of extraction time ( $X_1$ ), duty cycle ( $X_2$ ) and amplitude ( $X_3$ ) on the yield of andrographolide ( $Y$ ) were investigated by using response surface methodology. Experiments were designed according to 3-level 3-factor Box Behnken Design. The concentrations of andrographolide were quantified by high-performance liquid chromatography. Results of analysis of variance showed the data were well fitted with the 2<sup>nd</sup>-order polynomial equation with a  $R^2$  value of 0.9969 and  $p$ -value less than 0.005. Optimum extraction conditions were found to be at 5 min, 11 % duty cycle, and 66 A. A checkpoint experiment was carried out based on the optimum conditions, validated highest andrographolide was  $3.50 \pm 0.17$  w/w%. Response surface methodology was successfully applied to optimize the extraction processing parameters for yielding the andrographolide-rich *A. paniculata* extract. This study could be relevant for future ultrasonic-assisted extraction scale-up of andrographolide.

**Keywords:** *Andrographis paniculata*, Andrographolide, Probe ultrasonic-assisted extraction, Response surface methodology

### Introduction

*Andrographis paniculata* (Burm. P.) Nees, known as King of Bitter, is a member of the plant family Acanthaceae. This ancient medicinal plant has an extensive ethnobotanical history and can be easily found in South-East Asia, China and India. The established pharmacological effects of *A. paniculata* extract are anticancer and immunostimulatory activity [1,2], antimalarial [3,4], antiinflammatory [5], antioxidative [6], antimicrobial [7], antiviral [8,9], respiratory system benefits [10], neuroprotective [11], gastroprotective [12], hepatoprotective activity [13]. Due to its versatile bioactivities, *A. paniculata* was included in the World Health Organization (WHO) monograph of 2002 as an endorsed medicinal plant [14].

Andrographolide, a simple diterpene lactone, is the primary and most abundant bioactive constituent in *A. paniculata*. It has high antioxidant and anti-inflammatory properties [6,15]. Therefore, andrographolide is of interest to the pharmaceutical and food industries. Due to the limitation of natural resources and increasing demand for the plant-derived active compounds, the extraction optimization studies have become very crucial. Sharma and Sharma [16] compared the andrographolide yield by applying various conventional and nonconventional extraction methods. The yield of andrographolide extracted using Soxhlet extraction, reflux extraction, cold extraction, sonication assisted extraction and microwave-assisted extraction were 1.790, 2.040, 1.727, 1.867 and 1.717 % respectively. From Sharma and Sharma [16], using the reflux extraction method gave the highest yield of andrographolide followed by ultrasonic-assisted extraction method. However, the ultrasonic-assisted extraction method had a much shorter extraction time, roughly 8 times lesser, than the conventional Soxhlet extraction method. Another

study carried out by Rao and Rathod [17] combined microwave-assisted extraction with 3 phase partitioning technique to separate andrographolide from *A. paniculata*. This new extraction method yielded 3.853 w/w% of andrographolide in just 6 min compared to the conventional Soxhlet extraction method, which yielded 4.225 w/w% of andrographolide in 240 min. Therefore, the nonconventional extraction methods exhibited better extraction efficiency than the conventional methods. Other andrographolide extraction studies using nonconventional methods were supercritical fluid extraction [18], pressurized liquid extraction [19], probe ultrasonic-assisted extraction and microwave-assisted extraction [20].

Ultrasonic-assisted extraction (UAE) is a greener alternative to conventional extraction methods to extract natural products [21]. UAE requires much shorter extraction duration, higher efficiency and less power consumption than conventional extraction methods [22]. Besides, UAE has relatively lower auxiliary power, preventing bio-active compounds like andrographolide from thermal degradation during the extraction process [20]. There are 2 approaches to induce ultrasonic waves into extraction mass. One of them is by using an ultrasonic water bath where the ultrasonic waves are transmitted indirectly onto the extraction mass through the water bath. The other approach is ultrasonic probe method, which immerses the ultrasonic horn to employ the ultrasonic waves directly into the extraction mass. The latter method gives a uniform distribution of ultrasound energy, larger acoustic amplitude and power, resulting in higher extraction yield [23]. However, probe ultrasonic irradiation sharply increases the extraction temperature and induces the formation of free radicals under extreme operations, causing degradation of bio-active compounds [22]. Therefore, an optimization study on essential variables such as solvent type, duty cycle, sonication amplitude, and extraction time is critical in achieving better extraction results. Over the last few decades, numerous *A. paniculata* extraction optimization studies had been carried out, but meager scientific data available on the interaction effect of probe ultrasonic-assisted extraction variables on the yield of andrographolide [21-23].

Response surface methodology (RSM) is a powerful statistical-based experimental design tool to optimize processes. The advantages of using RSM are the reliability to reduce the number of experimental trials needed, ability to evaluate multiple parameters and analyze their interactions on response variables. Thus, it is less laborious and time-saving. In addition, RSM establishes mathematical equations, generates and portrays the data in 3-dimensional graphs, and locates the region where the extraction is optimized. This study attempted to apply RSM to optimize the probe UAE processing parameters of *A. paniculata* by investigating the combined influence of 3 independent variables, namely extraction time ( $X_1$ ), duty cycle ( $X_2$ ) and ultrasonic amplitude ( $X_3$ ), on the andrographolide yield as the dependent variable ( $Y$ ).

## Materials and methods

### Chemicals and materials

Methanol (ACS grade, HPLC grade) was purchased from Merck, 95 % ethanol (ACS grade) was purchased from QreC and dimethyl sulfoxide (ACS grade) was purchased from RCI Labscan and were used without any purification. The crystallized andrographolide 98 % from Sigma-Aldrich was used as standard. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Life Technologies. *Andrographis paniculata* was purchased from a local supplier in Johor, Malaysia. The voucher specimen (SBID 002/13) was deposited in the Herbarium of Forest Research Institute Malaysia (FRIM). The aerial part was dried at  $50 \pm 2$  °C oven and pulverized into powder with size  $< 0.05$  mm.

### HPLC analysis

Analyses of andrographolide content in the extract were performed by high-performance liquid chromatography (HPLC), equipped with Waters 600 controller as pump and Waters 2484 Photodiode Array Detector as the detection system. LiChrosorb RP-18, ODS column was used (5  $\mu$ m particle size, 250 $\times$ 4.6 mm, Merck, Germany). The mobile phase was methanol/water (volume ratio 3:2) at isocratic elution with a constant flow rate of 0.7 mL/min. The sample volume injected was 20  $\mu$ L and the eluent was detected at 223 nm. The calibration curve was constructed using a series of andrographolide standard solutions of known concentration. Five mg of crude extract was dissolved in 5 mL of methanol/water (volume ratio 3:2) and was filtered through a 0.45  $\mu$ m nylon membrane prior injected into the HPLC system [24].

### Ultrasonic-assisted extraction of *Andrographis paniculata*

The extraction of *A. paniculata* was done using the method described by Foujdar *et al.* [25] with slight modification. A ½ inch ultrasonic probe sonicator (40 kHz, Fisher FB705 sonic dismembrator) was used. The setting of amplitude of the equipment was from minimum 10 A to maximum 100 A. Three g of powdered *A. paniculata* was added in 50 mL of extraction solvent (solid to liquid ratio of 1:17) in a 100 cm<sup>3</sup> beaker. The horn of the ultrasonic probe was submerged 3 mm under the surface of the solution. After ultrasonic irradiation, the solution was filtered using vacuum filtration. The filtrate was concentrated using a rotary evaporator followed by drying in an oven at 50 ± 2 °C.

### Single-factor extraction study

Four extraction parameters were studied in this single factor preliminary extraction study, namely types of solvent, the amplitude of ultrasonic power, extraction duration and duty cycle. The dependent parameter is the yield of andrographolide. The effect of each parameter was determined by varying 1 parameter and keeping the others constant. All experiments were triplicated. A 2-way analysis of variance was performed to determine the significance of the parameters. The threshold for *p*-values is 0.05.

In order to examine the effect of solvent, different solvents such as water, methanol and ethanol at several concentrations (95, 75 and 45 %), were tested for optimum yield of andrographolide. The effect of amplitude (10, 30, 50, 70 and 90 A) on the extraction yield was examined using 75 % ethanol, 5 min sonication and 100 % duty cycle. The effect of extraction duration was conducted by using 75 % ethanol as extraction solvent under ultrasonic irradiation for 1, 2, 3, 4 and 5 min with 100 % duty cycle and amplitude at 40 A. The effect of duty cycle was done under 10, 30, 50, 70 and 100 % duty cycle with 10 s cycle time at 40 A using 75 % ethanol under ultrasonic irradiation for 5 min.

### Experimental design

Response Surface Methodology (RSM) along with Box-Behnken design (BBD) (Design-Expert software, version 12.0.9.0, Stat-Ease, Inc., Minneapolis, MN) was applied to study the regression and graphical analysis of experimental data and optimize the ultrasonic-assisted extraction of *A. paniculata* by investigating the combined influence of 3 independent variables which are extraction time ( $X_1$ ), duty cycle ( $X_2$ ) and ultrasonic amplitude ( $X_3$ ) on the andrographolide yield as the dependent variable. Each factor varied over 3 levels, consisting a set of points lying at the midpoint of each edge and 5 replicates of the design center point for experimental error determination, giving a total of 17 runs (Table 1). This experiment was designed by adapting the set-up used by [24]. The experiment order was randomized to minimize the effects of unknown variability in the response due to extraneous factors. The optimized extraction conditions simulated by the software was used to run as checkpoint analysis. The experimental results were further verified and compared with the predicted results.

### Statistical analysis

All experiments were triplicated and the results were expressed in mean ± standard deviation. A statistical analysis of variance (ANOVA) was performed for statistical analysis. The threshold for *p*-values is 0.05. Other statistics were calculated using Microsoft Excel 2007 (Microsoft, Redmond, WA).

**Table 1** Box-Behnken Design with the actual and predicted values of andrographolide yield.

Run	Independent variables and levels			Yield of andrographolide (w/w%)	
	$X_1$ , time (min)	$X_2$ , duty cycle (%)	$X_3$ , amplitude (A)	Actual values	Predicted values
1	-1 (1)	-1 (10)	0 (55)	2.57 ± 0.14	2.54
2	1 (5)	-1 (10)	0 (55)	3.56 ± 0.22	3.6
3	-1 (1)	1 (100)	0 (55)	2.18 ± 0.34	2.14
4	1 (5)	1 (100)	0 (55)	3.38 ± 0.22	3.41
5	-1 (1)	0 (55)	-1 (10)	1.94 ± 0.04	1.97
6	1 (5)	0 (55)	-1 (10)	3.17 ± 0.25	3.14
7	-1 (1)	0 (55)	1 (100)	2.09 ± 0.22	2.12
8	1 (5)	0 (55)	1 (100)	3.32 ± 0.28	3.28
9	0 (3)	-1 (10)	-1 (10)	3.08 ± 0.13	3.08
10	0 (3)	1 (100)	-1 (10)	2.75 ± 0.23	2.75
11	0 (3)	-1 (10)	1 (100)	3.19 ± 0.09	3.19

Run	Independent variables and levels			Yield of andrographolide (w/w%)	
	$X_1$ , time (min)	$X_2$ , duty cycle (%)	$X_3$ , amplitude (A)	Actual values	Predicted values
12	0 (3)	1 (100)	1 (100)	2.93 ± 0.32	2.94
13	0 (3)	0 (55)	0 (55)	3.23 ± 0.22	3.22
14	0 (3)	0 (55)	0 (55)	3.23 ± 0.23	3.22
15	0 (3)	0 (55)	0 (55)	3.20 ± 0.20	3.22
16	0 (3)	0 (55)	0 (55)	3.25 ± 0.22	3.22
17	0 (3)	0 (55)	0 (55)	3.18 ± 0.29	3.22

Results were expressed in mean ± S.D, n = 3.

## Results and discussion

### Single-factor extraction study

A single factor preliminary extraction study on *A. paniculata* was carried out to refine the intervention and evaluate the extraction efficiency of different types of solvent, amplitude, extraction duration and duty cycle for yielding high andrographolide extract. All 4 parameters in the screening experiments were significant ( $p < 0.05$ ).

From **Figure 1(a)**, the crude extract using water produced the highest yield of  $14.24 \pm 0.28$  %. The higher the concentration of ethanol or methanol as extraction solvent, the lower the yield of the crude extract. The crude extract yields for methanol and ethanol extraction were almost similar. In contradiction, among the 3 different extraction solvents, water extraction only yielded  $0.11 \pm 0.019$  w/w% of andrographolide, which was the lowest among all. Methanol and ethanol solvent extraction yielded  $0.65 \pm 0.019$  and  $0.56 \pm 0.012$  w/w% of andrographolide for 95 % methanol and 95 % ethanol respectively,  $0.53 \pm 0.024$  and  $0.64 \pm 0.012$  w/w% for 75 % methanol and 75 % ethanol respectively, and  $0.33 \pm 0.035$  and  $0.38 \pm 0.031$  w/w% by using 45 % methanol and 45 % ethanol as extraction solvent respectively. The crude extracts extracted using a higher percentage of methanol or ethanol were greenish. As the percentage of water in the extraction solvent increases, the crude extracts turned brownish-green while the crude extracts extracted using water were observed as dark brown. Kumoro *et al.* [26] reported the same observation with this study.

Andrographolide is a highly polarized molecule because of the aromatic delocalized electrons. Efficiencies of these extraction solvents were attributed to their polarities. The smaller the gap of polarity difference between the solvent and andrographolide, the better the molecular affinity between them, resulting in a greater extraction yield of the targeted phytochemical. According to Kumoro *et al.* [26], the Hildebrand solubility parameter ( $\delta$ ) of andrographolide was 14.80,  $\delta$  values of methanol, ethanol and water were 14.45, 12.90 and 23.40, respectively. Based on the  $\delta$  value, andrographolide is more soluble in methanol than in ethanol and hardly dissolve in water.

Furthermore, the lower yield of andrographolide in aqueous extract presumably because water has a higher boiling point than other organic solvents, so a higher temperature was achieved during the extraction process, causing degradation of the extracted compound. High pressure and temperature during ultrasonic cavitation generated reactive species such as hydroxyl radicals in the presence of water, causing possible oxidative degradation of andrographolide [27]. Degradation of andrographolide usually occurs by opening the lactone ring, but different solvents will cause different structural destruction mechanisms. Ultrasonic extraction using water as solvent opens the lactone ring by hydrolysis, but ethanol and methanol open the lactone ring by transesterification [26,28]. Hydrolysis is a faster reaction than transesterification. Therefore, all of the factors mentioned above had resulted in a lower extraction yield of andrographolide using water as an extraction solvent.

The Hildebrand value of a solvent mixture can be calculated by averaging the Hildebrand value of the individual solvents by its volume [29]. Adding a small amount of water in ethanol will alter the  $\delta$  of the mixture to be nearer or equal to  $\delta$  value of andrographolide hence enhancing the solute solubility and increasing the concentration of andrographolide in the extract [30]. As for methanol, the addition of water changes the  $\delta$  of the mixture to be higher than andrographolide hence lowering the solute solubility and yield of andrographolide. In [30] study, extraction of *A. paniculata* with 85 % ethanol yielded the most andrographolide compared to 20 and 52.5 % ethanol.

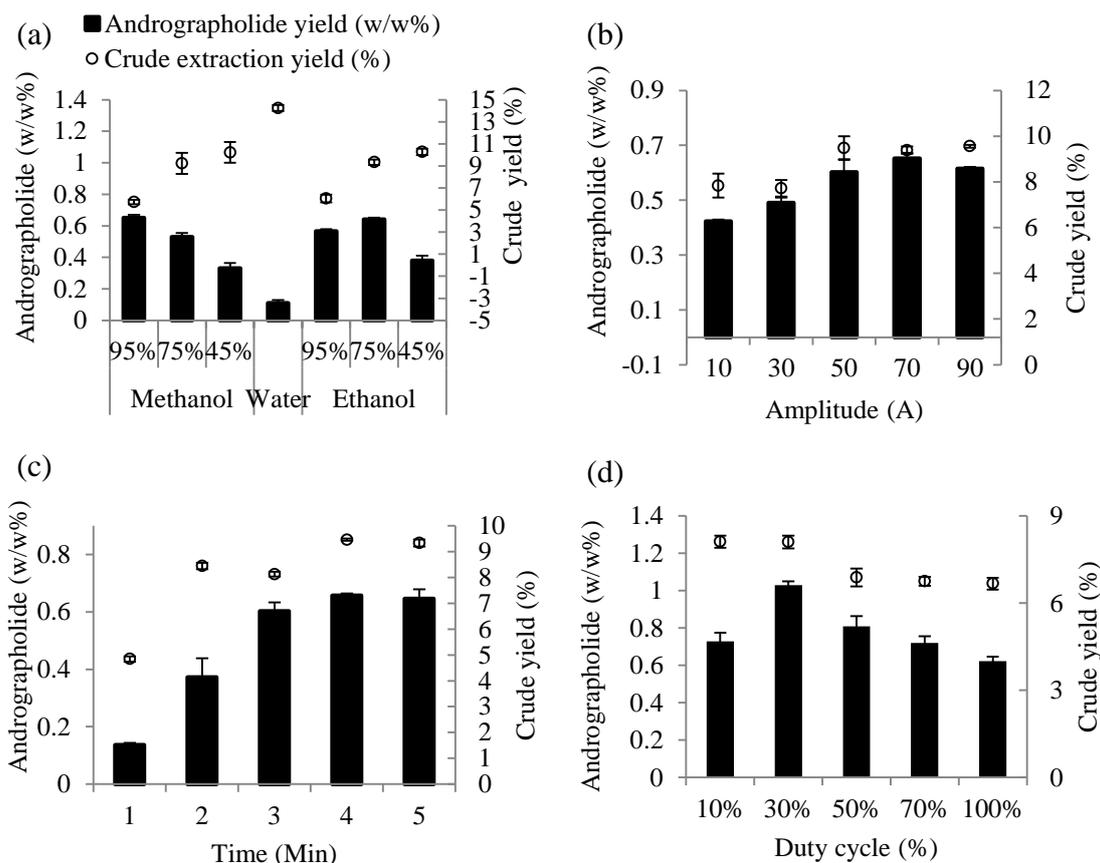
Andrographolide is considered a pharmaceuticals compound, therefore, the amount and types of solvent used are tightly regulated to prevent environmental hazards and toxicity in human during production. According to the Guideline for Residual Solvent (Q3C) published by The International

Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH), solvents are divided into 4 groups, from the 1<sup>st</sup> group (Class 1) being the most toxic and carcinogenic to the last group (Class 4) being no adequate toxicology data. Methanol is classified under Class 2 solvents with limited usage because of their inherent toxicity, whereas ethanol is classified under Class 3 with low toxic potential [31]. Wongkittipong *et al.* [32] concluded that the percentage of ethanol and water mixture did not affect the kinetics of extraction but only the concentration of andrographolide in the extracts. Under such considerations, 75 % ethanol was chosen as the best extraction solvent in this study. It was used for later RSM study because it is a relatively greener solvent with better environmental, health and safety properties while not compromising the target compound extraction yield.

**Figure 1(b)** shows the yield of the crude extract and andrographolide extracted under different ultrasonic amplitudes. 10 A and 30 A gave almost similar crude extract yields, and reached a plateau after 50 A. Andrographolide yield marked an increase from  $0.42 \pm 0.007$  w/w% at 10 A to  $0.65 \pm 0.020$  w/w% at 70 A, subsequently dropped slightly to  $0.62 \pm 0.006$  w/w% at 90 A. The extraction temperature recorded in this study from 10 A to 70 A was below  $75 \pm 2$  °C, while amplitude above 70 A resulted in extraction temperature elevated above  $75 \pm 2$  °C. Moderate increase in temperature was found to accelerate the extraction process by increasing the solubility of the solute. Moreover, sonication under moderate temperature formed lesser bubbles that imploded at a relatively higher intensity, causing microturbulence in the vicinity of solid. This phenomenon increases the mass transfer efficiency of the extraction [33,34]. In contrast, when sonication under temperature near to the boiling point of the solvent, formation of bubbles was easier but imploded with less intensity, eventually gave rise to lower extraction efficiency [35]. Similar findings were reported by [36]. The optimum yield of andrographolide was achieved at extraction temperature of 70 °C and any further increase beyond this temperature decreased the yield [36].

The effect of extraction time on crude extract and andrographolide yield is shown in **Figure 1(c)**. Extraction yield typically increases with the duration of extraction up to a certain threshold. The optimum yield of crude extract and andrographolide were both achieved under 4 min of extraction, and the extraction duration beyond 4 min did not significantly change the extraction yield. During the extraction process, the ultrasonic irradiation created a cavitation effect to mechanically rupture the plant cell, increasing the solvent's permeability to extract the active compounds from *A. paniculata*, until an equilibrium between the solute concentrations in a solution and the solid matrix had reached. Hence, a further increase in extraction time will not increase the yield. This phenomenon obeyed Fick's 2<sup>nd</sup> law of diffusion [39]. A control experiment was carried out to validate the effect of UAE of andrographolide by maceration for 5 min under the same extraction conditions. The yield of andrographolide from maceration extract was only  $0.05 \pm 0.021$  w/w%, proving that UAE significantly improved the andrographolide extraction rate.

**Figure 1(d)** illustrates the effect of UAE duty cycle on crude extract and andrographolide yield. The highest crude extraction yield was at 10 % duty cycle, and the crude extract yield gradually decreased with the increasing duty cycle. Meanwhile, the highest andrographolide yield ( $1.03 \pm 0.025$  w/w%) was recorded at 30 % duty cycle. Increasing duty cycle from 30 % decreased the andrographolide yield. Pulsed sonication with proper resting intervals was found to be more efficient to extract bioactive compounds than continuous sonication. Pulsed sonication is more energy efficient, has better control in temperature to avoid thermal degradation and less erosion to the ultrasound probe tip [40]. During pulsed sonication, degassing bubbles were suppressed during the resting period, as a result, less crowded and bigger cavitation bubbles were formed. These bigger bubbles stored higher energy, causing more violent shear force acting on the plant cells during its implosion [41]. This finding was in agreement with the foregoing reports by [40,42].



**Figure 1** Single factor screening results for (a) effect of extraction solvent at fixed extraction time 5 min, 100 % duty cycle and amplitude 40 A; (b) effect of amplitude at fixed extraction time 5 min, 100 % duty cycle and 75 % ethanol; (c) effect of extraction time at fixed extraction solvent 75 % ethanol, 100 % duty cycle and amplitude 40 A; (d) effect of extraction duty cycle at fixed extraction solvent 75 % ethanol, extraction time 5 min and amplitude 40 A, on the UAE of *A. paniculata*. Data are presented as mean standard errors of triplicate measurements.

#### Extraction optimization by Response Surface Methodology (RSM)

The results are shown in **Table 1**. Experimental data were well fitted with regressed quadratic polynomial model and the equation with a regression coefficient of the coded parameters was shown in Eq. (1).

$$Y = 3.22 + 0.5828X_1 - 0.1445X_2 + 0.074X_3 + 0.0525X_1X_2 - 0.0002X_1X_3 + 0.0169X_2X_3 - 0.3277X_1^2 + 0.0327X_2^2 - 0.2635X_3^2 \quad (1)$$

Where  $Y$  is the yield of andrographolide (w/w%);  $X_1$  is time of extraction;  $X_2$  is duty cycle and  $X_3$  is amplitude.

#### Model fitting

**Table 2** shows analysis of variance (ANOVA) of the regression model. The  $F$ -value of 247.11 and  $p$ -value less than 0.0001 implied that this model was significant. There was only 0.01 % probability of error occurring due to noise. Lack of fit analysis showed that  $F$ -value was 3.71 and  $p$ -value was 0.1187. The result revealed that the lack of fit was insignificant relative to the pure error.

The correlation coefficient ( $R^2$ ) of the model was 0.9969, indicated the regression models provided excellent model fitting to explain all the relationships between the independent factors and response. The model could explain about 99.69 % of the total variability within the studied range. Adjusted  $R^2$  is the corrected value for  $R^2$  after eliminating the unnecessary model terms. The remarkably close values, < 0.2 difference, of predicted  $R^2$  values (0.9618) to the adjusted  $R^2$  (0.9928) advocating both of the  $R^2$  was in

reasonable agreement. Likewise, the value of adjusted  $R^2$  was very close to the value of  $R^2$ , indicating a statistically good fitted model. Moreover, the C.V. value of 1.38 % was within the acceptable range. This exceptionally low C.V. value indicated a very high degree of precision and a good deal of reliability of the experimental values. The ratio value of “Adeq Precision” is another way to evaluate a model. The “Adeq Precision” measures the signal (detection) to noise (estimated standard deviation) ratio. Based on the correlation of coefficient value, a ratio greater than 4 is desirable. In this model, the ratio was 51.8738, indicating an adequate signal. This model can be used to navigate the design space and predict the optimum UAE conditions to maximize the yield of andrographolide.

The regression coefficients on the response variable of the linear and quadratic effects of independent variables are shown in Eq. (1). The positive and negative sign preceding the terms represent synergistic and antagonistic effects on the extraction yield of andrographolide. The linear effect of extraction time, duty cycle and amplitude were significant to affect the extraction yield of andrographolide from *A. paniculata* with  $p$ -value  $< 0.0001$ . Extraction time and amplitude showed a positive effect on the extraction yield while duty cycle had a significant negative linear effect on the extraction yield. A longer sonication enables more cavitation to disrupt the plant cell wall, enhancing the internal diffusion and mass transfer of andrographolide from the plant into the extraction solvent. A higher sonication amplitude, on the other hand, enables intense cavitation with stronger shear forces exerted on plant cells during extraction. However, there is an optimal point for both variables. The lower the percentage of duty cycle, the longer the resting period to prevent overheating scenario. Interaction terms of  $X_1X_2$ ,  $X_1^2$  and  $X_3^2$  were significant to impact the yield of andrographolide.

**Table 2** Analysis of variance (ANOVA).

Sources of variations	Sum of squares	Degree of freedom	Mean square	F-value	p-value
Model	3.73	9	0.414	247.11	$< 0.0001^{***}$
$X_1$ – Time	2.72	1	2.72	1622.04	$< 0.0001^{***}$
$X_2$ – Duty Cycle	0.1669	1	0.1669	99.65	$< 0.0001^{***}$
$X_3$ – Amplitude	0.0443	1	0.0443	26.47	0.0013**
$X_1X_2$ – Time×Duty Cycle	0.011	1	0.011	6.57	0.0373*
$X_1X_3$ – Time×Amplitude	1.81E-07	1	1.81E-07	0.0001	0.992
$X_2X_3$ – Duty Cycle×Amplitude	0.0011	1	0.0011	0.6835	0.4356
$X_1^2$ – Time×Time	0.4522	1	0.4522	269.95	$< 0.0001^{***}$
$X_2^2$ – Duty Cycle× Duty Cycle	0.0045	1	0.0045	2.68	0.1455
$X_3^2$ – Amplitude×Amplitude	0.2923	1	0.2923	174.48	$< 0.0001^{***}$
Residual	0.0117	7	0.0017	-	-
Lack of Fit	0.0086	3	0.0029	3.71	0.1187
Pure Error	0.0031	4	0.0008	-	-
Cor Total	3.74	16	-	-	-

Note:  $***p < 0.0001$ ,  $**p < 0.005$  and  $*p < 0.05$ .

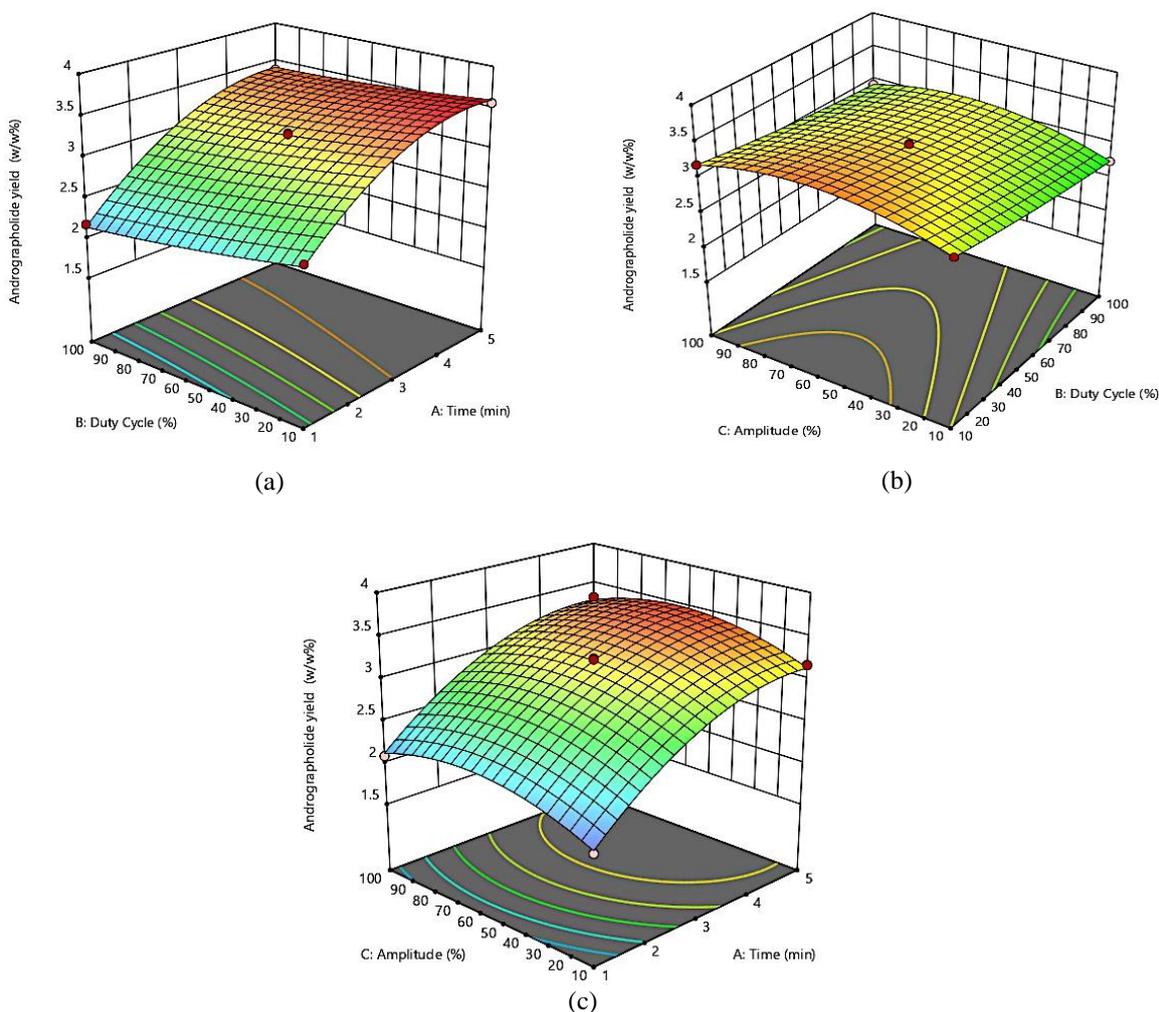
### Analysis of response surface plots

The effects of the parameters and their interactions on response are displayed in the 3-dimensional response surface and contour plot in **Figures 2(a)** to **2(c)**. Only interactions between time and duty cycle were significant ( $p < 0.05$ ) on the yield of andrographolide. **Figure 2(a)** shows the relationship between time and duty cycle on andrographolide extraction yield and amplitude constant at 55 A. The yield of andrographolide was substantially elevated with increasing UAE time from 1 to 5 min. However, the andrographolide yield was negatively correlated with extraction duty cycle. The maximum yield of andrographolide given was 3.56 w/w% at 5 min extraction duration and 10 % duty cycle. The minimum yield of andrographolide predicted was 2.18 w/w% at 1 min and 100 % duty cycle. With duty cycle 100 %, the yield of andrographolide reaches a plateau after 4 min of extraction time.

After 4 min of UAE, the system reaches its equilibrium. However, when the duty cycle was set at 10 %, the equilibrium of the UAE was shifted to yield higher andrographolide concentration extract. In continuous sonication, ultrasound propagation was disturbed by a large cluster of degassing bubbles formed, hence reducing the cavitation efficiency [41]. Intensified cavitation resulted in a more efficient

erosion and rupture of the plant cells to enable higher solute concentration in the extract. Many previous reports showed pulsed sonication resulted in better extraction yield of bioactive compounds, but the optimum duty cycle reported varied from 25 up to 80 %. The optimum duty cycle found to extract bio-compounds reported by [40,42] was 25 to 30 %, [43,44] was 60 % and [38,45] was 80 %.

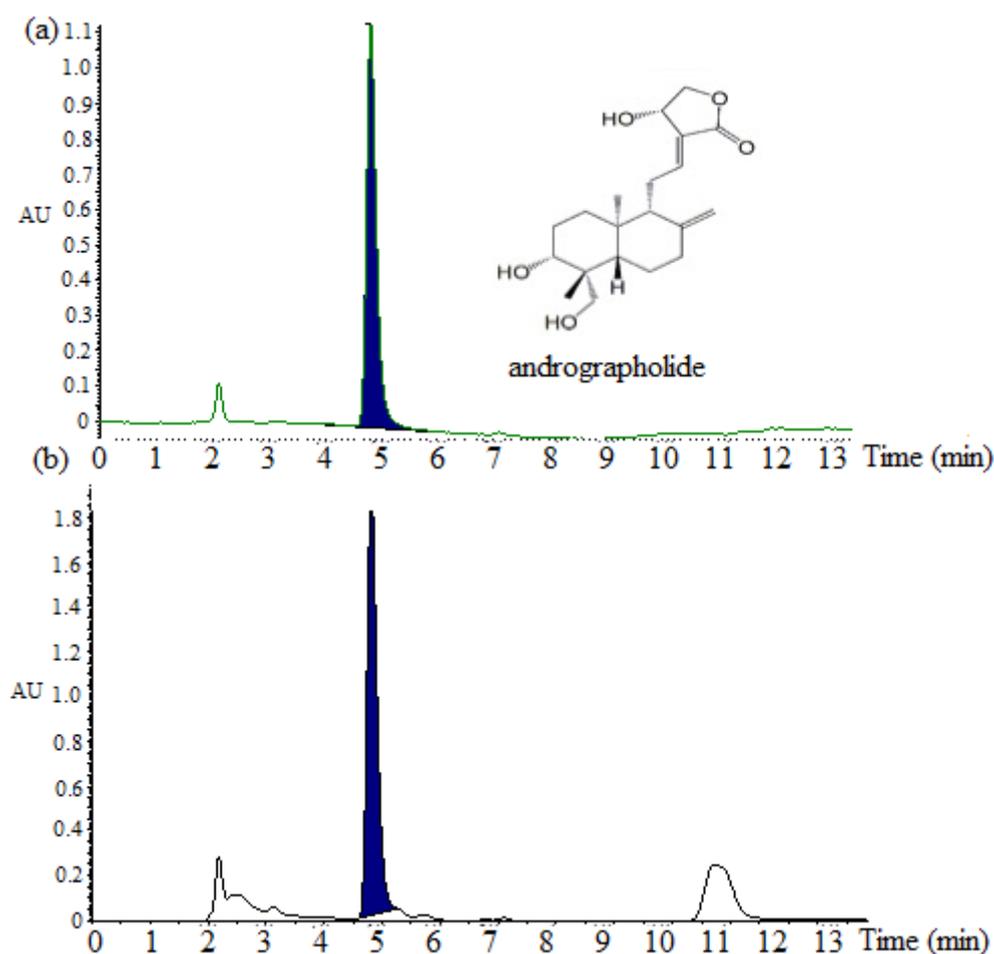
**Figure 2(b)** illustrates the interaction between duty cycle and ultrasonic power amplitude on andrographolide extraction yield with time kept constant at 3 min. Meanwhile, **Figure 2(c)** illustrates the relationship between ultrasonic power amplitude and time on andrographolide extraction yield with duty cycle constant at 55 %. Both contour plots showed elliptical shape. From **Figure 2(b)**, the UAE system with 10 % duty cycle and 60 A yielded the highest andrographolide at 3.39 w/w%, while 100 % duty cycle and 10 % amplitude yielded the least at 2.75 w/w%. From **Figure 2(c)**, ultrasonic amplitude at 60 A and 5 min extraction duration yielded a maximum concentration of andrographolide at 3.48 w/w%. Extraction conditions at 1 min and 10 A yielded the lowest andrographolide of 1.94 w/w%. Despite the independent terms for all the studied parameters being significant to affect the yield of andrographolide, but the interactions in **Figures 2(b)** and **2(c)** were insignificant ( $p > 0.05$ ). Hence, no further discussion on insignificant terms.



**Figure 2** Response surface and contour plots of andrographolide extraction yield at variables: (a) Duty cycle and extraction time; (b) Duty cycle and ultrasonic power amplitude and (c) Time of extraction and ultrasonic power amplitude.

### Model verification

A checkpoint analysis was carried out to ensure the BBD model predicted results agreed with the experimental value. The optimum *A. paniculata* extraction conditions to yield the highest andrographolide proposed by this model with the highest desirability value was 4.6 min extraction duration, 11 % duty cycle and 65.83 A to yield 3.605 w/w% of andrographolide. The validation experiment was carried out with slight modification to the extraction conditions. The extractions were performed in triplicate under 5 min extraction time with 11 % duty cycle and 66 A. The maximum andrographolide yield from the experiment was  $3.50 \pm 0.17$  w/w%, found to be very close to its predicted value of 3.605 w/w%, amounting to only 2.78 % of deviation. The amount of andrographolide in the extract was quantified using a standard response curve of andrographolide standard and the HPLC chromatograms are shown in **Figure 3**. The optimized conditions generated from this model to predict the UAE andrographolide from *A. paniculata* were statistically reliable and accurate. The andrographolide yield in this study was higher than the result published by [21] using the same extraction technique. 2.01 w/w% andrographolide was obtained under solid-liquid ratio of 1:20, 50 % aqueous ethanol, the amplitude of 50 % for 10 min by [21] as compared to  $3.50 \pm 0.17$  w/w% yield under solid-liquid ratio of 1:17, 75 % aqueous ethanol, 66 A and 5 min extraction time with 11 % duty cycle in this study. However, the study by [21] did not perform extraction optimization.



**Figure 3** HPLC chromatogram of (a) andrographolide standard and (b) crude extract of *A. paniculata*.

## Conclusions

This study has shown that response surface methodology (RSM) along with Box-Behnken design (BBD) was successfully applied to optimize the UAE processing parameters that produced a higher andrographolide yield. This greener UAE method achieved  $3.50 \pm 0.17$  w/w% andrographolide from *A. paniculata* under 75 % ethanol as extraction solvent, 5 min extraction time with 11 % duty cycle and 66 A. This study lays the groundwork for the future UAE scale-up of andrographolide from *A. paniculata*.

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