Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
www.phcog.com | www.phcog.net

ORIGINAL ARTICLE

Optimization of Ultrasonic-Assisted Extraction of Flavonoids and Anti-oxidant Capacity from the Whole Plant of *Andrographis echioides* (L.) Nees by Response Surface Methodology and Chemical Composition Analysis

Jayalakshmi Ramasamy, Ruckmani Kandasamy, Selvamani Palanisamy, Subramanian Nadesan

National Facility for Drug Development (NFDD), Department of Pharmaceutical Technology, Anna University, BIT Campus, Tiruchirappalli, Tamil Nadu, India

Submitted: 20-12-2018 Revised: 31-01-2019 Published: 19-09-2019

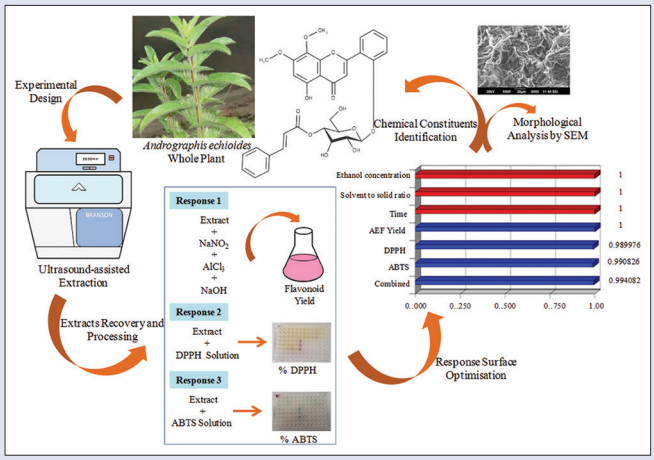
ABSTRACT

Background: *Andrographis echioides* (L.) Nees is an annual herb mainly distributed in India and Sri Lanka. In traditional medicine system, the plant is used for treating various ailments such as fevers, skin diseases, stomach ache, toothache, snake bite, and eczema. The whole plant of *A. echioides* was reported as the rich source of flavonoids. Ultrasound-assisted extraction (UAE) is an effective extraction method used for secondary metabolite extraction from various plant materials over conventional methods. Today, the response surface methodology (RSM) is a successful statistical tool used to optimize the various extraction conditions of the secondary metabolite from various sources. **Objective:** The objective of this study is to optimize the UAE conditions such as ethanol concentration (50%–100%), solvent-to-solid ratio (10–50 mL/g), and sonication time (20–60 min) for the extraction of flavonoids and anti-oxidant capacity (AOC) from *A. echioides* (L.) Nees whole plant (AEWP) using the RSM strategy with Box–Behnken design (BBD). **Materials and Methods:** UAE conditions, i.e. ethanol concentration, solvent-to-solid ratio, and sonication time, were optimized with the corresponding responses of flavonoid yield and $\%DPPH_{AOC}$ and $\%ABTS_{AOC}$ by RSM. The effect of ultrasound on plant material was analyzed using Scanning electron microscope (SEM). The efficiency of the optimized extract was analyzed using Fourier-transformed infrared spectroscopy (FTIR) and liquid chromatography-mass spectra (LC-MS). **Results:** The BBD provided adequate mathematical models that accurately describe the behavior of the technique and help to predict the flavonoid yield, $\%DPPH_{AOC}$ and $\%ABTS_{AOC}$ from AEWP. The optimized UAE conditions were 77% of ethanol concentration, 35 mL/g of solvent-to-solid ratio, and 41 min of sonication time. Under these extraction conditions, UAE would obtain a maximum of 10.91 ± 0.04 mg CE/g for flavonoid yield, 87.36 ± 0.06% for $\%DPPH_{AOC}$, and 85.14 ± 0.03% for $\%ABTS_{AOC}$. The obtained experimental results of all the responses are in good agreement with the predicted values. SEM analysis explores the effect of UAE compared with the conventional extraction. The FTIR and LC-MS analysis revealed that the optimized extract of AEWP is rich in flavonoids; apart from the known flavonoids, five new flavonoids were identified from this optimization study. **Conclusion:** The study confirmed that UAE was the effective extraction method for the extraction of flavonoids from AEWP with ethanol as a solvent of choice with a low solvent usage in a reasonable time.

Key words: *Andrographis echioides*, anti-oxidant capacity, Box–Behnken design, flavonoids, optimization, ultrasound-assisted extraction

SUMMARY

- The response surface optimization study states that ultrasound-assisted extraction was the effective method for flavonoids extraction from *Andrographis echioides* (L.) Nees whole plant.



Abbreviations used: RSM: Response surface methodology; BBD: Box–Behnken design; AEWP: *Andrographis echioides* (L.) Nees whole plant; AOC: Anti-oxidant capacity; SEM: Scanning electron microscope; FTIR: Fourier-transform infrared spectroscopy; LC–MS: Liquid chromatography–mass spectroscopy; ESI: Electron spray ionization; ANOVA: Analysis of variance; CL: Confidence level; CE:(+)-catechin; DPPH: 2,2-diphenyl- 1-picrylhydrazyl; ABTS: 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid).

Correspondence:

Dr. Selvamani Palanisamy,
National Facility for Drug Development (NFDD),
Department of Pharmaceutical Technology, Anna University, BIT Campus,
Tiruchirappalli - 620 024,Tamil Nadu, India.
E-mail: selva.autpharma@gmail.com

DOI: 10.4103/pm.pm_647_18

Access this article online
Website: www.phcog.com
Quick Response Code:

INTRODUCTION

Andrographis echioides (L.) Nees (Acanthaceae), also known as *Indoneesiella echioides* (L) Nees or *Justicia echioides*, is an annual herb mainly distributed in India and Sri Lanka.[1] Conventionally, it has been used to treat various ailments including fevers, skin diseases, stomachache, toothache, snake bite, and eczema and it has remarkable pharmacological activity as it is hepatoprotective, antipyretic, antiulcer, anti-inflammatory, antimicrobial,

Cite this article as: Ramasamy J, Kandasamy R, Palanisamy S, Nadesan S. Optimization of ultrasonic-assisted extraction of flavonoids and anti-oxidant capacity from the whole plant of *Andrographis echioides* (L.) nees by response surface methodology and chemical composition analysis. Phcog Mag 2019;15:547-56.

anti-oxidant and an analgesic as well. All these beneficial special effects are currently believed to be connected to the fact that *A. echioides* contain medicinally active phytoconstituents.[2-5] Earlier, the phytochemical profile of this genus was studied quite well in the perspective of its importance in traditional Indian medicine and has been reported to be rich in flavonoids.[6,7] Recently, several flavonoid compounds have been reported as found in the whole plant extract of *A. echioides*. It has been revealed that flavonoids are the major constituents of this species similar to other species of this genus and the above-mentioned beneficial effect may be because of the flavonoid composition.[8-12] Hence, it is necessary to utilize the most efficient extraction method for flavonoid extraction.

Recent studies reported that ultrasound-assisted extraction (UAE) highly enhances the extraction efficiency of secondary metabolites from various plant materials, such as polyphenols from olive leaf,[13] flavonoids from *Portulaca oleracea*,[14] anthraquinones from *Morinda citrifolia* roots,[15] carnosic acid from rosemary leaves,[16] and isoflavones from soybeans.[17] The extraction efficiency has been greatly improved with the help of ultrasonics by decreasing the processing time and organic solvent usage. In addition, the UAE can be performed at a lower temperature; preventing thermal degradation of compounds in the sample extracts, and this enhances the extraction yield with its preserved functional properties. These augmentations in the UAE have been connected to cavitation forces generated from ultrasonics because the mechanical effects produced by acoustic cavitation damage the cell walls of plant tissues as well as improve the mass transfer rate of the cell's contents to the extraction medium.[18-20] Apart from these, the extraction efficiency of the UAE has been influenced by some common parameters, such as the selection of solvent, percentage of solvent used, solvent to sample ratio, time contact, and operating temperature.[21] Therefore, statistical optimization is required to determine the most significant parameters on the UAE.

Response surface methodology (RSM) is a successful statistical technique that has been used to optimize the UAE from various plant materials, such as melatonin from red rice,[22] phenolics from yarrow *Achillea beibrestinii*,[23] anthocyanins from fully ripened haskap berries,[24] phenolics, flavonols, and anti-oxidant capacity (AOC) from grape pomace[25] and polysaccharide content from *Paeonia emodi*.[26] This phenomenon is described so that RSM can be applied to analyze the effect of more than two process variables and their interactions on the response function in the UAE. It has the advantages of being in expensive and less time consuming with the statistical interpretation of data over the conventional methods.[27] Thus, RSM is the foremost method adapted in optimization studies today.

At present, the isolation and identification of new drug lead from natural source as the emerging file in medicine. In the present work, the main objective was to determine the optimal UAE conditions for the extraction of flavonoid and AOC from *A. echioides* using RSM. The efficacy of UAE was evaluated using scanning electron microscope (SEM). Further, the flavonoids present in the optimized extract were analyzed spectroscopically.

MATERIALS AND METHODS

Plant material

A. echioides (L.) Nees whole plant (AEWP) was collected from the Sri Adhivaraganallur village in Cuddalore District, Tamil Nadu, India, in August 2015 and identified and authenticated by Dr. M. Palanisamy, Botanical Survey of India (BSI), Southern Regional Centre, Coimbatore, India (voucher specimen no. BSI/SRC/5/23/2013-14/Tech/1921) and the voucher specimens were deposited at the university department. The collected plant material was cut into small pieces, dried at room temperature under a shaded area for 7–8 days, and then ground to a powder using an electrical mill. Finally, the powdered plant material was sieved (60-mesh) and used for further experiments.

Chemicals and reagents

Ethanol and methanol used in this study were of analytical grade. Potassium persulphate ($K_2S_2O_8$) was procured from HiMedia Company (Mumbai, India). 2,2-diphenyl-1-picrylhydrazyl (DPPH), (+)-catechin, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), sodium nitrite ($NaNO_2$), sodium hydroxide (NaOH), aluminum chloride ($AlCl_3$), and a membrane filter, 0.45 µm, were acquired from Sigma-Aldrich Company (Bengaluru, India). Whatman No. 1 filter paper was sourced from local suppliers.

Ultrasound-assisted extraction

UAE was conducted using a rectangular Branson ultrasonic cleaning bath CPX3800H-E (Branson, USA) device. The ultrasonic device consists of a rectangular container with the overall size of 40.64 cm × 30.48 cm × 36.83 cm, internal tank size of 29.21 cm × 15.24 cm × 15.24, fixed working frequency of 40 kHz (transducers annealed to the bottom), the input power of 230 W and equipped with digital operating control. Table 1 shows the experimental values and coded levels used in the UAE and Table 2 shows the experimental design used in the UAE optimisation. As per the experimental design [Table 2] the UAE was performed, that is, 1 g of AEWP powder was transferred into a screw-capped glass tube, mixed with varying ethanol concentration and solvent-to-solid ratio. Then, the mixture was immersed in water in the ultrasonic device and the extraction was carried out at 40 kHz frequency and the 230 W input power with varying ultrasonic extraction time. All the experiments were carried out at room temperature as replicate. Then, the extracts were filtered by filter paper (Whatman No. 1) and centrifuged at 6000 rpm for 15 min at 4°C (5430R Centrifuge, Eppendorf, Belgium). Following the centrifugation, the supernatant was filtered with 0.45 µm syringe filter and stored in a refrigerator at 4°C for further analysis.

Determination of flavonoid yield

The flavonoid yield was determined spectrophotometrically according to the method described by Zhishen *et al.*[28] and then expressed as milligram (+)-catechin equivalent (CE) per gram of AEWP dry weight (mg CE/g). In a test tube, 1 mL of sample extract, 4 mL of distilled water, and 0.3 mL of $NaNO_2$ (1:20, w/v) were added and incubated for 5 min. Then, 0.3 mL of $AlCl_3$ (1:10, w/v) was added and again incubated for 6 min. Then, 2 mLof 1 M NaOH was added and made up to 10 mL with distilled water. Immediately, the absorbance was measured at 510 nm using a spectrophotometer (UV-2600, Shimadzu Asia Pacific Pte. Ltd., Singapore). The flavonoid yield was calculated from the standard (+)-catechin calibration curve regression equation of $Y = 0.02636x + 0.06486$ with the $R^2 = 0.9973$. All the tests were conducted in triplicate, and the data were given as mean ± standard deviation (SD).

Anti-oxidant capacity

Assay of DPPH$_{AOC}$

The $DPPH_{AOC}$ of the AEWP extracts was performed using the method of Zielinski *et al.*[29] with some alterations. The methanolic solution containing (190 µL, 0.004%) DPPH free radical was added to 10 µL of AEWP sample extracts and incubated for 30 min at room temperature in the dark. Subsequent incubation the absorbance was measured at 517 nm using a spectrophotometer, microplate reader (Perkin Elmer, USA). The inhibition percentage of the sample extract on DPPH radical cations was determined by Equation (1):

$$\%\ DPPH_{AOC} = \frac{A_c - A_s}{A_c} \times 10 \quad (1)$$

Table 1: Experimental values and coded levels of the process variables used for optimisation

Process variables	Units	Symbol	Coded levels		
			−1	0	+1
Ethanol concentration	%	X_1	50	75	100
Solvent to solid ratio	mL/g	X_2	10	30	50
Sonication time	min	X_3	20	40	60

Where, A_s is the absorbance of the sample extracts and A_c is the absorbance of the control solution containing the methanolic DPPH free radical. All the experiments were performed in triplicate, and the data were given as mean ± SD.

Assay of ABTS$_{AOC}$

The $ABTS_{AOC}$ was carried out according to the method of Dahmoune *et al.*[30] with some modification. For the production of ABTS radical cations, the equal volume of 7 mM ABTS and 2.45 mM potassium persulfate were mixed and allowed to stand in the dark for 13 h at room temperature before use. Further, the concentrated ABTS stock solution was diluted with methanol to obtain 0.700 ± 0.005 absorbance at 734 nm. Then, 10 µL of diluted sample extract and 190 µL diluted ABTS stock solution were added to a 96-well plate, and the decrease in the absorbance was measured exactly after 5 min at 734 nm using a multimode microplate reader (Perkin Elmer, USA). The inhibition percentage of the sample extracts on ABTS radical cations was determined using Equation (2):

$$\%\ ABTS_{AOC} = \frac{A_c - A_s}{A_c} \times 100 \quad (2)$$

where, A_s is the absorbance of the sample extracts and A_c is the absorbance of the control solution containing ABTS radical cations. All the experiments were performed in triplicate, and the data were given as a mean ± SD.

Experimental design

RSM with Box–Behnken design (BBD)[31] was applied statistically to optimize the UAE with high flavonoid yield, $DPPH_{AOC}$ and $ABTS_{AOC}$ from AEWP. The ethanol concentration ($X1$), solvent-to-solid ratio ($X2$) and sonication time ($X3$) were taken as the process variables coded according to the following equation,

$$x_i = \frac{X_i - X_0}{\Delta X_i}, x_i = 1,2,3 \quad (3)$$

where x_i and X_i are the dimensionless and the real value of the process variable i; X_0 is the real value of the process variable i at the center point; and ΔX_i is the increment/step change of X_i corresponding to a one-unit variation of xi.

The experimental design was constructed using Design Expert (v. 7.1.3 trial version) software and the data are shown in Table 2. A second-order polynomial regression equation was used to correlate the relationship existing between the three, and they were coded at three levels: 1, 0, and +1 [Table 1]. The three process variables were process variables and the three response functions and the quadratic equation is given by,

$$Y = \beta_0 + \sum_{i=1}^{3}\beta_i X_i + \sum_{i=1}^{3}\beta_{ii} X_i^2 + \sum_{\substack{i=1 \\ i<j}}^{2}\sum_{j=2}^{3}\beta_{ij} X_i X_j + \varepsilon \quad (4)$$

where Y is the response function, β_0 is the intercept, β_i, β_{ii} and β_{ij} are the linear, quadratic and cross-product/second-order interaction coefficients of the coded variables, and ε is a random error. The final polynomial equation used for the optimization process is,

$$Y_n = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (5)$$

Where Y_n predicted response functions (Y_1 to Y_3), X_1, X_2 and X_3 are the process variables, β_0 is the constant, β_1, β_2, β_3, β_{11}, β_{22}, β_{33} and β_{12}, β_{13}, β_{23} are the linear, quadratic and cross-product coefficients, respectively. The significance of the model was evaluated by the coefficient of the determinant (R^2) and the analysis of variance (ANOVA). Based on these statistical analyses, the nonsignificant terms ($P > 0.05$) of the model were eliminated and only the significant terms of the model with $P < 0.05$ were fitted. Finally, Derringer and Suich (1980)[32] desirability function were used for the simultaneous optimization.

Validation of the model

The final optimized conditions of the process variables for the response functions were validated using the same experimental procedure as mentioned earlier. All the experiments were performed in triplicate and the obtained experimental values were compared with the theoretical predicted values of the model to establish the validity of the model.

Scanning electron microscope

The SEM analysis was performed to study the morphological changes in the AEWP powder before and after UAE compared with the conventional extraction (by heat reflux extraction in 80% (v/v) of ethanol, at 60°C for 5 h). The investigation was carried out using scanning electron microscope (JEOL JSM-6390 LV, JEOL Ltd., USA). Samples were mounted on stubs and coated with a thin layer of gold then the analysis was performed with an accelerating voltage of 20 kV under high vacuum condition.

Fourier-transform infrared spectra

Preliminary characterization of the optimized AEWP extract was performed by FTIR. KBr powder was mixed with the optimized extract in order to make 1% (w/v) concentration of slurry and the KBr pellet was prepared by pressuring around 5.5 tons for 3 min. Then the measurements were carried out on a JASCO FT/IR-6300 instrument (JASCO Corporation, Tokyo, Japan) at a resolution of 4 cm^{-1} and the spectra were recorded over the IR range of 400–4,000 cm^{-1}.

Liquid chromatography–mass spectra

Qualitative analysis of the optimized AEWP extract was performed using a Waters Acquity TQD Mass spectrometer coupled to Waters Acquity QSM pumps (Waters, Milford, MA, USA). The separation was performed using an Accucore C_{18} UHPLC column (100 mm × 3 mm i.d., 2.6 µm d; Accucore) and a Waters Acquity PDA detector (UPLC LG 500 nm) was used. The secondary metabolites present in the extract were identified using electrospray ionization in positive ion mode at the scan range of 50-1000 m/z. The mobile phase compositions were: (A) water + 0.5% formic acid (B) methanol: acetonitrile: formic acid (50:50:0.5% (v/v)). The linear gradient elution was started at 90:10 (A: B) and changed to 10:90 (A: B) with the flow rate of 0.2 mL/min for a total run time of 20 min. The column temperature was held at 30°C. The approximate sample volume injected into the column was 20 µl. Other parameters include source temperature of 120°C and desolvation temperature of 350°C. Capillary voltage, extractor voltage and cone voltage were set at 3.50 kV, 3 V, and 30 V, respectively.

Table 2: Experimental design with observed and predicted values of flavonoid yield and anti-oxidant capacity of extracts from *Andrographis echioides*

Run number	Process variables (coded values with actual values)			Response functions					
	X_1 (%)	X_2 (mL/g)	X_3 (min)	Response 1 (Y_1) Flavonoid yield (mg CE/g)		Response 2 (Y_2) DPPH (%)		Response 3 (Y_3) ABTS (%)	
				Observed*	Predicted	Observed*	Predicted	Observed*	Predicted
1	0 (75)	0 (30)	0 (40)	10.96±0.01	10.93	86.83±0.04	86.64	85.64±0.04	84.68
2	−1 (50)	0 (30)	−1 (20)	5.23±0.12	5.30	72.36±0.12	72.03	68.75±0.02	68.76
3	0 (75)	0 (30)	0 (40)	10.88±0.01	10.93	86.52±0.01	86.64	84.31±0.05	84.68
4	0 (75)	−1 (10)	1 (60)	3.47±0.03	3.52	72.48±0.03	72.06	66.74±0.08	66.71
5	0 (75)	−1 (10)	−1 (20)	4.95±0.05	4.86	74.57±0.03	74.60	70.90±0.04	71.33
6	−1 (50)	0 (30)	1 (60)	4.84±0.04	4.77	73.95±0.04	74.06	71.52±0.09	71.98
7	1 (100)	−1 (10)	0 (40)	2.81±0.06	2.83	68.50±0.08	68.58	64.24±0.03	64.27
8	−1 (50)	1 (50)	0 (40)	6.27±0.07	6.25	72.10±0.07	72.02	70.18±0.09	70.15
9	1 (100)	0 (30)	−1 (20)	4.96±0.04	5.03	74.86±0.05	74.75	73.01±0.06	72.55
10	1 (100)	1 (50)	0 (40)	5.26±0.05	5.25	78.69±0.04	78.38	76.80±0.05	77.23
11	0 (75)	1 (50)	−1 (20)	7.25±0.02	7.20	75.78±0.07	76.19	73.14±0.03	73.16
12	0 (75)	0 (30)	0 (40)	11.01±0.01	10.93	87.15±0.06	86.64	85.06±0.06	84.68
13	0 (75)	0 (30)	0 (40)	10.85±0.02	10.93	86.76±0.05	86.64	83.82±0.01	84.68
14	0 (75)	0 (30)	0 (40)	10.90±0.07	10.93	85.94±0.11	86.64	84.55±0.11	84.68
15	1 (100)	0 (30)	1 (60)	3.74±0.11	3.66	74.00±0.05	74.33	72.56±0.07	72.55
16	−1 (50)	−1 (10)	0 (40)	3.20±0.10	3.21	71.64±0.06	71.95	67.42±0.02	66.99
17	0 (75)	1 (50)	1 (60)	6.55±0.03	6.64	80.37±0.08	80.34	81.43±0.06	81.00

*Response data are expressed as the mean (n=3)±SD. X_1 (%): Ethanol concentration (%); X_2 (mL/g): Solvent to solid ratio (mL/g); X_3 (min): Sonication time (min). SD: Standard deviation; DPPH: 2,2-diphenyl-1-picrylhydrazyl; ABTS: 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)

Table 3: Regression coefficients of predicted quadratic polynomial models for flavonoid yield and anti-oxidant capacity from *Andrographis echioides*

Model term	Regression coefficients estimated[a]		
	Flavonoid yield (mg CE/g)	DPPH (%)	ABTS (%)
β_0	10.93***	86.64***	84.68***
β_1	−0.35***	0.75**	1.09**
B_2	1.36***	2.47***	4.03***
β_3	−0.47***	0.40*	0.81*
β_{11}	−3.71***	−7.96***	−8.3***
β_{22}	−2.84***	−5.95***	−6.71***
β_{33}	−2.54***	−4.89***	−4.91***
β_{12}	−0.16*	2.43***	2.45***
β_{13}	−0.21**	−0.61*	−0.81*
β_{23}	0.19**	1.67***	3.11***
Adequacy of the mathematical model			
R^2 [b]	99.96	99.76	99.64
p (LOF)	0.1424	0.3903	0.5578
CV	1.40	0.62	0.89

[a]Significant at *P<0.05, **P<0.01, ***P<0.001, [b]Coefficient of multible variation. LOF: Lack of fit; CV: Coefficient of variation (%); DPPH: 2,2-diphenyl-1-picrylhydrazyl; ABTS: 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)

RESULTS AND DISCUSSION

Optimization of ultrasound-assisted extraction process and model suitability

A RSM strategy was performed in order to optimize the effect of ethanol concentration (X_1), solvent-to-solid ratio (X_2), and sonication time (X_3) on the flavonoid yield, $\%DPPH_{AOC}$ and $\%ABTS_{AOC}$ of AEWP during the UAE. The results of 17 runs using BBD design are presented in Table 2 that contains the experimental design with observed and predicted values of the three response functions. It shows that the observed values of the three response functions are in close agreement with the predicted values. In addition, it can be seen that the flavonoid yield ranged from 3.20 to 11.01 (mg CE/g) and AOC ranged from 71.64% to 87.15% and from 67.42% to 85.64% for $\%DPPH_{AOC}$ and $\%ABTS_{AOC}$, respectively. All the response maximum points were observed at the midpoint of the process variables (ethanol concentration of 75%, solvent-to-solid ratio of 30 mL/g, and sonication time of 40 min) indicating that the low- and high-level values of the process variables have a negative effect on the responses but the mid-point values of the process variables have a positive effect on the responses.

With the experimental data listed in Table 2, the regression analysis was performed and the second order polynomial equation (Eq. 6-8) was derived using the estimated regression coefficients presented in Table 3. The adequacy of the fitted model was validated by ANOVA and R^2 values [Table 3]. The Fisher's test P value helped to analyze the fitness of the model terms; $P < 0.05$ implying the model terms are significant. Table 3 shows that the linear, quadratic and cross-product terms of ethanol concentration, solvent-to-solid ratio and sonication time on the flavonoid yield, $\%DPPH_{AOC}$ and $\%ABTS_{AOC}$ are found to be significant ($P < 0.05$). From our analysis 99.96%, 99.76%, and 99.64% R^2 values were obtained for flavonoid yield, $\%DPPH_{AOC}$ and $\%ABTS_{AOC}$, respectively. In general, the R^2 values are statistically acceptable at a 95% confidence level (CL) but our results are significant with >95% CL, which indicates the developed model exhibits a significant adequacy. The low percentage of coefficient of variation (1.40%, 0.62%, and 0.89%) clearly suggested that the experimental values are highly precise and reliable [Table 3]. The 14.24%, 39.03%, and 55.78% of lack of fit F-values could occur due to noise and this nonsignificant lack of fit is good. To examine the interactive effects of process parameters on the responses, the three-dimensional (3D) response surface plots are shown in Figure 1. The plots help to visualize the effect of the process variables on the response functions at all levels. The shape of the plot itself indicates that the process parameters have good interactive effect between them.

Effect of ultrasound-assisted extraction conditions on flavonoid yield

Figure 1a-c shows the 3D response surface plots of flavonoid yield. The three extraction conditions on the UAE were screened, exhibiting significant ($P < 0.05$) linear, quadratic, and interactive effects on flavonoid

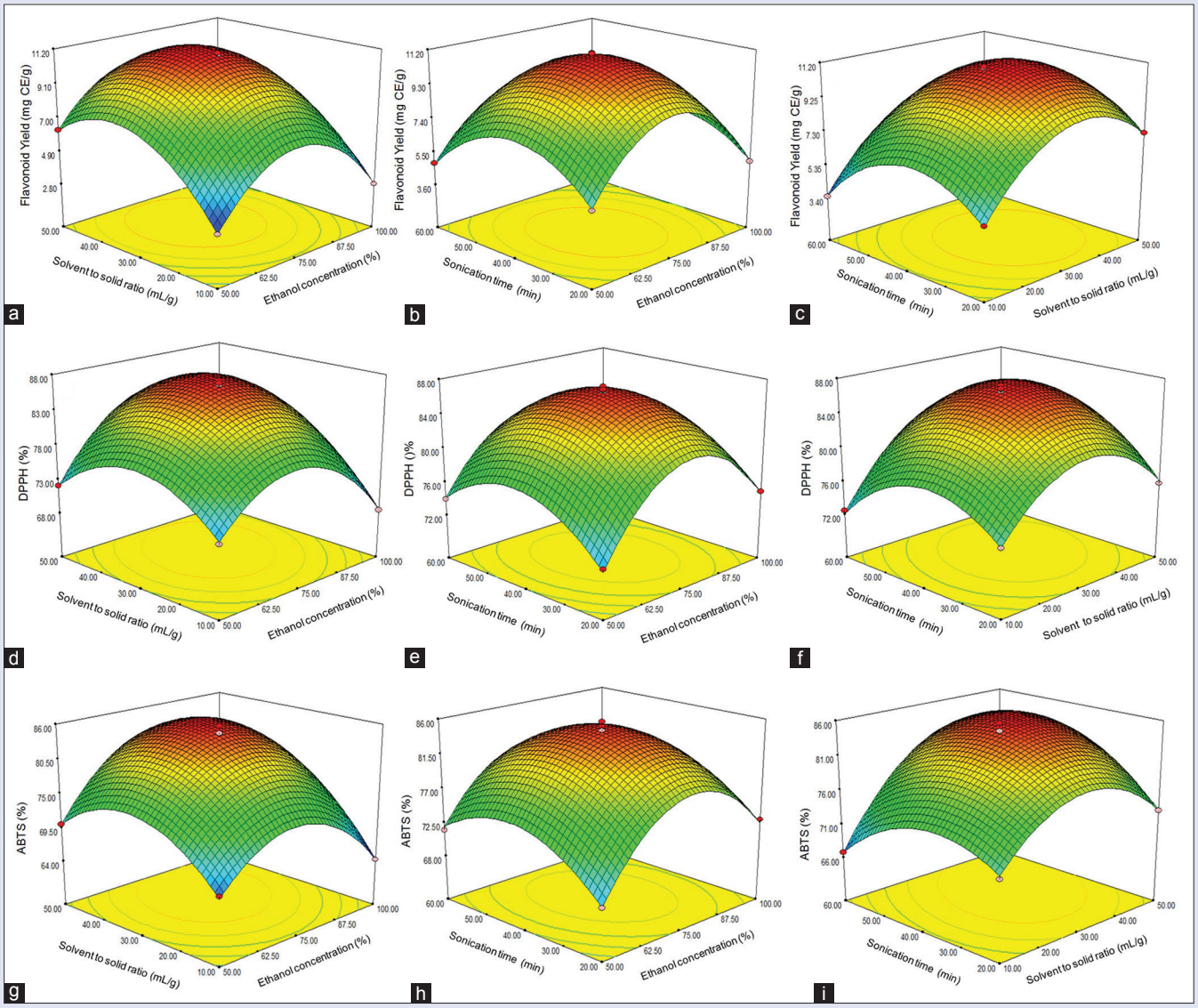


Figure 1: Three-dimentional response surface plots showing the effect of process variables on the flavonoid yield (a-c) and %DPPH (d-f) and %ABTS (g-i) anti-oxidant capacity from *Andrographis echioides*

yield. Based on the polynomial equation, the highest flavonoid yield of AEWP was predicted to be 11.01 (mg CE/g) under the UAE conditions involving 75% ethanol, a solvent-to-solid ratio of 30 mL/g with a process time of 40 min. The predicted model for the flavonoid yield of AEWP is given in the following Equation (6),

$$\text{Flavonoid yield} = 10.93 - 0.35X_1 + 1.36X_2 - 0.47X_3 - 0.16X_1X_2 - 0.21X_1X_3 + 0.19X_2X_3 - 3.71X_1^2 - 2.84X_2^2 - 2.54X_3^2 \quad (6)$$

The flavonoid yield was increased, on increasing the ethanol concentration from 50% to 75% and decreased when the concentration increased beyond 75% [Figure 1a and b]. It reveals that midpoint concentration (75%) influences the maximum flavonoid yield from AEWP. Pan *et al.* (2012) reported that 72% of ethanol was suitable for flavonoids extraction from hawthorn seed.[33] Wang *et al.* (2014) reported that the 39.01% ethanol was adequate for effective flavonoid extraction from *P. oleracea* L.[14] These differences in the solvent concentration are because of the nature of the flavonoid compounds present in the plant materials. Previously, Shen *et al.* (2013) used 85% aqueous MeOH for flavonoid extraction from AEWP and identified the number of flavonoid compounds from the extract.[12]

According to Figure 1a and c, flavonoid yield was significantly influenced by the solvent-to-solid ratio in the extraction medium; when the solvent ratio was increased from 10 to 30 mL/g the flavonoid yield was positively influenced, and further increasing the solvent ratio from 30 to 50 mL/g in the extraction medium had a negative influence on the flavonoid yield. The maximum flavonoid productivity was obtained at 30 mL/g of solvent-to-solid ratio in the extraction medium and could be due to the mass transfer process (diffusivity) in the liquid: solid UAE.[20,21]

Ultrasonic time is another crucial factor in the flavonoid extraction from AEWP. Figure 1b and c illustrates the effect of ultrasonic time on the flavonoid yield from AEWP. When the sonication time was increased from 20 to 40 min, the flavonoid yield increased, and further increasing the sonication time from 40 to 60 min the flavonoid yield decreased. Our results are supported by Wong Paz *et al.* (2015) in which 40 min of sonication time provides the maximum polyphenols productivity from Mexican desert plants.[20] Generally, the sonication time is taken as an important factor for the secondary metabolite extraction because it helps to minimize the process time with high productivity. However, when the process time exceeds a

certain limit, degradation of the compounds occur and the response is reduced.[18,21,24] This phenomenon was observed in our study too, and this may be the reason for the lower flavonoid yield at 60 min of process time.

Effect of ultrasound-assisted extraction conditions on anti-oxidant capacity

The UAE conditions greatly affect the AOC of *A. echioides*. The three-dimensional response surface plots for $\%DPPH_{AOC}$ and $\%ABTS_{AOC}$ are depicted in Figure 1d-i. In terms of the linear, quadratic and interactive effects of UAE conditions, such ethanol concentration, solvent-to-solid ratio and sonication time on $\%DPPH_{AOC}$ and $\%ABTS_{AOC}$ were found to be statistically significant ($P < 0.05$) and they were enhanced in a way similar to that observed for the flavonoid yield. According to the second order polynomial equation, the highest $\%DPPH_{AOC}$ and $\%ABTS_{AOC}$ of AEWP was predicted to be 87.15% and 85.64%, respectively, under UAE conditions involving 75% ethanol, a solvent-to-solid ratio of 30 mL/g with a process time of 40 min. The predicted models for $\%DPPH_{AOC}$ and $\%ABTS_{AOC}$ of AEWP are presented in the following equations (7 and 8),

$$\%DPPH_{AOC} = 86.64 + 0.75X_1 + 2.47X_2 + 0.40X_3 + 2.43X_1X_2 - 0.61X_1X_3 + 1.67X_2X_3 - 7.96X_1^2 - 5.95X_2^2 - 4.89X_3^2 \quad (7)$$

$$\%ABTS_{AOC} = 84.68 + 1.09X_1 + 4.03X_2 + 0.81X_3 + 2.45X_1X_2 - 0.81X_1X_3 + 3.11X_2X_3 - 8.30X_1^2 - 6.71X_2^2 - 4.91X_3^2 \quad (8)$$

Ethanol concentration significantly influences the AOC of AEWP. When the ethanol concentration increased from 50% to 75% (up to midpoint) the AOC also increased. However, further increasing the ethanol concentration from 75% to 100% (beyond the midpoint) the AOC decreased. In the case of solvent-to-solid ratio and sonication time, a response pattern similar to that of the ethanol concentration was observed on AOC. The increase of solvent-to-solid ratio and sonication time up to the midpoint value (-1 to 0) (from 10 to 30 mL/g and from 20 to 40 min) the AOC also increased and further increasing the extraction conditions beyond the midpoint (0 to + 1) (from 30 to 50 mL/g and from 40 to 60 min) the AOC decreased. Figure 1d-i explains the effect of ethanol concentration, solvent-to-solid ratio and sonication time on $\%DPPH_{AOC}$ and $\%ABTS_{AOC}$ at all levels. These observations have relative correlation with the flavonoid yield. The experimental run with the lower flavonoid yield shows the lower AOC and the experimental run with the higher flavonoid yield shows the higher AOC. Thus, the presence of flavonoids may play a significant role in AOC of AEWP.

Validation of optimized conditions and predictive model

In order to validate the suitability of the predictive model, the results of the optimized conditions were used for the extraction test of the flavonoid yield and AOC. The optimized condition to attain the maximum flavonoid yield from AEWP as well as maximum AOC, were ethanol concentration of 77%, solvent to solid ratio of 35 mL/g and sonication time of 41 min. Under the optimized conditions, the experimental values obtained for flavonoid yield, $\%DPPH_{AOC}$ and $\%ABTS_{AOC}$ were 10.91 ± 0.04 mg CE/g, 87.36 ± 0.06%, and 85.14 ± 0.03%, respectively. The obtained experimental values are in good agreement with the predicted values, which correspond to 11.01 mg CE/g, 86.96%, and 85.44%.

Morphological analysis

The AEWP cell morphology was examined by Scanning electron microscope. The significant differences observed between the untreated Sample, sample after UAE and sample of conventional solvent extraction were illustrated in Figure 2a-c. In untreated samples stomata are broadly elliptical, stomatal pore wide and elongated, epidermal cells are fairly intact and subsidiary cells are intact [Figure 2a]. Where in the UAE the stomata are affected, stomatal opening wide due to shrinkage of the guard cells, epidermal cells shrunken and their shape and size much affected, the outer surface not seen properly it was highly losing its nature [Figure 2b]. In conventional extraction, the stomata are affected and stomatal pore not seen properly, epidermal cells are affected and the outer surface of the plant cell was not seen clearly [Figure 2c]. These results suggested that UAE (41 min) are much effective than the conventional extraction (5 h).

Spectral analysis of the optimized extract

Figure 3 shows typical IR spectra for wavenumbers 400–4000 cm^{-1} of the optimized extract of AEWP. It showed a broad hydroxyl absorption band at 3391 cm^{-1}, carbonyl absorption band at 1651 cm^{-1}, aliphatic C-H stretching band at 2923 cm^{-1}, OH deformation band at 1358 cm^{-1}, and C-O stretching vibration band at 1078 cm^{-1}. Distinctive bands at

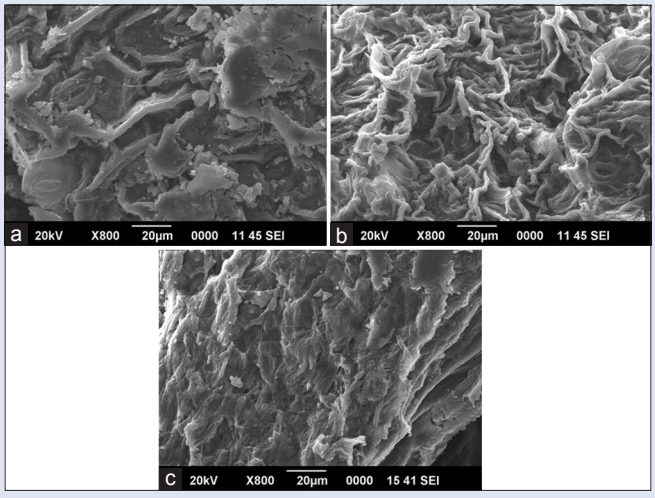


Figure 2: SEM image of *Andrographis echioides* (L.) Nees whole plant sample, (a) a untreated Sample, (b) a sample after ultrasound-assisted extraction and (c) a sample of conventional solvent extraction

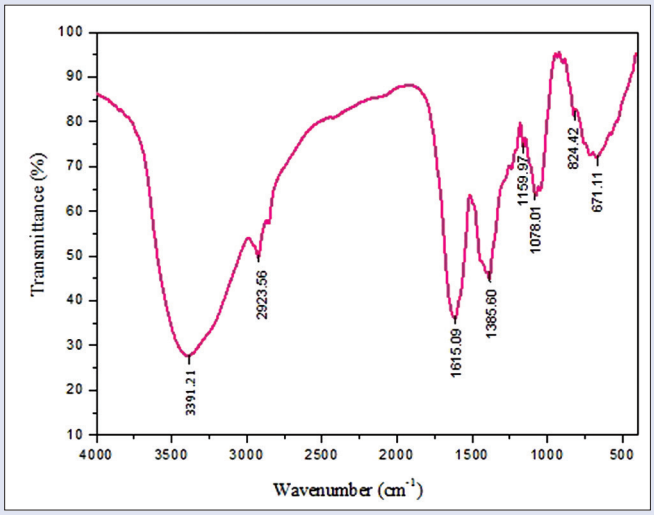


Figure 3: FTIR characterization of optimized extract of *Andrographis echioides* (L.) Nees whole plant

1159 and 824 cm^{-1} are related to sugars in the plant tissue, whereas the absorption bands between 500 and 900 cm^{-1} completely rely on the glycosylation pattern. The absorption band at 671 cm^{-1} may relate to the glycosylation pattern of flavonoid compounds in the sample extract.[12,34] According to these results, the observed AOC of AEWP may be because of the presence of these functional groups.

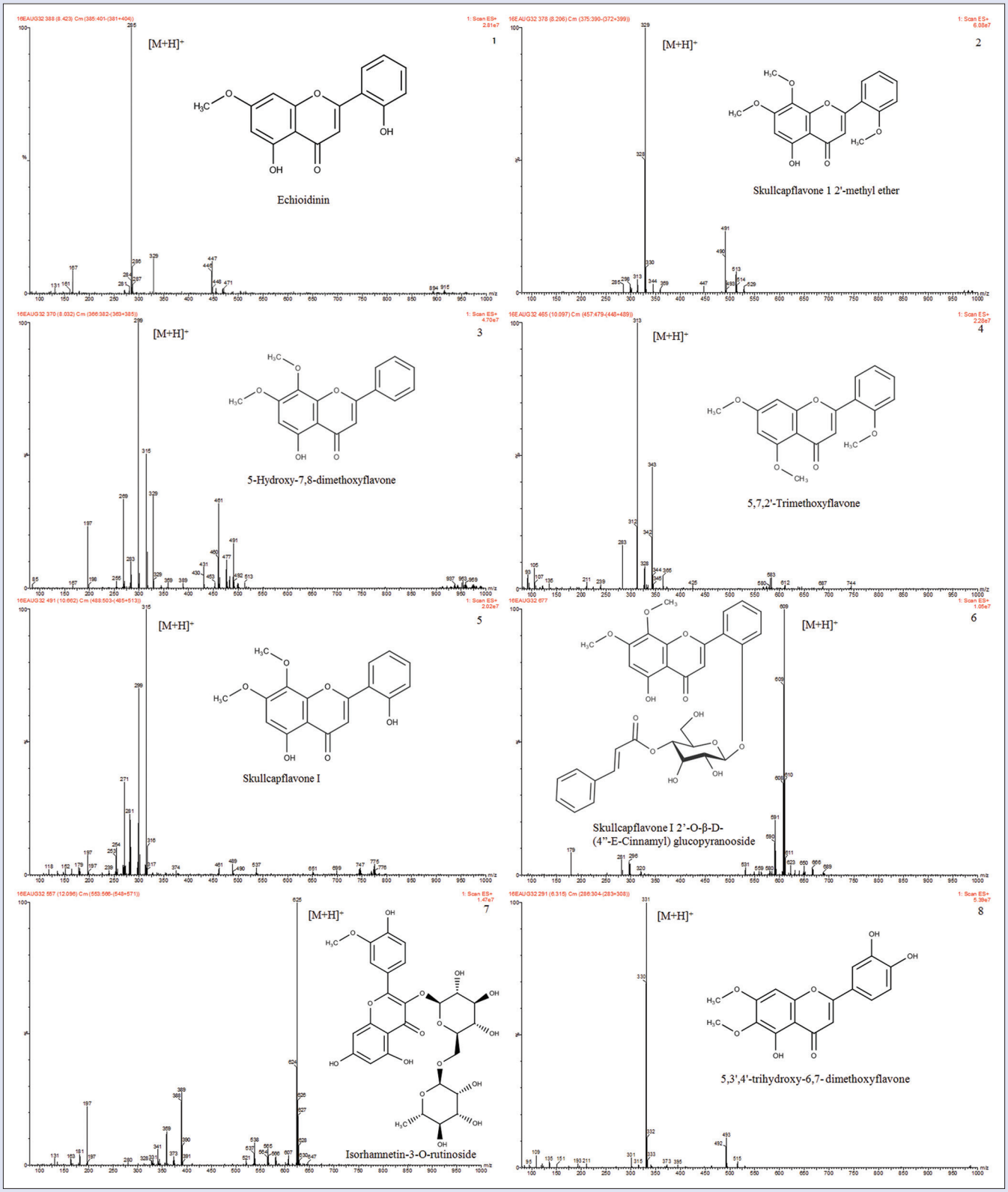


Figure 4: Mass spectrogram of optimised extract of *Andrographis echioides* whole plant viz., (1) echioidinin, (2) skullcapflavone I 2′-methyl ether, (3) 5-hydroxy-7,8-dimethoxyflavone, (4) 5,7,2′-trimethoxyflavone, (5) skullcapflavone I (6) skullcapflavone I 2′-O-β-D-(4″-E-cinnamyl) glucopyranooside, (7) Isorhamnetin-3-O-rutinoside and (8) 5,3′,4′-trihydroxy-6,7- dimethoxyflavone

Table 4: ESI-MS and ESI-MS/MS data of compounds 1–8

Compound/ peak number	t_R (min)	MW	$[M+H]^+$	MS^2 ions	Tentative structural assignment	Molecular formula	Intensity	References
1	7.53	284	285	284, 281, 255, 166, 149, 138, 107	Echioidinin	$C_{16}H_{12}O_5$	100	[8]
2	8.25	328	329	328, 313, 285,181,153,135	Skullcapflavone I 2'-methyl ether	$C_{18}H_{16}O_6$	100	[10,35]
3	8.08	298	299	298, 283, 255, 181, 153, 105, 102	5-Hydroxy-7,8-dimethoxyflavone	$C_{17}H_{14}O_5$	100	[36]
4	10.10	312	313	312, 311, 283, 266, 180, 151, 131	5,7,2'-Trimethoxyflavone	$C_{18}H_{16}O_5$	100	[37]
5	8.08	314	315	314 , 299, 284, 271, 254, 196,181, 168, 153, 121, 118	Skullcapflavone I	$C_{17}H_{14}O_6$	100	[12,36]
6	17.53	608	609	609,608,607, 477, 315	Skullcapflavone I 2'-*O*-*β*-D-(4"-E-cinnamyl) glucopyranooside	$C_{32}H_{30}O_{12}$	100	[36]
7	12.10	625	625	647, 331, 301, 179, 151	Isorhamnetin-3-O-rutinoside	$C_{28}H_{32}O_{16}$	100	[38]
8	6.36	330	331	332, 330, 353	5,3',4'-Trihydroxy-6,7-dimethoxyflavone	$C_{17}H_{14}O_7$	100	[39]

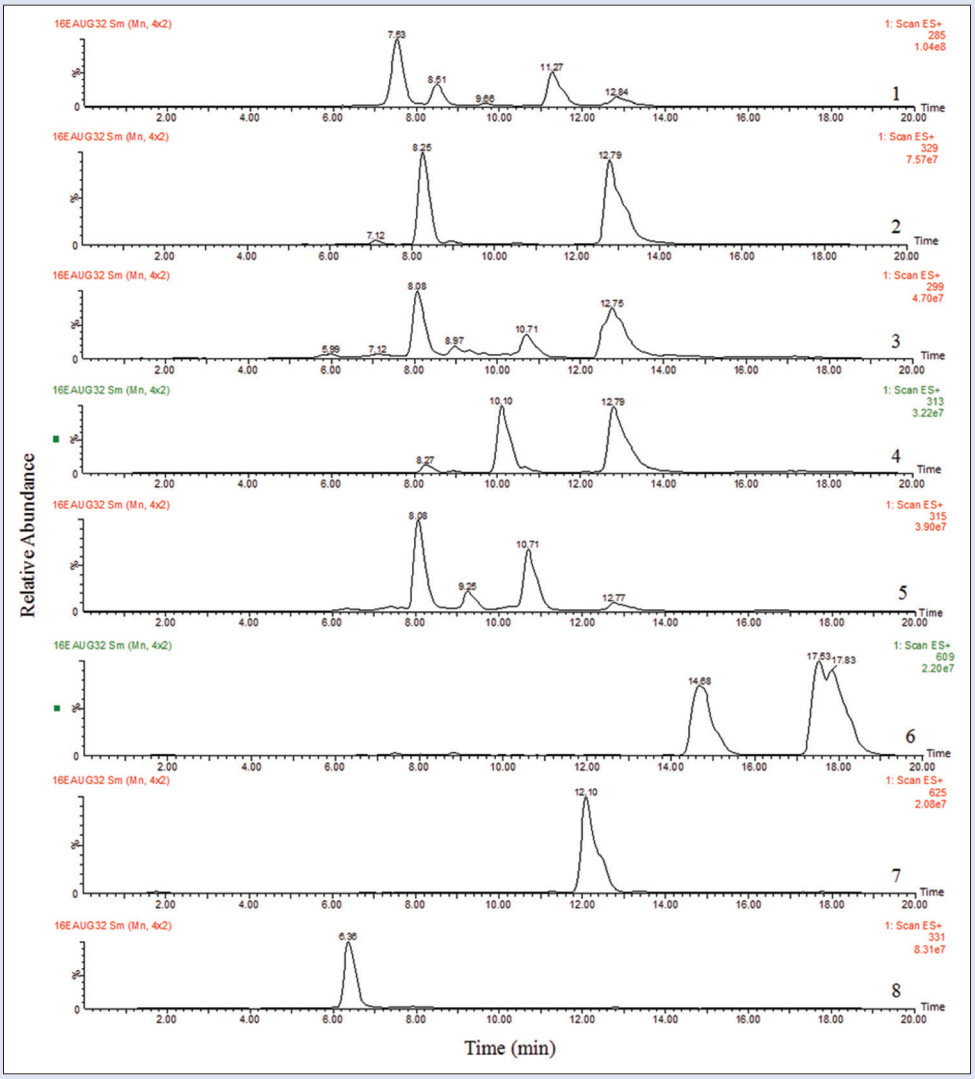


Figure 5: Reconstructed extracted ion chromatograms of the optimised extract of AEWP. The labelling of the peaks corresponds to Figure 4 (1) to (8), respectively

Flavonoid profile of the optimized extract

In this analysis, 8 flavonoid compounds were identified in positive ionization mode. The tentative identification of the mass spectrum of optimized extract was based on the retention time and ESI–MS/MS data [Table 4 and Figures 4, 5]. The identified flavonoid compounds are (1) echioidinin, (2) skullcapflavone I 2'-methyl ether, (3) 5-hydroxy-7,8-dimethoxyflavone, (4) 5,7,2'-trimethoxyflavone, (5) skullcapflavoneI, (6) skullcapflavone I 2'-*O*-*β*-D-(4"-E-cinnamyl) glucopyranooside, (7) Isorhamnetin-3-O-rutinoside, and (8) 5,3',4'-trihydroxy-6,7-dimethoxyflavone. Our results are in good agreement with the literature. The compound (1), (2) and (5) were

previously reported in petroleum ether, n-hexane, Me_2CO and MeOH extract of AEWP.[8,10,12] The other 5 compounds are newly reported from this optimization study. Compound (3), (4), and (6) were already reported in the same genus of *A. elongate* and *A. viscosula*.[36,37] Mass spectrogram, extracted ion chromatograms of the compounds 1–8 are shown in Figures 4 and 5. ESI-MS and ESI-MS/MS data of compounds 1–8 are given in Table 4. Thus, the qualitative analysis states that the optimized AEWP extract is rich in flavonoids. Therefore, the AOC of this plant may be because of these bioactive compounds in the sample extract.

CONCLUSION

This optimization study provides direct evidence that UAE is a successful technique for the extraction of flavonoid and AOC from AEWP using ethanol as the solvent of choice with low solvent usage and in a reasonable time. The optimal extraction conditions were acquired by RSM for the UAE of flavonoid and AOC from AEWP: ethanol concentration 77%, solvent to solid ratio 35 mL/g and sonication time 41 min. Under the optimized conditions, the experimental values are in good agreement with the predicted values. In addition, FTIR and LC–MS analysis reveal that the optimized extract is rich in flavonoids and provides additional evidence that the UAE is an efficient method for flavonoid extraction from AEWP.

Acknowledgements

The authors would like to acknowledge the National Facility for Drug Development for Academia, Pharmaceutical & Allied Industries (NFDD) at Department of Pharmaceutical Technology, Anna University, BIT Campus, Tiruchirappalli, Tamil Nadu, India for support and laboratory facilities to carry out this study.

Financial support and sponsorship

This study was funded by the Department of Science and Technology (DST) of Ministry of Science and Technology, Government of India (DST letter No.VI-D&P/349/10-11/TDT Dt: 21.10.2010).

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Elangovan K, Elumalai D, Anupriya S, Shenbhagaraman R, Kaleena PK, Murugesan K. Phyto mediated biogenic synthesis of silver nanoparticles using leaf extract of *Andrographis echioides* and its bio-efficacy on anticancer and antibacterial activities. J Photochem Photobiol B 2015;151:118-24.
2. Basu SK, Rupeshkumar M, Kavitha K. Studies on the anti-inflammatory, anaigesic and antipyretic properties of *Andrographis echioides* nees. Int J Pharmacol 2009a; 5:251-6.
3. Basu SK, Rupeshkumar M, Kavitha K. Hepatoprotective and antioxidant effect of *Andrographis echioides* nees against acetaminophen induced hepatotoxicity in rats. J Biol Sci 2009b; 9:351-6.
4. Kumar UA, Manjunath C, Thaminzhmani T, Gnana Shekaran D, Ravi Kiran Y, Brahmaiah Y. Evaluation of antiulcer activity of methanolic extract of *Andrographis echioides*. Pharmacologyonline 2011;3:917-24.
5. Kanchana G, Nirubama K, Rubalakshmi G. Phytochemical screening and antimicrobial activity of *Andrographis echioides* (L.) Nees – An indigenous medicinal plant. World J Pharm Sci 2014;3:702-10.
6. Harborne JB. The Flavonoids: Advances in Research Since 1986. London, UK: Chapman and Hall; 1994. p. 280-90.
7. Iinuma M, Mizuno M. Natural occurrence and synthesis of 2'-oxygenated flavones, flavonols, flavanones and chalcones. Phytochem 1989;28:681-94.
8. Govindachari TR, Parthasarathy PC, Pai BR, Subramaniam PS. *Andrographis echioides*. I. Structure and synthesis of echioidinin. Tetrahedron 1965a; 21:2633-40.
9. Govindachari TR, Parthasarathy PC, Pai BR, Subramaniam PS. *Andrographis echioides*. II. Structure and synthesis of echoidin. Tetrahedron 1965b; 21:3715-20.
10. Jayaprakasam B, Damu AG, Gunasekar D, Blond A, Bodo B. Dihydroechioidinin, a flavanone from *Andrographis echioides*. Phytochem 1999;52:935-7.
11. Jayaprakasam B, Gunasekar D, Rao KV, Blond A, Bodo B. Androechin, a new chalcone glucoside from *Andrographis echioides*. J Asian Nat Prod Res 2001;3:43-8.
12. Shen DY, Juang SH, Kuo PC, Huang GJ, Chan YY, Damu AG, *et al.* Chemical constituents from *Andrographis echioides* and their anti-inflammatory activity. Int J Mol Sci 2012;14:496-514.
13. Şahin S, Samlı R. Optimization of olive leaf extract obtained by ultrasound-assisted extraction with response surface methodology. Ultrason Sonochem 2013;20:595-602.
14. Wang C, Li Y, Yao L, Wu G, Chang J, Shu C, *et al.* Optimization of ultrasonic-assisted extraction of flavonoid from *Portulaca oleracea* L. by response surface methodology and chemical composition analysis. J Korean Soc Appl Biol Chem 2014;57:647-53.
15. Hemwimol S, Pavasant P, Shotipruk A. Ultrasound-assisted extraction of anthraquinones from roots of *Morinda citrifolia*. Ultrason Sonochem 2006;13:543-8.
16. Albu S, Joyce E, Paniwnyk L, Lorimer JP, Mason TJ. Potential for the use of ultrasound in the extraction of antioxidants from *Rosmarinus officinalis* for the food and pharmaceutical industry. Ultrason Sonochem 2004;11:261-5.
17. Rostagno MA, Palma M, Barroso CG. Ultrasound-assisted extraction of soy isoflavones. J Chromatogr A 2003;1012:119-28.
18. Vinatoru M. An overview of the ultrasonically assisted extraction of bioactive principles from herbs. Ultrason Sonochem 2001;8:303-13.
19. Entezari MH, Hagh Nazary S, Haddad Khodaparast MH. The direct effect of ultrasound on the extraction of date syrup and its micro-organisms. Ultrason Sonochem 2004;11:379-84.
20. Wong Paz JE, Muñiz Márquez DB, Martínez Ávila GC, Belmares Cerda RE, Aguilar CN. Ultrasound-assisted extraction of polyphenols from native plants in the Mexican desert. Ultrason Sonochem 2015;22:474-81.
21. Rodrigues S, Pinto GA, Fernandes FA. Optimization of ultrasound extraction of phenolic compounds from coconut (*Cocos nucifera*) shell powder by response surface methodology. Ultrason Sonochem 2008;15:95-100.
22. Setyaningsih W, Duros E, Palma M, Barroso CG. Optimization of the ultrasound-assisted extraction of melatonin from red rice (*Oryza sativa*) grains through a response surface methodology. Appl Acoust 2016;103:129-35.
23. Bashi DS, Mortazavi SA, Rezaei K, Rajaei A, Karimkhani MM. Optimization of ultrasound-assisted extraction of phenolic compounds from yarrow (*Achillea beibrestinii*) by response surface methodology. Food Sci Biotechnol 2012;21:1005-11.
24. Celli GB, Ghanem A, Brooks MS. Optimization of ultrasound-assisted extraction of anthocyanins from haskap berries (*Lonicera caerulea* L.) using response surface methodology. Ultrason Sonochem 2015;27:449-55.
25. González-Centeno MR, Knoerzer K, Sabarez H, Simal S, Rosselló C, Femenia A. Effect of acoustic frequency and power density on the aqueous ultrasonic-assisted extraction of grape pomace (*Vitis vinifera* L.) – A response surface approach. Ultrason Sonochem 2014;21:2176-84.
26. Ahmad A, Alkharfy KM, Wani TA, Raish M. Application of box-behnken design for ultrasonic-assisted extraction of polysaccharides from *Paeonia emodi*. Int J Biol Macromol 2015;72:990-7.
27. Feng S, Luo Z, Tao B, Chen C. Ultrasonic-assisted extraction and purification of phenolic compounds from sugarcane (*Saccharum officinarum* L.) rinds. LWT Food Sci Technol 2015;60:970-6.
28. Zhishen J, Mengcheng T, Jianming W. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food Chem 1999;64:555-9.
29. Zielinski AA, Granato D, Alberti A, Nogueira A, Demiate IM, Haminiuk CW. Modelling the extraction of phenolic compounds and *in vitro* antioxidant activity of mixtures of green, white and black teas (*Camellia sinensis* L. Kuntze). J Food Sci Technol 2015;52:6966-77.
30. Dahmoune F, Spingo G, Moussi K, Remini H, Cherbal A, Madani K. *Pistacia lentiscus* leaves as a source of phenolic compounds: Microwave assisted extraction optimized and compared with ultrasound-assisted and conventional solvent extraction. Ind Crops Prod 2014;61:31-40.
31. Box GE, Behnken DW. Some new three level designs for the study of quantitative variables. Technometrics 1960;2:455-75.
32. Derringer G, Suich R. Simultaneous optimization of several response variables. J Qual Technol 1980;12:214-9.
33. Pan G, Yu G, Zhu C, Qiao J. Optimization of Ultrasound-Assisted Extraction (UAE) of Flavonoids Compounds (FC) from Hawthorn Seed (HS). Ultrason Sonochem 2012;19:486-90.
34. Ozaki Y, Cho R, Ikegawa K, Muraishi S, Kawauchi K. Potential of near-infrared fourier

transform Raman spectroscopy in food analysis. Appl Spectrosc 1992;46:1503-7.

35. Jalal MA, Overton KH, Rycroft DS. Formation of three new flavones by differentiating callus cultures of *Andrographis paniculata*. Phytochem 1979;18:149-51.
36. Jayakrishna G, Arikishore P, Rao CV, Gunasekar D, Blond A, Bodo B. Two new 2′-oxygenated flavones from *Andrographis elongate*. Chem Pharm Bull 2001;49:1555-7.
37. Rao YK, Harikishore P, Rao CV, Gunasekar D, Blond A, Bodo B. Flavones from *Andrographis viscosula*. Phytochemistry 2002;61:927-9.
38. Wanga X, Zhao X, Gu L, Zhang Y, Bi K, Chen X. Discrimination of aqueous and vinegary extracts of Shixiao San using metabolomics coupled with multivariate data analysis and evaluation of anti hyperlipidemic effect. Asian J Pharmacol 2014;9:17-26.
39. Mustafa EH, Zarga MA, Sabri S, Abdalla S. Effects of cirsiliola, a flavone isolated from *Achillea fragantissima*, on rat isolated smooth muscle. Int J Pharmacogn 1995;33:204-9.