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Optimization for Ultrasonic-Assisted Extraction of *Aframomum melegueta* Phenolics Using Response Surface Methodology

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Abstract

Aframomum melegueta seeds (AMS) is an African spice with well known traditional and therapeutic values. Its pharmacological activities are attributable mainly to phenolics. This study aimed to investigate and optimize the parameters affecting the ultrasound-assisted extraction (UAE) of AMS major constituents and total phenolic content (TPC) using response surface methodology. 6-gingerol, 6-shogoal and 6-paradol were isolated from AMS and a Box-Behnken design (3 factors /3 levels) was used to determine the effect of three extraction parameters (extraction time, methanol concentration and liquid/solid ratio) on their extraction yield. The results showed that methanol concentration and liquid/solid ratio have positive and significant impact on the UAE of TPC and the three investigated compounds, while extraction time has no significant effect. Under optimal conditions, each 1 g dry sample provides 9.32±0.02 mg, 3.72±0.01 mg, 12.32±0.04 mg and 10.71±0.19 mg/GAE of 6-gingerol, 6-shogoal, 6-paradol and TPC, respectively. The optimized UAE conditions were validated and are recommended for the recovery of 6-gingerol, 6-shogoal, 6-paradol and TPC from AMS for further applications as alternative to conventional extraction method.

Keywords: *Aframomum melegueta*, optimization, phenolic compounds, Response surface methodology, Ultrasonic-assisted extraction

1. Introduction

Aframomum melegueta K. Schum seeds (AMS) is an African plant consumed not only as a spice but also for its valuable pharmacological activities such as antihyperlipidemic [1], antioxidant [2], antimicrobial [3], hepato-protective [4], anti-cancer [5], anti-diabetic [6] and aphrodisiac [7] effects. AMS contain a variety of bioactive phenolics, whereas 6-gingerol, 6-shogaol and 6-paradol were assigned as its major individual phenolic compounds [8]. 6-gingerol [9–11] and 6-shogaol [12] exhibit potent anti-inflammatory, antioxidant, anticancer, analgesic and antiemetic effects. Additionally, 6-paradol and 6-shogaol showed remarkable neuroprotective activity [13–15]. Despite the well documented therapeutic potential of these compounds and the positive impact of optimizing their extraction on the activity of the whole plant extract, there is no available data about maximizing their yield in AMS.

Extraction is the main step for the recovery and isolation of bioactive phytochemicals from plants. Among the different extraction techniques, ultrasonic-assisted extraction (UAE) was reported as eco-friendly, efficient, rapid and low-cost extraction method [16]. Combined physical mechanisms, mainly cavitation, are involved in AUE and result in increasing the material surface area as well as mass transfer process without affecting neither the structure nor the function of the extracts [17]. Additionally, AUE increases the extraction rate at low temperatures; thereby prevent instability of thermolabile active ingredients [18]. Recently, UAE has been successfully used for the extraction of phenolics from different plants [19, 20]. However, the extraction efficiency depends on the plant material [21]. Consequently, UAE should be studied for each individual plant. Different parameters such as liquid/solid ratio, solvent concentration, extraction time affect the extraction process [22]. Thus, optimizing these conditions is imperative to

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maximize the extraction yield of the bioactive compounds.

The response surface methodology (RSM) is the most widely used approach for process optimization [23]. It allows evaluating the effects of multiple factors and their interactions on one or more response variables with reducing number of experimental runs, cost and time [24]. Accordingly, the aim of this study is to develop and validate the optimal conditions for UAE of phenolics from AMS using RSM, which could provide a basis for large-scale extraction of these important phytoconstituents. Additionally, the optimized UAE is compared with a conventional extraction method.

2. Experimental

2.1. Chemicals and equipments

HPLC-grade methanol, Folin-Ciocalteu reagent and gallic acid were obtained from Sigma-Aldrich, Germany. All other chemicals were of highest available analytical grade. Shimadzu UV-1650 PC was used for spectrophotometric determination of TPC.

2.2. Plant material and compounds isolation

AMS were purchased from the herbal store Haraz, Cairo, Egypt and were identified by Prof. Dr. Abdelhaleem A. Mohamed, Flora and Phytotaxonomy research Department, Agriculture museum, Dokki, Egypt. A voucher specimen (No. 3.7.2019) was deposited in the Herbarium of the Pharmacognosy Department, Faculty of Pharmacy, Cairo University. Dried powdered AMS (1.5 kg) were extracted with methanol (3×3L) using Ultraturrax blender and the obtained extracts were concentrated using a rotatory evaporator (40°C). The methanol extract (78 g) was suspended in water (500 ml), partitioned with chloroform (500 ml ×5) and the pooled fractions were evaporated to yield chloroform fraction (55 g), which was further partitioned on a silica gel column (70 cm×8 cm). Gradient elution was performed with *n*-hexane:ethylacetate (5%~70% v/v) then chloroform:methanol (10%~70%v/v) to afford 10 fractions. Subfraction (95% *n*-hexane in ethyl acetate) was chromatographed on a silica gel column (35 cm × 3.5 cm) using isocratic elution (*n*-hexane: ethyl acetate, 95:5 v/v) to yield 4.5 g of compound (C1). Subfraction (90% *n*-hexane in ethyl acetate) was subjected to chromatography on silica gel column (25 cm×2.5 cm) and the elution was carried out using *n*-hexane:ethyl acetate (95:5 v/v). Similar fractions were collected and yielded upon concentration 1 g of impure residue, which was rechromatographed on Wakosil C-300 silica gel column (20 cm×1.5 cm). Isocratic elution was performed using *n*-hexane:ethyl acetate (98:2 v/v) and similar fractions showing pure spot yielded upon concentration 254 mg of compound (C2). Subfraction (70% *n*-hexane in ethyl acetate) was purified on silica gel column (35 cm×3.5 cm) using gradient elution with *n*-hexane:ethylacetate (95%~85% v/v) to yield 2 g of compound (C3).

2.3. Experimental design

Box-Behnken experimental design (3-factors/3-level) generated by Design Expert trial version 9.0 software (Stat-Ease, Inc., Minneapolis, MN, USA) was used. Fifteen experiments, including three replicates at the center point, were designed and carried out in a random order with different combinations of the independent variables; extraction time (A:20–60 min), methanol concentration (B:40–100%) and liquid/solid ratio (C:4 –10 mL/g).

2.4. Samples preparation

2.4.1. UAE method

Known weights (2–5 g) of powdered AMS were mixed with 20 ml solvent (40–100% HPLC-grade methanol) in falcon tubes. The samples were sonicated in a sonic bath (Elmasonic P, Elma Schmidbauer GmbH, Singen, Germany) with an ultrasonic frequency of 37 kHz and temperature at 35°C to avoid degradation of the investigated compounds. After varying time (20–60 min), the extracts were centrifuged for 2 min, filtered (Whatman No. 2, 8 μm) and stored at 20 °C for subsequent HPLC quantification and TPC estimation.

2.4.2. Conventional extraction method (maceration)

Two g powdered AMS was extracted with 70% methanol (10 mL) for two hours in water bath at room temperature. The extract was filtered and stored at 20 °C for subsequent phytochemical analysis. The experiment was performed triplicate and the results were expressed as mean ± SD.

2.5. HPLC quantification of the major compounds

Quantification of AMS major phenolics was carried out using reversed phase HPLC. After filtration through a 0.45-millipore membrane filter, An aliquot (20μl) of the each extract was injected into Hewlett Packard HPLC system (series1050) equipped with an autosampling injector, a solvent degasser, a quaternary HP pump (series 1050), a Lichrosorb RP-18column (4.0 x 250mm, 5μm, Merck, Darmstadt) and a DAD detector set at 230 nm. The column was maintained at room temperature and the flow rate was adjusted at 1.1 mL/min. The gradient program with water as mobile phase A and acetonitrile as mobile phase B was adjusted as described by [25] with modification: 0–1.5 min, 35% B; 1.5–2.2 min, 35% – 60% B; 2.2–6 min, 60% B; 6–10 min, 60%–100% B; 10–12 min, 100% B; 12–12.1 min, 100–35% B. Serial dilutions of 6-gingerol, 6-shogaol and 6-paradol were prepared from stock

solution (1 mg/ml) and used to establish their standard calibration curves.

2.6. Quantitative estimation of TPC

Spectrophotometric determination of the TPC was carried out by Folin-Ciocaltu reagent method according to the procedures reported in the European Pharmacopeia [26]. The absorbance was measured at λ_{max} 760 nm and TPC in the extract was expressed as mg of gallic acid equivalent per g of sample dry weight (mg GAE/g DW).

2.7. Statistical analysis

Analysis of variance (ANOVA) and RSM analysis, performed by Design Expert software, were used to determine the statistical significance of the model. The *p*-value less than 0.05 ($p < 0.05$) is considered as statistically significant. A paired comparison student's *t*-test was used to compare values of optimized UAE and conventional extraction method.

3. Results and discussion

The major isolates were identifies as C1, 6-paradol [8]; C2, 6-shogaol [27] and C3, 6-gingerol [28]. The structure, ^{1}H–NMR and ^{13}C–NMR spectral data of these compounds are shown in Table S1 and Figs. S1–S4. Box-Behnken design and the extraction yield of 6-gingerol, 6-shogaol, 6-paradol and TPC at different extraction conditions are presented in Table 1. Based on the experimental data, mathematical modeling was designed, ANOVA was performed and a first degree polynomial equation was established correlating each response with the selected factors.

The quadratic model was selected to predict the four responses because it exhibit the highest determination of coefficient (R^2) and show non-significant lack of fit. Values of $R^2 > 0.9$ (Table 2) showed good fitness of the models and indicated the statistical validity and significance of the designed polynomial equations for optimization [29]. Additionally, the adjusted R^2 values of 0.92–0.96 showed the lack of R^2 inflation effect due to introduction of insignificant variables [30]. Predicted R^2 values were in good agreement with the adjusted R^2 *i.e.* the difference is less than 0.2. Adequate precision, which measures the signal to noise ratio, represented an adequate signal as a ratio greater than 4 is desirable [31]. Low values of Coefficient of Variation indicated good precision and reliability of the experiments [32]. A good agreement between the predicted and actual values (Fig. 1) was observed as they approximately fit the line, which further indicated models validity.

For all responses a high significance ($p < 0.01$) for the model was shown (Table 3). Moreover, the lack of fit tests does not reach statistical significance ($p > 0.05$), which further verify the validity of the models [33]. The polynomial equations predicting the extraction yield of 6-gingerol (Y_1), 6-shogaol (Y_2), 6-paradol (Y_3) and TPC (Y_4) were represented by equations (1–4).

$$Y_1 = 5.04 + 0.3786A + 1.59B + 1.17C + 0.1099AB + 0.2782AC - 0.1435BC + 1.08A^2 + 0.0372B^2 + 0.3818C^2 \quad (1)$$

Table 1: Experimental results for the three-factor/three-levels Box-Behnken design

Run no.	Independent variables			6-gingerol content	6-shogaol Content	6-paradol content	TPC
	A	B	C				
1*	40	70	7	5.55	2.64	8.25	7.17
2	60	100	7	8.54	3.20	10.76	9.38
3	20	70	4	5.61	2.06	6.73	6.87
4*	40	70	7	4.81	1.92	5.91	6.51
5*	40	70	7	4.76	1.93	5.95	5.85
6	60	70	4	5.36	2.01	6.76	6.42
7	60	40	7	4.98	0.77	1.37	4.78
8	40	100	4	5.79	2.16	7.41	7.59
9	20	40	7	3.99	0.50	0.86	4.13
10	60	70	10	7.94	3.23	10.98	10.10
11	40	40	10	5.42	0.86	1.50	5.52
12	40	100	10	8.16	3.35	11.28	10.18
13	20	100	7	7.12	2.75	8.86	9.84
14	40	40	4	2.47	0.29	0.49	2.18
15	20	70	10	7.08	2.89	10.11	9.35

A (extraction time, min), B (methanol concentration, %), C (liquid/solid ratio, mL/g). 6-gingerol, 6-shogaol and 6-paradol contents are in mg/g DW and TPC is in mg/g GAE. *central points

$$Y_2 = 1.47 + 0.0476A + 0.4621B + 0.1742C - 0.0100AB + 0.0287AC - 0.0071BC + 0.0943A^2 - 0.3018B^2 + 0.0279C^2 \quad (2)$$

Y_3= 6.70 + 0.4137A + 4.26B + 1.56C + 0.3475AB + 0.2100AC + 0.7150BC +1.12A^2 – 2.36B^2 + 0.8246C^2 (3)

Y_4= 6.51+ 0.0612A+ 2.55B + 1.51C – 0.2775AB + 0.3000AC – 0.1875BC + 1.17A^2 – 0.6475B^2 + 0.5050C^2 (4)

Table 2: Statistical parameters calculated after implementation of Box-Behnken experimental design

Response	C.V. %	R^2	Adjusted R^2	Adequate Precision
Y_1	7.86	0.9727	0.9235	15.4155
Y_2	6.98	0.9810	0.9469	16.5178
Y_3	15.21	0.9761	0.9332	14.4654
Y_4	5.97	0.9889	0.9689	23.6106

Y_1 content of 6-gingerol (mg/g DW), Y_2 content of 6-shogaol (mg/g DW), Y_3 content of 6-paradol (mg/g DW), Y_4 TPC (mg/g GAE). C.V. Coefficient of Variation

It is noteworthy that positive sign of coefficient indicates a linear effect to increase the yield of the response, whereas negative sign of coefficient indicates a linear effect to decrease it [34].

The results showed that methanol concentration (B) and liquid/solid ratio (C) have significant impact on all investigated responses ($p < 0.01$).

The most prominent effect was that of factor B (methanol concentration) as revealed from its high F-value (Table 3). Both factors (B and C) showed positive sign of coefficient which indicates that increasing the methanol concentration and liquid to solvent ratio leads to significant increase in the extraction yield of total and individual AMS phenolics. This could be also observed in the generated 3D response surface graphs (Fig. 2). Increasing extraction yield with increasing methanol concentration could be explained by the non-polar nature and low molecular weights of AMS phenolics. Moreover, our result is in consistent with previous studies [35], which reported direct proportional relationship between solvent/sample ratio and the extraction yield of phenolic compounds. A possible explanation is that with higher liquid/solid ratio, the contact area between plant and solvent increase and subsequently enhance solubility of extractable compounds [36].

The results also indicated that linear term of time (A) has no significant effect on the extraction yield of AMS phenolics ($p > 0.05$). No interactive effects between the three investigated extraction parameters were observed. The effect of quadratic term of methanol concentration (B^2) on the extraction of 6-shogaol and 6-paradol was significant while the quadratic term of time (A^2) positively and significantly influence the extraction yield of 6-gingerol and TPC.

Table 3: ANOVA for the quadratic response surface models of all responses

Source	Sum of Squares				df	*F*-value				*p*-value			
	Y_1	Y_2	Y_3	Y_4		Y_1	Y_2	Y_3	Y_4	Y_1	Y_2	Y_3	Y_4
Model	37.53	2.37	198.71	78.92	9	19.78	28.75	22.72	49.46	* 0.0021	** 0.0009	* 0.0015	** 0.0002
A	1.15	0.0181	1.37	0.0300	1	5.44	1.98	1.41	0.1693	0.0670	0.2184	0.2885	0.6978
B	20.31	1.71	145.27	51.92	1	96.33	186.74	149.50	292.82	** 0.0002	*** 0.0001	*** 0.0001	*** 0.0001
C	11.00	0.2428	19.47	18.27	1	52.19	26.54	20.04	103.05	** 0.0008	* 0.0036	* 0.0065	** 0.0002
AB	0.0483	0.0004	0.4830	0.3080	1	0.2293	0.0434	0.4971	1.74	0.6522	0.8432	0.5123	0.2446
AC	0.3097	0.0033	0.1764	0.3600	1	1.47	0.3598	0.1815	2.03	0.2797	0.5747	0.6878	0.2135
BC	0.0824	0.0002	2.04	0.1406	1	0.3907	0.0218	2.10	0.7931	0.5594	0.8884	0.2066	0.4140
A^2	4.29	0.0329	4.61	5.05	1	20.33	3.59	4.74	28.51	* 0.0063	0.1166	0.0814	* 0.0031
B^2	0.0051	0.3362	20.53	1.55	1	0.0242	36.75	21.13	8.73	0.8824	* 0.0018	* 0.0059	0.0317
C^2	0.5382	0.0029	2.51	0.9416	1	2.55	0.3133	2.58	5.31	0.1710	0.5998	0.1689	0.0694
Residual	1.05	0.0457	4.86	0.8865	5								
Lack of Fit	0.6646	0.0076	1.27	0.0153	3	1.14	0.1329	0.2358	0.0117	0.4994	0.9322	0.8665	0.9977
Pure Error	0.3895	0.0381	3.59	0.8712	2								
Cor Total	38.59	2.41	203.57	79.81	14								

Significant difference at *p<0.01, **p<0.001, ***p<0.0001; A, B and C: linear regression coefficients for time, methanol concentration and liquid\solid ratio; AB, AC and BC: regression coefficients for interaction between time × methanol concentration, time × ratio, methanol concentration × ratio; A, B and C: quadratic regression coefficients for time, methanol concentration and liquid\solid ratio, df (degree of freedom).

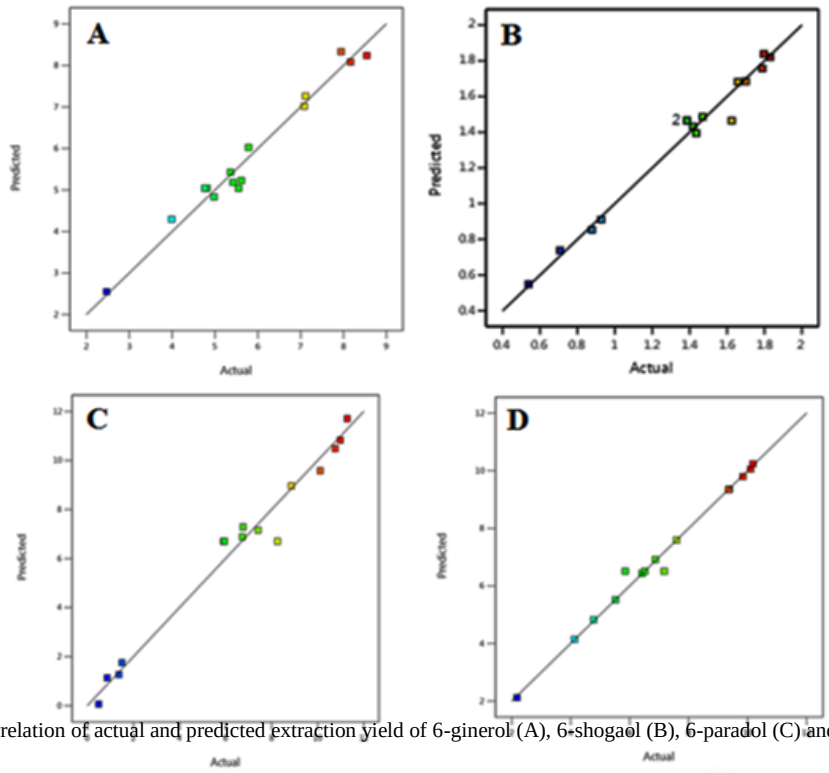

Fig. 1: Correlation of actual and predicted extraction yield of 6-ginerol (A), 6-shogaol (B), 6-paradol (C) and TPC (D)

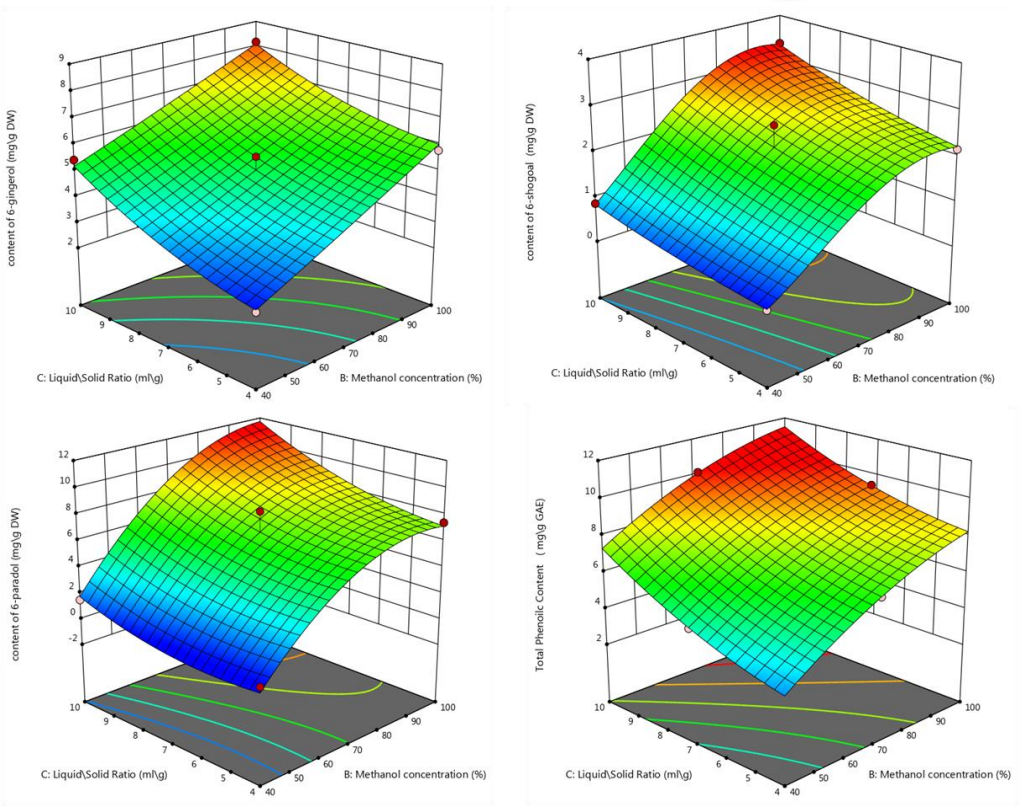

Fig. 2: 3D graphs for the effect of methanol concentration and liquid/solid ratio on the extraction yield of 6-ginerol, 6-shogaol, 6-paradol and TPC at constant time (40 min)

3.2. Optimization of extraction by RSM

In current study, the optimal UAE conditions for AMS phenolics are 59.3 min, 99.8% methanol and 9.8 mL/g solvent to sample. The predicted values under these conditions are 9.74 mg/g DW 6-gingerol, 3.86 mg/g DW 6-shogaol, 13.53 mg/g DW 6-paradol and 11.31 mg/g GAE TPC, desirability = 1.0.

3.3. Model validation study

Three experiments with the predicted optimum conditions were conducted to verify the adequacy of the developed extraction model. The experimental values (9.32±0.02 mg/g DW 6-gingerol, 3.72±0.01 mg/g DW 6-shogaol, 12.32 ±0.04 mg/g DW 6-paradol and 10.71±0.19 mg/g GAE TPC) were in consent with the predicted values obtained by RSM because acceptable percentage error values (<10%) were observed [37]. Thus, the validation study further confirmed the accuracy and adequacy of the designed model for predicting the responses. HPLC chromatogram of AMS optimized extract is shown in Fig. 3.

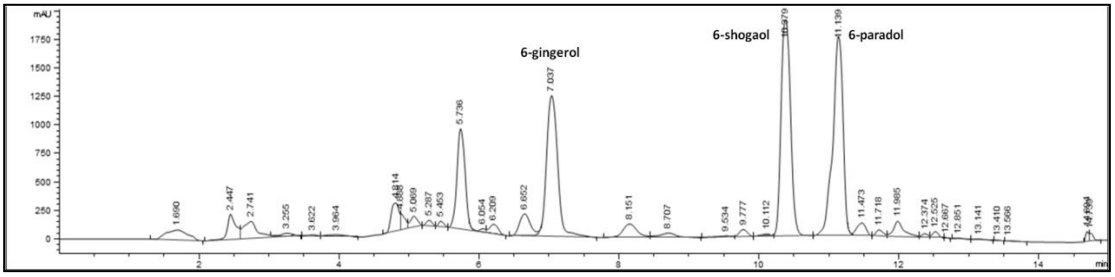

Fig. 3: HPLC chromatogram of the AMS optimized extract

3.4. Comparison of UAE with maceration method

Compared to conventional method of extraction, optimized UAE has significant higher capacity for AMS phenolics (Table 4). The results were in agreement with published studies on the extraction of phenolic compounds from peaches and pumpkin [38]. This could be attributed to that UAE allows disruption of plant cell walls, which enhances solvent penetration and facilitates the migration of the extractable compound from plant matrix into solvents [16, 39].

Table 4: Content of AMS individual phenolics and TPC using optimized UAE and maceration extraction

Method	6-gingerol mg/g DW	6-shogaol mg/g DW	6-paradol mg/g DW	TPC mg/g GAE
Optimized UAE	9.32±0.02	3.72±0.01	12.32±0.04	10.71±0.19
Maceration extraction	7.94±0.23	3.23±0.06	10.89±0.08	9.35±0.26

4. Conclusion

This study successfully developed valid and accurate UAE protocol to optimize TPC and three major constituents from AMS. The designed model assigned methanol concentration and liquid/solid ratio as the major parameters affecting the yield of target responses. For extraction of AMS phenolics, the optimized UAE conditions are recommended as more efficient alternative to conventional method. This study could provide a preliminary basis for large scale extraction of the pharmacologically valuable AMS phenolics.

Conflicts of interest

There are no conflicts to declare.

Supplementary materials

Table S1 and Figures S1–S4 are provided as supplementary materials.

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