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ORIGINAL ARTICLE

Factorial Design-Guided Optimization of Extraction of Therapeutically Active Furanocoumarin Khellin from *Ammi majus* L. Fruits

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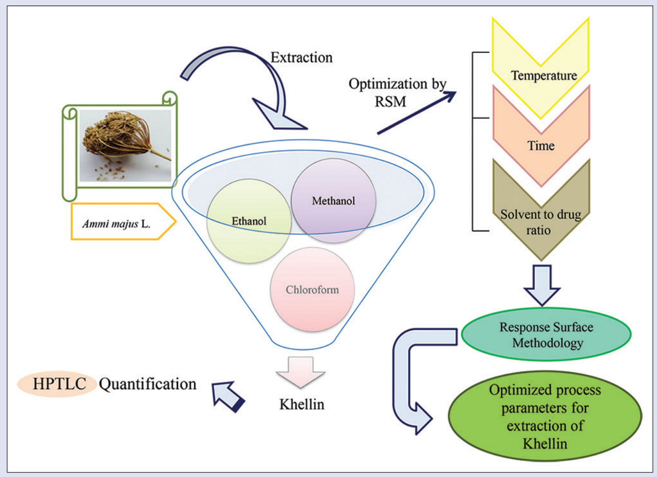
ABSTRACT

Background: The use of *Ammi majus* L. for the treatment of various diseases has been reported by many. Its umbelliferous fruits possess khellin that is responsible for various pharmacological activities and used in treating psoriasis and vitiligo. **Objectives:** The study objective was to analyze the optimization of extraction parameters of khellin from *A. majus* L. by response surface methodology (RSM). **Materials and Methods:** Box–Behnken Design (BBD) of RSM was used for optimization needs. Quantification of khellin in extracts was done by high performance thin-layer chromatography using a mix of ethyl acetate: toluene: formic acid, and the peaks were monitored at 254 nm. **Results:** Among the explored traditional modes and ultrasound-assisted extraction (UAE), UAE was found to be the most ideal for khellin extraction, with methanol being the most suitable solvent. The applied BBD established a quadratic model for the experimental setup with the regression coefficient of 0.998. The optimal conditions were set up as – extraction temperature: 63.84°C, extraction time: 29.51 min. and solvent-to-drug ratio: 21.64 v/w, which yielded 6.21% w/w of khellin. Contrarily, under the modified experimental conditions, 6.86% w/w of khellin was extracted. **Conclusion:** It was concluded that all the variables studied significantly affected khellin yield. Moreover, a contemporary green extraction mode stood out to be the best for khellin extraction.

Key words: *Ammi majus* L., Box–Behnken Design, khellin, Response Surface Methodology (RSM), ultrasound assisted extraction(UAE)

SUMMARY

- Plants have been utilized by human as medicines since ages. One such plant is *Ammi majus* L. which is credited with a wide range of biological activities owing to its phytochemical makeup. A furanocoumarin named khellin forms a major constituent of *A. majus* L. fruits. Because this phytocompound bears a long list of pharmacological activities, optimization of its extraction becomes indispensible. Unlike the traditional optimization methods, here optimization was done using response surface methodology (RSM) which is a statistical technique and helps avoiding problems faced with traditional optimization. Through prefatory and single-factor trials, it became evident that ultrasound-assisted extraction is the most desirable method for extraction of khellin. Further, RSM was applied which yielded 17 runs. Through the multiple regression analysis and point prediction analysis, the optimized conditions for khellin extraction were laid down as follows: extraction temperature – 63.84°C, extraction time – 29.51 min, and solvent-to-drug ratio – 21.64% v/w, which extracted 6.21% w/w of khellin. Under similar experimental conditions (extraction temperature – 64°C, extraction time – 30 min, solvent-to-drug ratio – 22% v/w), 6.86% w/w of khellin was observed which was slightly higher than the predicted value. The quadratic model established by RSM was found to be satisfactory and validated.



Abbreviations used: 3D: Three-dimensional; %w/w: Percent weight by weight; %v/w: percent volume by weight, °C: Degree Celsius; ANOVA: Analysis of variance; BBD: Box–Behnken Design; G: Gram; HPTLC: High performance thin layer chromatography; RSM: Response surface methodology; UAE: Ultrasound-assisted extraction.

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INTRODUCTION

Use of plants by human to fulfill health-care needs is not new. It dates back to the times of early man and is still continuing. As per the World Health Organization, 80% of the developing countries population depend on plants for their health-care needs.[1] Among the twenty largest plant families, 12 have higher number of medicinal plants.[2] One such family is *Apiaceae,* commonly called the carrot family. It consists of about 4079 medicinal plants, with coumarins being the key compounds found in this family.[2] *Ammi majus* L., known by the name bishop's weed, is a

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herb belonging to the same family and has been traditionally used in the treatment of bronchial asthma, coronary diseases, renal colic, vertigo, diabetes, kidney stones, and certain skin diseases such as psoriasis and vitiligo and as an emmenagogue for menstrual regulation.[3-7] Its rich phytochemical makeup makes it as multifaceted medicinal herb containing γ-pyrones and coumarins such as khellin, visnagin, khellol, ammiol, 4-norvisnagin, khellinol, visamminol, khellinin, khellinone, visnaginone, and 5,7-dihydroxy-2-methyl-γ-pyrone-7-*O*-glucoside.[7-13] Khellin, a furanocoumarin [Figure 1b], forms a major portion of its phytochemical profile[7-9] and is a known bronchodilator and vasodilator.[14-16]

Presence of any phytocompound in a plant is varied by various factors such as temperature, rainfall, humidity, type of soil, moisture, fertilizers, manures and topography. Similarly, the extraction of the phytocompound from any plant or plant material is governed by certain factors or conditions such as extraction temperature, extraction time, solvent used, polarity of solvent, pH of extracting solvent, liquor-to-drug ratio, extraction mode used, and others. Thus, optimization of the extraction process becomes crucial so that therapeutically active compounds can be extracted out in maximum amount from the plant. At present, statistical techniques have come up for optimization such as response surface methodology (RSM). RSM is a mathematical software which is better than traditional means because it is economical, is time saving, is less laborious, and helps to give interactive effects among different variables upon the extraction process which traditional optimization methods fail to give.[17,18] RSM was originally given in 1951 by Box and Wilson for studying any process which is affected by three or more variables.[18,19] Here, changes in variables are done to study their effect on the response. Phytocompound extraction field has exploited RSM to optimize the extraction process of various phytocompounds such as lawsone from *Lawsonia inermis*,[18] karanjin from *Pongamia pinnata*,[20] flavonoids from *Vitis vinifera*,[21] embelin from *Embelica ribes*,[22] quercitrin from *Herba Polygoni capitati*,[23] luteolin from *Vitex negundo*,[24] glycyrrhizic acid from *Glycyrrhiza glabra*,[25] carthamin from *Carthamus tinctorious*,[26] lupeol from *Ficus racemosa*,[27] and atropine from *Atropa balledona*.[28] Box–Behnken design (BBD) is one design of RSM which is a three-level factorial design. It aims to fit the experimental data into quadratic model and desires to have better prediction near the center points rather than at extremes. Fit of the polynomial equation and empirical model building decides whether the experiment fits into the model or not. Graphical representation of the model is given by three-dimensional (3D) curves, which gives a clear picture of interactions between various variables.

Use of any phytocompound starts with its extraction from plant material using specific solvents and an effective method. Sonication or ultrasound-assisted extraction (UAE) is a method of extraction of phytocompounds, which uses ultrasound energy (20 to 20,000 kHz) and works on the cavitation theory. When ultrasound passes through a solvent, refraction and compression phases are formed, leading to the formation of cavitation bubbles. These cavities rupture near the plant matrix, which causes release of the constituents of plant due to physical pressure.[29-31] Moreover, in the cavitation region, plant matrix and solvent are aggressively mixed, which causes breaking of the plant material into submicron sizes. Small sizes lead to increased surface areas and thus more mass transfer.[32] UAE is counted among the green extraction methods and is known for being time, solvent, and power saving. Apart from providing better extraction efficiencies, UAE can be used for extraction of thermo labile compounds.[33,34]

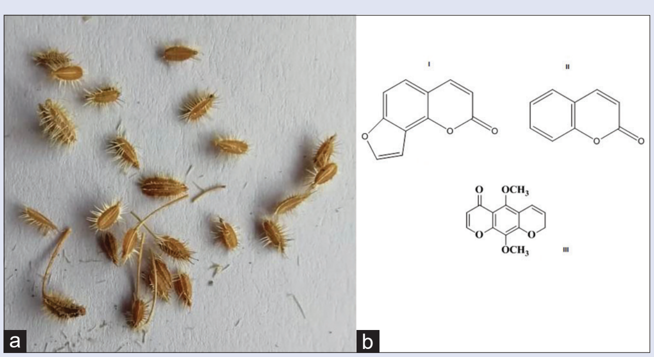

Figure 1: *Ammi majus* L. fruits (a) and chemical structure of furanocoumarin (i), coumarin (ii), and khellin (iii)

The present work aims at optimizing the extraction parameters of khellin from *A. majus* L. fruits utilizing RSM and concomitant quantification of khellin in extracts by high performance thin layer chromatography (HPTLC).

MATERIALS AND METHODS

Plant material

A. majus L. plant was collected from Herbal Garden, Jamia Hamdard, New Delhi, India, and authenticated by a taxonomist, Department of Botany, Jamia Hamdard, New Delhi, India. Respective specimen was preserved in the School of Pharmaceutical Education & Research (SPER), Jamia Hamdard, New Delhi-110062, India.

Chemicals

Standard khellin was purchased from Sigma-Aldrich, St. Louis, Missouri, USA. Solvents used were of analytical grade and purchased from S.D. Fine Chemicals, Mumbai, Maharashtra, India. Aluminum-backed HPTLC plates were purchased from T. C. I. Chemicals, Chennai, Tamil Nadu, India.

Extraction methods/prefatory experiments

The plant material obtained was properly cleaned and shade dried. Fruits were separated from the plant and cleaned again from any remaining earthly matter. Extraction was then carried out employing four different methods – maceration, hot solvent extraction by reflux technique, soxhlet technique, and UAE. Solvents of varying polarity were chosen for extraction including methanol, ethanol, and chloroform.

Quantitative analysis of khellin by high performance thin-layer chromatography

Preparation of standard and sample solutions

Stock solution of standard khellin was prepared at a concentration of 1 mg/mL in methanol. Aliquots were prepared from the stock solution varying from 6.25 to 100 μg/ml. In addition, accurately weighed 10 mg of each extract was dissolved in methanol. All the solutions were filtered through 0.2-μm membrane filter (Axiva Sichem Biotech, New Delhi, India) and then stored at −20°C till further use, and all were bought to room temperature prior to use.

Chromatographic conditions

Spot application

The aluminum-backed TLC plates (E. Merck, Darmstadt, Germany, 20 × 10 cm), coated with silica gel 60 F_{254}, were washed with methanol and dried in an oven at 105°C–110°C before spot application. Spots were applied 10 mm above the base of plate as 4.0-mm wide bands, under constant nitrogen flow using Camag's Linomat 5 applicator (CAMAG Scientific USA, Muttenz, Switzerland).

Mobile phase

Separation was done using mobile phase constituting of ethyl acetate: toluene: formic acid (5.4:4:0.5% v/v).[35]

Development of plate

This was done in a CAMAG® twin trough chamber (CAMAG Scientific USA, Muttenz, Switzerland) 200 mm × 100 mm covered with a steel lid. The chamber was saturated for about 30 min before placing the spotted plate in the chamber, and development was done till 80.0 mm in an ascending manner. This was followed by air-drying the plate and its densitometric scanning at 254 nm with the help of CAMAG TLC scanner III (CAMAG Scientific USA, Muttenz, Switzerland) equipped with Win CATS (CAMAG Scientific USA, Muttenz, Switzerland) software (V1.2.1). Peak area versus concentration calibration plot was utilized for quantification of khellin in different extracts.

Single-factor experiments

Based on the results of initial trials where different modes and solvents were tested for khellin extraction, single-factor experiments were conducted. As the name suggests, in these experiments, a single factor was varied to understand its effect on the response. In this piece of work, extraction temperature, extraction time, and solvent-to-drug ratio were taken as independent variables. Out of the three independent variables, one factor was varied at a time by keeping others constant to study its effect on khellin content. Khellin content in each extract was determined by HPTLC as described in the above section.

Software application and statistical optimization

Design-Expert software (version 11, Stat-Ease, MN, USA) was used to carry out optimization. BBD was chosen because it lacks any embedded factorial designs, which makes it more convenient.[17] Design of experiments consisted of 12 factorial experiments and five replicas of the center points. Independent variables were coded as per the below equation:

$$x_i = \frac{(X_i - X_o)}{\Delta_X}$$

where x_i is the coded value of the variable X_i, while X_o is the value of X_i at the center point and Δ_x is the step change of an independent variable. The coded values of the variables and the BBD experiments are given in Tables 1 and 2, respectively.

RESULTS AND DISCUSSION

Prefatory experiments

These experiments indicated the supremacy of UAE over other modes. Khellin content obtained using UAE was the highest, and the time taken with this technique was the lowest as compared to other methods. Moreover, methanol stood out to be the most effective extracting solvent for khellin. Chloroform extracted the least amount of khellin [Figure 2a].

Table 1: Actual and coded values of different variables

Independent variable	Coded levels		
	−1	0	+1
Extraction temperature (°C) – A	55	60	65
Extraction time (min) – B	20	25	30
Solvent-to-drug ratio (v/w) – C	15:1	20:1	25:1

A quantitative analysis of khellin in each extract was done through HPTLC. Five dilutions of standard were made and subjected to separation via HPTLC. Sharp peaks were obtained [Figure 3a]. Peak area versus concentration plot gave a good regression coefficient (R_2 = 0.990). The corresponding linear regression equation ($y = 1734.7x + 2887.2$) was used to calculate khellin content in each extract [Figure 2b].

Single-factor experiments

Holding on the best mode of extraction and most effective solvent for extraction of khellin, single-factor runs were conducted [Figure 3b]. These runs served as a mentor for selecting the ranges of different parameters for BBD.

Statistical optimization

These runs were conducted in accordance to the design of experiments furnished by BBD.

Table 2 tabulates the khellin content (experimental and predicted) in different extracts. A considerable variation of khellin content depending on the different extraction conditions was observed.

These 17 runs had different combination of variables A, B, and C. Through the multiple regression analysis application of RSM, a second-order polynomial equation was obtained which defined the relationship between response and variables as per the equation given below:

$Y = +6.86 + 1.06A - 0.4237\ B - 0.0188C + 0.2950AB - 0.1600AC + 0.5775BC - 1.66A^2 - 0.4413B^2 - 0.5962C^2$

where Y is the khellin content and A, B, and C are extraction temperature, extraction time, and solvent-to-drug ratio, respectively.

Model fitting and analysis of variance

Coefficient of regression (R^2) was found to be 0.998, which tells about the proximity of the data with fitted regression. A difference of < 0.2 between adjusted R^2 and predicted R^2 advocated excellent fit of the model. Coefficient of variance (%CV) which is the ratio of standard deviation to mean gives the comparison between distributions of values. The lower the %CV, the more precise is the model which in this case is 0.4624. The signal-to-noise ratio of the model is checked by adequate precision (195.819) which patroned that the model can be used to navigate the design space [Table 3].

Analysis of variance was applied for further fitting of the model. Variation of the data around the fitted model is given by lack of fit test. A large *F* value and small *P* value make a model good.[36] Here, *F* value of 4.90 and *P* value of 0.0794 make lack of fit insignificant, which is good for the model. Moreover, the *P* values of A, B, AB, AC, BC, A^2, B^2, and C^2 were found to be < 0.05, indicating that these parameters are significant, thereby demonstrating their interactive effects [Table 4].

Response surface curves

3D response surface plots help to give a visual outlook of the relationship between response and the variable. Figure 4a gives the interaction between A and B. It was observed that as temperature is increased, khellin content too increases till 58°C after which depreciation in the response is observed. A similar trend is observed with extraction time till 24 min of extraction. Interaction between A and C is depicted in Figure 4b, which shows that khellin content increases as temperature is increased from 55°C to 58°C after which a downhill pattern is seen. Similarly, with solvent-to-drug ratio, khellin content is enhanced till 18 mL/g and then it decreases. In the case of B and C [Figure 4c], both variables show

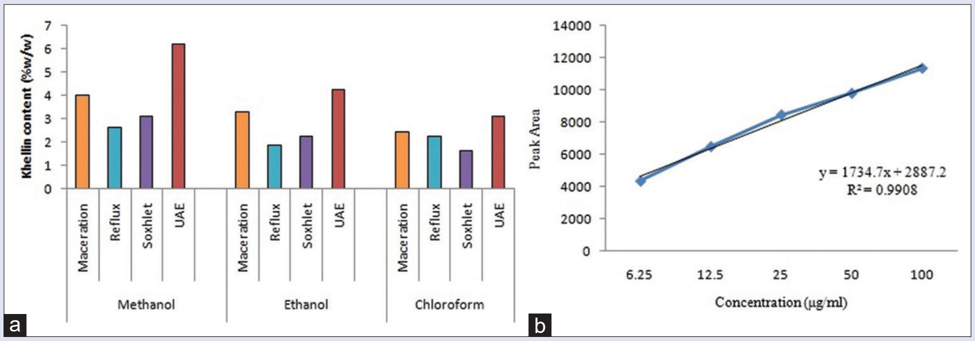

Figure 2: Results of prefatory experiments (a) and calibration plot for pure khellin (b)

Table 2: Design of experiments given by Box–Behnken Design

Run	A Extraction temperature (°C)	B Extraction time (min)	C Solvent-to-drug ratio (v/w)	Khellin content (%w/w)	
				Experimental value	Predicted value
1	60	30	25	5.98	5.96
2	65	25	15	5.86	5.84
3	55	25	15	3.42	3.40
4	60	25	20	6.84	6.86
5	60	30	15	4.82	4.84
6	60	20	25	5.67	5.65
7	55	25	25	3.66	3.68
8	55	20	20	4.42	4.42
9	60	25	20	6.85	6.86
10	60	25	20	6.87	6.86
11	60	25	20	6.88	6.86
12	65	25	25	5.46	5.48
13	65	30	20	5.68	5.68
1	60	20	15	6.82	6.84
15	55	30	20	2.98	2.98
16	65	20	20	5.94	5.94
17	60	25	20	6.86	6.86

Table 3: Fit statistics for the given model

Parameter	Value
R^2	0.9998
Adjusted-R^2	0.9996
Predicted-R^2	0.9978
Adequate precision	195.8118
CV%	0.4624

CV: Coefficient of variance

maximum khellin content at 21 min and 18 mL/g, respectively. Further inflation in both variables shows a dip in khellin content.

Validation of the model

Point prediction analysis and inverse matrix of polynomial equation presented the optimal conditions for khellin extraction which are as follows: extraction temperature – 63.84°C, extraction time – 29.51 min, and solvent-to-drug ratio – 21.64% v/w. However, to verify the suitability of the model, check runs were done with similar conditions as predicted by BBD where the khellin content was found to be 6.86% w/w (extraction temperature –64°C, extraction time –30 min, and solvent-to-drug ratio – 22% v/w). This was slightly higher than the predicted value (6.21% w/w), but no significant difference was observed between the predicted yield and experimental one when the Student's *t*-test ($n = 3$) was conducted, emphasizing that the model was satisfactory.

DISCUSSION & CONCLUSION

A wide range of extraction methods are available for the extraction of phytocompounds, with every method having its pros and cons. In this piece of work, we investigated both conservative and contemporary methods for the extraction of khellin. Further, its extraction parameters were optimized employing a modern-day statistical technique named RSM. This method of optimization was chosen because it provides certain advantages over conventional optimization methods. Aourabi *et al*. through their work have optimized the extraction process of phenolic compounds from *A. visnaga,* but for khellin has not been reported yet.[37]

Through our investigation, it was noted that UAE is the best mode for khellin extraction and methanol is the most effective solvent.

Table 4: Analysis of variance for the given quadratic model

Source	Sum of squares	Mean square	*F-value*	*P-value*	Remarks
Model	27.16	3.02	4518.65	<0.0001	
A – Temperature	8.95	8.95	13395.75	<0.0001	Significant
B – Time	1.44	1.44	2150.93	<0.0001	
C – Solvent-to-drug ratio	0.0028	0.0028	4.21	0.0793	
AB	0.3481	0.3481	521.22	<0.0001	
AC	0.1024	0.1024	153.33	<0.0001	
BC	1.33	1.33	1997.47	<0.0001	
A^2	11.66	11.66	17451.35	<0.0001	
B^2	0.8198	0.8198	1227.50	<0.0001	
C^2	1.50	1.50	2241.35	<0.0001	
Residual	0.0047	0.0007			
Lack of fit	0.0037	0.0012	4.90	0.0794	Not significant
Pure error	0.0010	0.0002			
Corrected Total Sum of Squares	27.16				

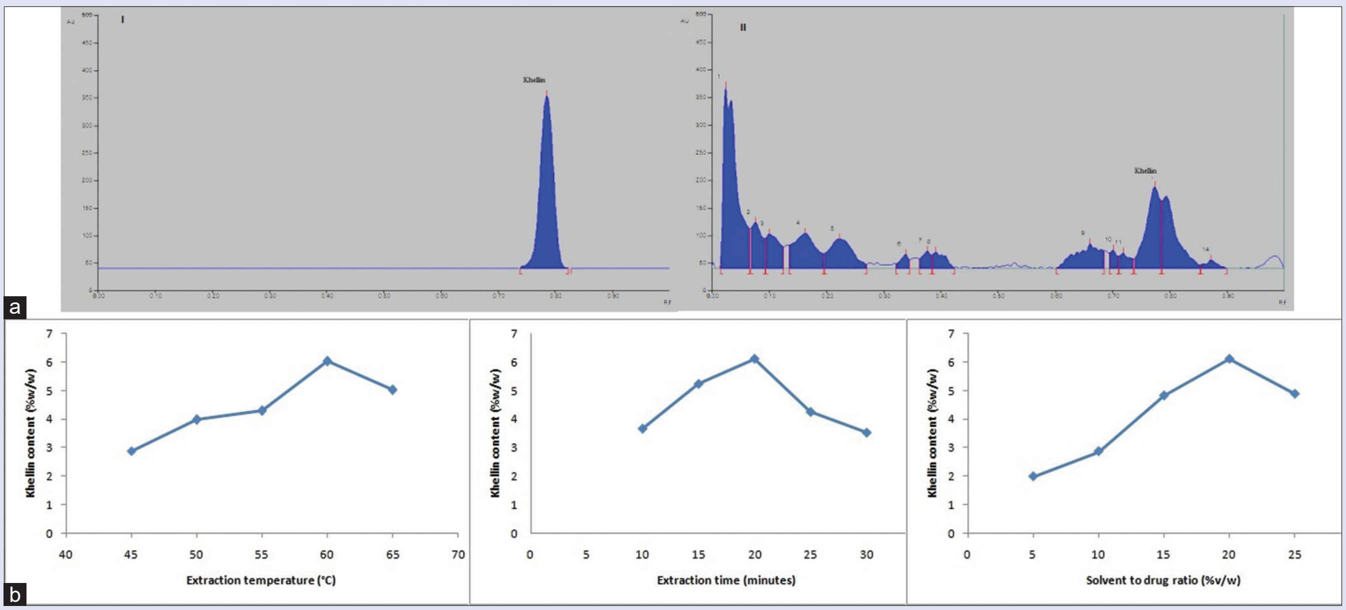

Figure 3: (a) High performance thin layer chromatography (i) chromatogram of pure khellin and (ii) *Ammi majus* L. methanolic extract and (b) results of single factor trials

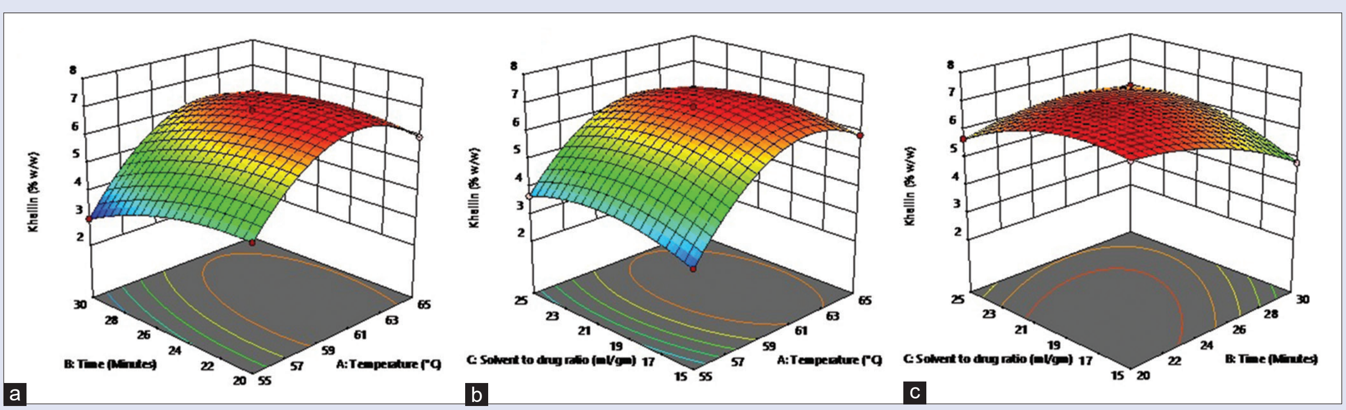

Figure 4: Three-dimensional response surface plots for A and B (a), A and C (b), and B and C (c)

Moreover, the optimal conditions for khellin extraction through UAE were laid down as follows: extraction temperature – 63.84°C, extraction time – 29.51 min, and solvent-to-drug ratio – 21.64% v/w. Under such conditions, 6.21% w/w of khellin was extracted.

In a nutshell, it can be said that a contemporary, green, and a non-thermal mode, that is, UAE, stood out to be best for khellin extraction. Such modes provide the additional benefit of being eco-friendly apart from being economical. This work is a perfect example of collaboration of statistics and pharmacognosy which might be of benefit for the upcoming researchers as well as pharmaceutical industries who wish to extract out khellin in maximum amount from *A. majus* L. fruits.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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