Pharmacogn. Mag.
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ORIGINAL ARTICLE

Statistical Approach towards Optimization of Extraction Process of Karanjin from *Pongamia pinnata* Seeds

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Submitted: 03-12-2018 Revised: 19-06-2019 Published: 28-11-2019

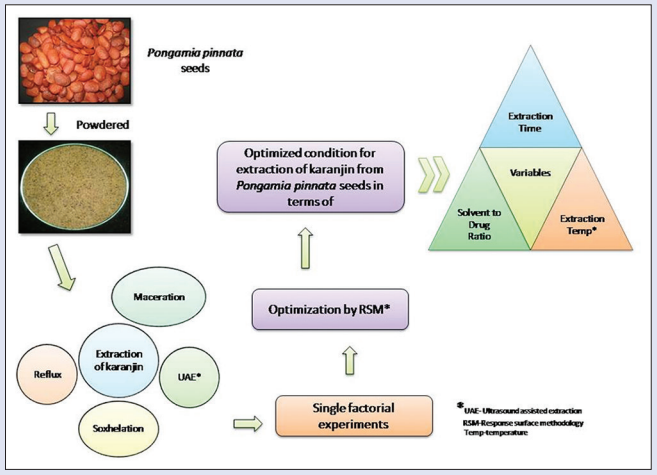
ABSTRACT

Background: *Pongamia pinnata* is a valuable herb with loads of pharmacological activities owing to its phytochemical profile. Karanjin is one phytocompound found in the seeds of *P. pinnata*. Optimization of karanjin extraction process becomes a high priority task because of its high significance. **Objective:** Use of Box–Behnken design for optimization of extraction of karanjin from *P. pinnata* seeds. **Materials and Methods:** Design expert software was used for optimization purpose. Extraction temperature, extraction time and solvent-to-drug ratio were taken as input variables which affected the karanjin content. Quantification of karanjin in different extracts was done through high-performance liquid chromatography using methanol and water (80:20% v/v) as mobile phase. **Results:** Ultrasound-assisted extraction stood out to be the most efficient mode for extraction of karanjin using methanol as solvent. Extraction temperature of 57.85°C, extraction time of 25.45 min, and solvent-to-drug ratio of 86.4709% v/w were established as optimum conditions for extraction of karanjin from *P. pinnata* seeds. Under such extraction conditions, 8.33% w/w karanjin was extracted. **Conclusion:** From our study, it was concluded that non-thermal methods are a better choice for extraction of karanjin and methanol is the most efficient solvent for the same. All the three input variables significantly affected karanjin content which was confirmed by model fitting and analysis of regression coefficients. Our research shows the relevance of a statistical approach in phytocompound research area which makes the extraction process cheap and less laborious.

Key words: Box-behnken design, furanoflavonoid, karanjin, *Pongamia pinnata*, response surface methodology

SUMMARY

- Karanjin is a furanoflavonoid possessed with innumerable biological actions. However, its presence in *Pongamia pinnata* seeds is very small quantity. In this piece of work, we have optimized the extraction conditions of karanjin from *P. pinnata* seeds. Computer-aided Box–Behnken design was used for optimization purpose and extraction temperature, extraction time and solvent-to-drug ratio were taken as independent parameters. Among the various modes studied, ultrasound-assisted extraction extracted karanjin in maximum quantity, whereas methanol was seen as the most effective extracting solvent. Extraction temperature of 57.85°C, extraction time of 25.45 min and solvent-to-drug ratio of 86.4709% v/w were established as optimum conditions for extraction of karanjin from *P. pinnata* seeds. Under such extraction conditions, 8.33% w/w karanjin was extracted. This work stood out to be a successful collaborative venture of statistics and pharmacognosy, whereby making the optimization process economical.



Abbreviations used: BBD: Box-Behnken design; HPLC: High-performance liquid chromatography; RSM: Response surface methodology; UAE: Ultrasound-assisted extraction.

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DOI: 10.4103/pm.pm_609_18

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INTRODUCTION

Extraction of any phytocompound means separating it from plant matrix and interstices employing a suitable solvent and extraction method, which may vary from conventional methods like maceration to modern extraction approaches such as microwave-assisted extraction, supercritical fluid extraction or ultrasound-assisted extraction (UAE). Every extraction process is impacted by solvent type, extraction process, temperature, light, pH, etc.[1] However, solvent type and strength play the most important role in extraction depending on the polarity of compound being isolated.[1] In the modern-day scenario, not only extraction of the phytocompound but also optimization of its extraction process is being researched widely to obtain the maximum quantity of the phytocompound. In 1951, Box and Wilson introduced a collection of mathematical and statistical techniques called response

Cite this article as: Ansari SA, Abidin L, Gupta T, Mujeeb M, Ahmad A, Mir SR. Statistical approach towards optimization of extraction process of karanjin from *Pongamia pinnata* seeds. Phcog Mag 2019;15:S419-25.

surface methodology (RSM) for empirical model building aiming at careful designing of experiments to obtain optimal response.[2] RSM can be successfully used where different combinations of input variables (like extraction temperature, extraction time, pH etc.) are given, and its response (quantity of phytocompound) is studied. Being economical, time-saving and effective in providing interactive effects of input variables are some of the advantages of using RSM.[3] Various instances of the use of RSM for optimization of extraction of phytocompounds are available like sinigrin from *Brassica juncea,*[4] phenolic compounds from *Sesamum indicum,*[5] embelin from *Embelica ribes*[6] betulinic acid from *Tacomella undulate*,[7] quercetin from *Herba polygoni*,[8] luteolin from *Vitex negundo*,[9] gymnemic acids from *Gymnema sylvestre*,[10] baicalein and pinostrobin from *Scutellaria violacea*,[11] lycopene from *Citrullus lanatus*,[12] phenolic acids from *Melissa officinalis*,[13] polysaccharides from *Suillus granulates*,[14] quercetin arabino pyranosyl rhamnopyranoside from *Kalanchoe pinnata*,[15] and polysaccharides from the root of *Limonium sinense.*[16]

Karanjin, a marker compound of Pongamia species is a furanoflavonoid and credited with numerous pharmacological properties such as antibacterial,[17] antioxidant,[18] anti-inflammatory,[18] α-glucosidase inhibitory,[19] and many more. The seed oil of the plant is used in the treatment of ulcers, rheumatism, leukoderma, and scabies.[20] Muthu *et al.* reported its use in wound and gastric treatment, gonorrhea, cleaning gums, teeth, and ulcers and in vaginal and skin diseases.[21] Being such a therapeutically active compound, optimization of its extraction process becomes crucial.

The study was directed towards optimizing the extraction process of karanjin from the seeds of *Pongamia pinnata* employing RSM and its simultaneous quantitative analysis by high-performance liquid chromatography (HPLC). The study was divided into three portions as follows:

1. Preliminary examination to find out the best mode and most effective solvent for extraction of karanjin
2. Single factorial experiments
3. Optimization using RSM.

MATERIALS AND METHODS

Plant material

The seeds of *P. pinnata* were obtained from Herbal Garden, Jamia Hamdard, New Delhi, India. The seeds were authenticated by a taxonomist from the Department of Botany, School of Chemical and Life Sciences, Jamia Hamdard, New Delhi, India, and a specimen is retained in the School of Pharmaceutical Education and Research, Jamia Hamdard, New Delhi, India.

Chemicals

Standard karanjin was purchased from Yucca Enterprises, Wadala, Mumbai, India. HPLC grade methanol and water were purchased from S. D Fine Chemicals, India. All other reagents used were of analytical grade and purchased from S. D. Fine Chemicals, India.

Statistical technique

Design-Expert Software (Version 11, Stat-Ease) was utilized for optimization requirements. Box–Behnken design (BBD) was selected for the same as it does not include any embedded factorial design.[3]

Experimental

Preliminary examination for the best mode and most effective solvent for extraction of karanjin

The seeds were properly cleaned, air-dried, and the dried samples were crushed and powdered through grinder (Sujata Supermix, 900W). Extraction of karanjin was done by different modes such as maceration, hot solvent extraction by reflux technique, hot solvent extraction by soxhlet technique, and UAE. Solvents of varying polarity were chosen for extraction such as methanol, chloroform, petroleum ether, and acetone. Extraction parameters for different techniques are described in Table 1. The residues obtained were weighed, and appropriate dilutions were made for quantitative estimation of karanjin in the extracts through HPLC.

Analysis of karanjin by high-performance liquid chromatography

Preparation of standard solutions

Stock solution of karanjin was prepared in HPLC grade methanol at a concentration of 1 mg/1 mL from which different dilutions ranging from 10 to 100 μg/mL and stored at −20°C. Before filtering ([0.2-μm membrane filter (Axiva]), solutions were bought to room temperature and then subjected to HPLC analysis. The calibration plot was made for concentration (μg/mL) versus peak area. The linear equation from the standard plot was used to determine the concentration of karanjin in sample solutions.

Preparation of sample solution

Ten milligrams of each solvent extract of *P. pinnata* prepared by different extraction methods were weighed and dissolved in HPLC grade methanol to obtain a final concentration of 1 mg/mL. The solutions were then filtered through a 0.2-μm membrane filter (Axiva), and 20 μL of the resulting solution was subjected to HPLC analysis. The final concentration of karanjin in the extracts was calculated by using the linear equation for the calibration curve.

Chromatographic conditions

HPLC analysis of the extracts was performed on HPLC Quaternary System (Shimadzu, Japan) consisting of LC10AT VP pumps (Shimadzu, Japan), a single wavelength programmable ultraviolet-visible detector and a system controller. Samples were injected by using a rheodyne injector fitted with a 20 μL fixed loop. The separation was achieved by using a column with 25 mm × 4.6 mm, particle size 5 μm, Lichrosphere C_{18} reverse-phase column (Merck, Germany). Determination of karanjin was carried out with the mobile phase composed of a mixture of methanol and water in a ratio of 80:20 v/v[22] at a flow rate of 1.0 mL/min. The optimum separation in HPLC was achieved at 30°C, and absorbance was measured at 260 nm.

Single factorial experiments

Single factorial experiments help to establish a range over which a particular variable can be studied. This is done by varying a particular variable while keeping other variables constant during a trial. Three variables were studied, namely extraction temperature (°C) and extraction time (min) and solvent-to-drug ratio (mL/g). Karanjin content in each extract was determined by HPLC as discussed in section 2.5.

Table 1: Different extraction techniques along with different parameters

Extraction techniques	Extraction time (h)	Extraction temperature (°C)	Solvent to drug ratio (mL/g)
Maceration	72	Room temperature	25:1
Reflux	5	50	25:1
Soxhlet	5	50	25:1
UAE	0.5	50	25:1

UAE: Ultrasound-assisted extraction

Optimization by response surface methodology

BBD experimental design consisted of seventeen runs-twelve factorial experiments and five replicates of the center points. Variables were coded according to the below given equation:

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

Where x_i is coded value of an independent variable, X_i is the actual value of the independent variable, X_o is actual value of independent variable at center point and ΔX is step change value of independent variable. The three variables were designated as X_1, X_2, and X_3 and were prescribed into three levels coded as +1, 0, and −1 for high, intermediate, and low levels, respectively. Coded and actual values of variables are depicted in Table 2, and the BBD runs are given in Table 3. All extracts were quantified by HPLC, as discussed in previous section.

RESULTS

Extraction of karanjin by various extraction techniques

Karanjin was extracted by four different extraction techniques, namely maceration, soxhlet, reflux and UAE employing methanol, chloroform, petroleum ether, and acetone as solvents. All the variables such as extraction temperature, extraction time, and solvent-to-drug ratio were kept constant in the experiments. In general, methanol extracts were found to show an appreciable amount of karanjin. However, of all the extraction techniques, UAE was found to be the most suitable mode of extraction [Figure 1].

Quantitative estimation of karanjin by high-performance liquid chromatography method

The standard and the sample solutions were subjected to HPLC. The retention time for karanjin was seen at 7.7 min [Figures 2 and 3]. 6 different dilutions were taken for preparing calibration curve (concentration ranged from 10 to 60 µg/mL).Linear regression was obtained (y = mx + c) where y and x correspond to the area under the curve and concentration, respectively [Figure 4]. The correlation coefficient (R^2) of 0.9927 was obtained. Quantitative analysis of karanjin in different extracts was calculated from the regression equation obtained from the calibration plot (y = 20645x + 211881).

Quantification of extracts unveiled that methanol is the best solvent for the extraction of karanjin. Karanjin content in methanol extract obtained through maceration, reflux, soxhelation, and UAE techniques was found to be 5.73% w/w, 6.14% w/w, 6.47% w/w, and 7.09% w/w respectively [Figure 1]. Petroleum ether also showed an appreciable amount of karanjin but not as high as methanol. Moreover, among all the modes studied, UAE was found to be most productive.

Single factorial experiments

The effect of three variables, including extraction temperature (°C), extraction time (min), and solvent-to-drug ratio (mL/g), were studied on the content of karanjin by UAE using methanol as solvent. One variable was varied and two were kept constant during the experiment to study the effect of that particular variable on karanjin content.

Table 2: Coded levels of independent variables

Independent variables	Symbol		Coded levels		
	Coded	Uncoded	−1	0	+1
Extraction temperature (°C)	X_1	x_1	45	55	65
Extraction time (min)	X_2	x_2	10	20	30
Solvent-to-drug ratio (mL/g)	X_3	x_3	30:1	90:1	150:1

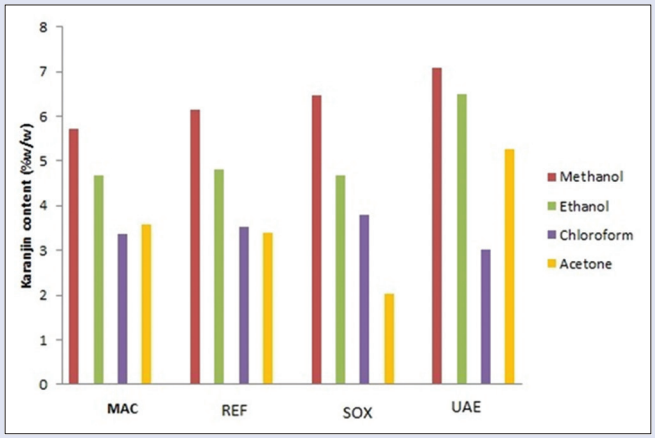

Figure 1: Graph depicting karanjin content in different solvent extracts through various extraction techniques. MAC: Maceration, REF: Reflux, SOX: Soxhelation, UAE: Ultrasound-assisted extraction

Table 3: Box-Behnken design matrix and the response values for ultrasound-assisted extraction extracted karanjin content

Run	Raw material: solvent ratio (mL/g) (x_3)	Extraction time (min) (x_2)	Extraction temperature (°C) (x_1)	Karanjin content (%w/w)		
				Experimental Ye	Predicted Y	Ye-Y
1	30	10	55	8.00	7.96	0.04
2	150	20	65	8.88	8.75	0.13
3	150	20	45	8.49	8.41	0.08
4	90	20	55	8.40	8.38	0.02
5	30	30	55	8.56	8.49	0.07
6	150	10	55	8.56	8.52	0.04
7	30	20	45	8.50	8.45	0.05
8	90	20	55	8.45	8.36	0.09
9	90	20	55	8.47	8.34	0.13
10	90	10	65	8.61	8.58	0.03
11	90	10	45	8.00	7.98	0.02
12	150	30	55	8.17	8.12	0.05
13	90	30	65	8.26	8.23	0.03
14	30	20	65	8.57	8.53	0.04
15	90	30	45	8.47	8.44	0.03
16	90	20	55	8.46	8.33	0.13
17	90	20	55	8.44	8.37	0.07

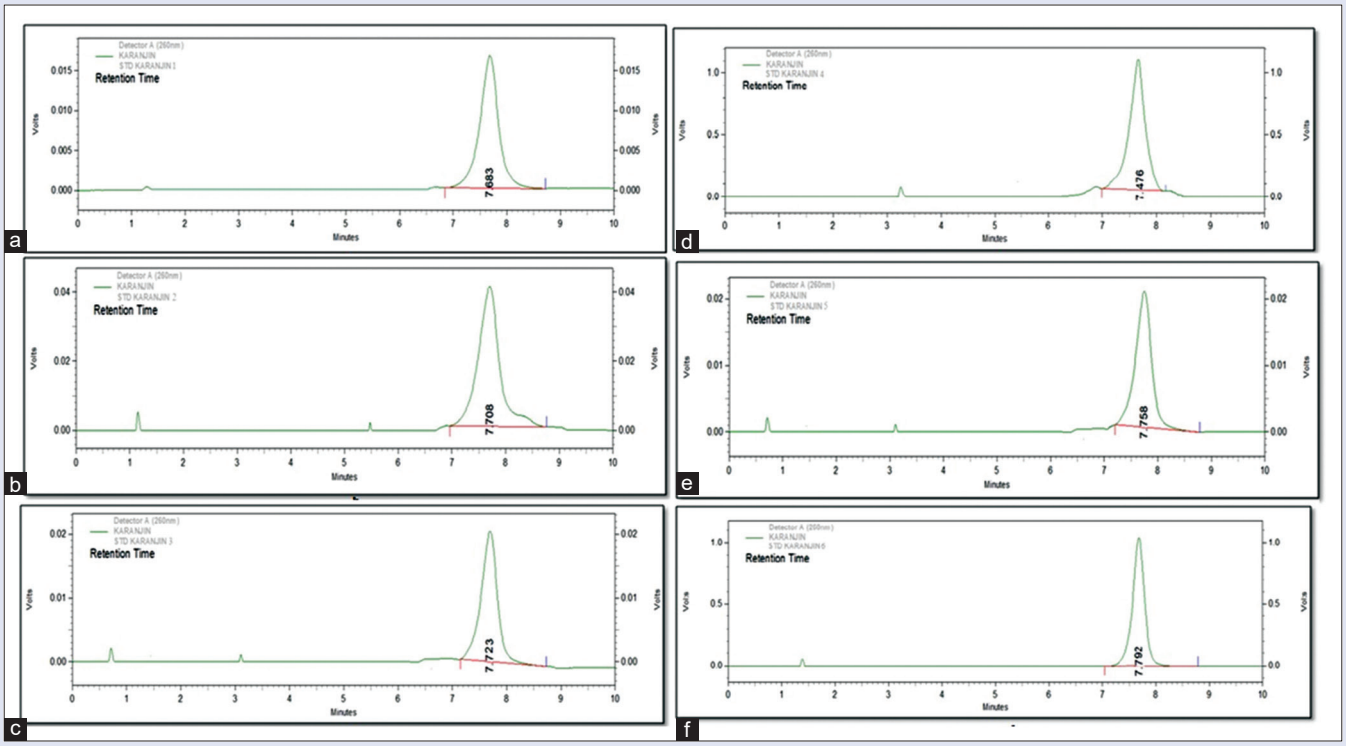

Figure 2: High-performance liquid chromatography chromatogram of standard karanjin at 10 µg/mL (a), 20 µg/mL (b), 30 µg/mL (c), 40 µg/mL (d), 50 µg/mL (e), and 60 µg/mL (f)

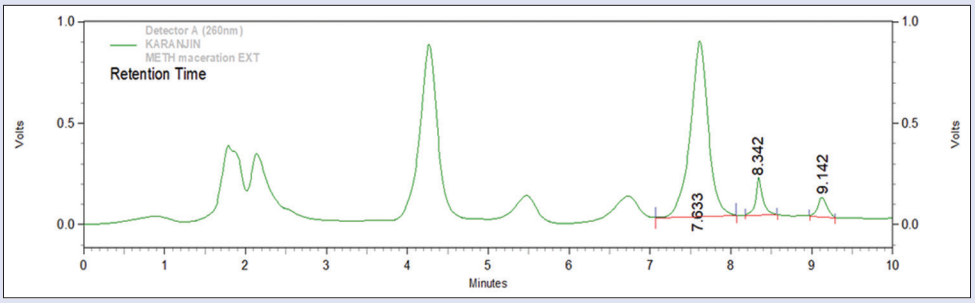

Figure 3: High-performance liquid chromatography chromatogram of methanolic extract of *Pongamia pinnata* seeds (ultrasound-assisted extraction)

Karanjin content in each extract was analyzed by HPLC, as discussed previously. These experiments helped to select a range of a variable to be studied by RSM. The results of single factorial experiments are depicted in Figure 5.

Optimization of extraction parameters by box-Behnken design

Results of single factorial experiments helped to select a range for BBD. BBD provided seventeen runs of different combinations of the three variables [Table 3]. Experiments were conducted accordingly and by statistical analysis of the experimental data, a second-order polynomial model was established, which correlated a relationship between karanjin yield and the extraction variables. The relationship could be expressed by the following equation:

$$Y = a_0 + a_1x_1 + a_2x_2 + a_3x_3 + a_{12}x_1x_2 + a_{13}x_1x_3 + a_{23}x_2x_3 + a_{11}x_{12} + a_{22}x_2^2 + a_{33}x_3^2$$

Where Y is the predicted response value; a_0 is the intercept term; x_1, x_2 and x_3 are independent variables; a_1, a_2 and a_3 are linear coefficients; a_{12}, a_{13} and a_{23} are cross-product coefficients; and a_{11}, a_{22} and a_{33} are the quadratic term coefficients.

Model fitting and analysis of regression coefficients and the response surface

Model fitting was tested by determinant coefficient (R^2) and adjusted determinant coefficient (adj-R^2). The value of Adj-R^2 (0.9935) was close R^2 (0.9918) emphasizing excellent fit of the model. Moreover, a high value of R^2 (0.9918) conveyed 99.18% of the variation could be illustrated by the fitted model. Failure of the model to represent the data in the experimental domain at points which were not included in the regression is represented by "Lack of fit" test. The *F*-value for the lack-of-fit was not significant ($P > 0.05$) (0.993), thereby validating the model. Signal-to-noise ratio is given by "Adequate Precision" which should be more than 4 for model fitting. The ratio of 62.108 indicates an adequate signal in this model. The analysis of variance (ANOVA) of quadratic regression model demonstrated that the model was highly significant, evident from the Fisher's *F*-test with a model *F*-value 273.70, but a very low *P* value ($P < 0.0001$).

By employing multiple regression analysis on the experimental data, the variable (Y) and the tested variables were related by the following second-order polynomial equation:

$$Y = 8.36 + 0.10x_1 + 0.030x_2 + 0.046x_3 - 0.20\,x_1x_2 + 0.065x_1x_3 - 0.23x_2x_3 + 0.11x_1^2 - 0.16x_2^2 + 0.072x_3^2$$

Summary of ANOVA for the fitted quadratic polynomial model was used to determine the goodness of the model [Table 4]. The coefficient of variation (CV = 0.20%) was a relatively small value advocating better reliability of the experimental values. Lack of fit tests was also helpful for determining the goodness of the model. *F* and *P* values were 0.14 and 0.0337, respectively, suggesting lack of fit to be nonsignificant and model to be satisfactory. *P* value of each coefficient helped to check the significance of each variable [Table 5]. All the terms were found to be significant model terms with $P < 0.001$ proposing that the model could be used to predict these responses.

Three-dimensional response surface curves

Three-dimensional (3D) response surface plots and contour plots help in understanding the interactions between the variables and the response more clearly.[5] The surface-confined in the smallest ellipse in the contour diagram is indicative of maximum predicted response. As the temperature was increased from 50°C to 55°C, karanjin yield was enhanced till 57°C beyond which karanjin yield was decreased. Moreover, increment in time also enhanced karanjin yield, but increment beyond 25 min showed a decreasing content of Karanjin. Karanjin content also increased as a solvent-to-drug ratio was increased, but an increment beyond 86.4 mL/g decreased the karanjin yield. Furthermore, as the temperature from 50°C to 55°C was increased, karanjin yield increased but enhancing the temperature beyond 57°C decreased the Karanjin content. With the elevation in extraction time, karanjin yield increased till 25 min beyond which a dip in the yield was observed. Similarly, as a solvent-to-drug ratio was increased, karanjin content also increased. However, increment

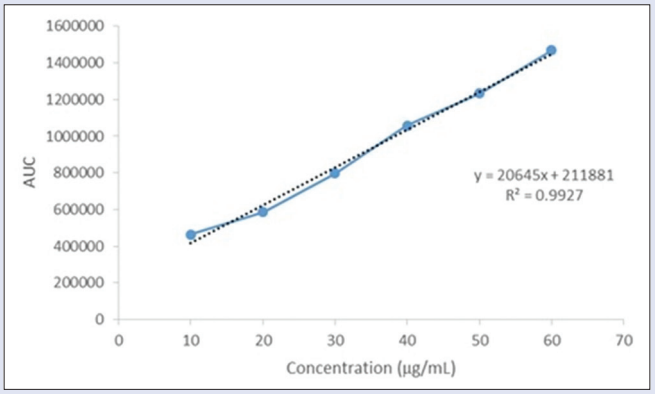

Figure 4: Calibration curve of standard karanjin (concentration versus area under the curve)

Table 4: Analysis of variance for the fitted quadratic polynomial model

Source	Sum of squares	Degree of freedom	Mean square	*F*	*P*>*F*
Model	0.67	9	0.074	273.70	<0.0001 (S)
Lack of fit	1.750	3	5.833	0.14	0.9337 (NS)
Pure error	1.720	4	4.300	-	-
Corrected total	0.67	16	-	-	

R^2=0.9972, R^2_{adj}=0.9935, R^2_{pred}=0.9918, C.V.(%)=0.20. NS: Not significant; S: Significant

Table 5: Analysis of variance for second-order polynomial model and co-efficient values

Model term	Sum of square	Degree of freedom	Mean square	*F*	*P*>*F*
Intercept					
x_1	0.082	1	0.082	302.95	<0.0001
x_2	0.019	1	0.019	26.60	<0.0001
x_3	0.017	1	0.017	63.21	<0.0001
x_1x_2	0.16	1	0.16	605.90	<0.0001
x_1x_3	0.017	1	0.017	62.43	<0.0001
x_2x_3	0.22	1	0.22	798.72	<0.0001
x_1^2	0.048	1	0.048	178.07	<0.0001
x_2^2	0.10	1	0.10	376.08	<0.0001
x_3^2	0.022	1	0.022	80.63	<0.0001

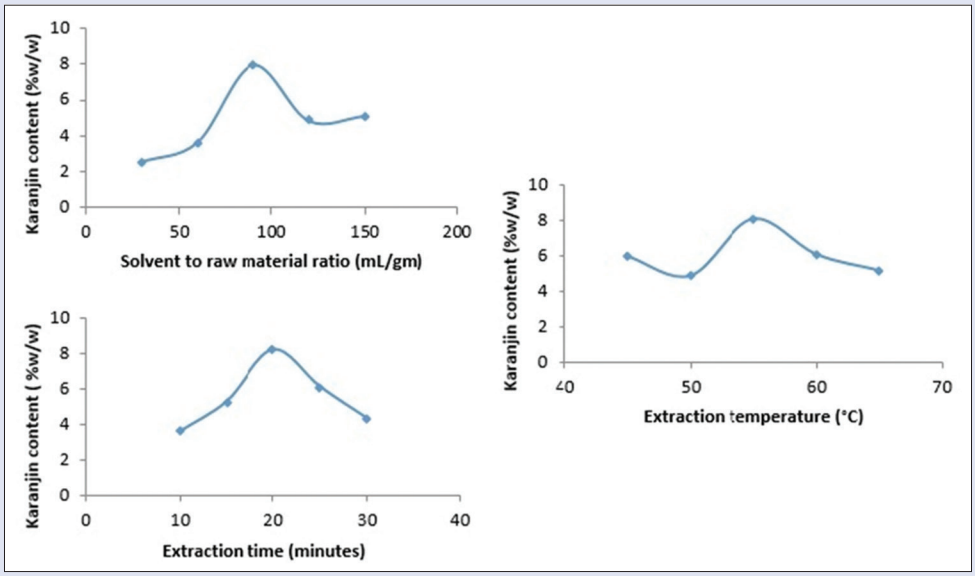

Figure 5: Effect of extraction time, temperature, and solvent-to-drug ratio on karanjin content

beyond 86.4 mL/g showed a decrease in karanjin yield. The 3D response surface and contour plots are given in Figure 6.

Model validation

To validate the acceptability of the model

By solving the inverse matrix of regression polynomial equation and point prediction analysis, the optimum values of the tested parameters in uncoded units were obtained as given in Table 6. Under such optimum conditions, the maximum Karanjin yield was predicted to be 8.33% w/w.

Table 6: Predicted and experimental set up

	Optimal conditions predicted by BBD	Experimental Conditions
Mode of extraction	Ultrasound-assisted extraction	
Solvent	Methanol	
Solvent to raw material ratio	86.4709% v/w	86% v/w
Extraction temperature	57.857°C	58°C
Extraction time	25.4557 min	25 min
Karanjin yield	8.33608% w/w	8.224%w/w*

*n=3, no significant difference observed when Student's t-test was performed

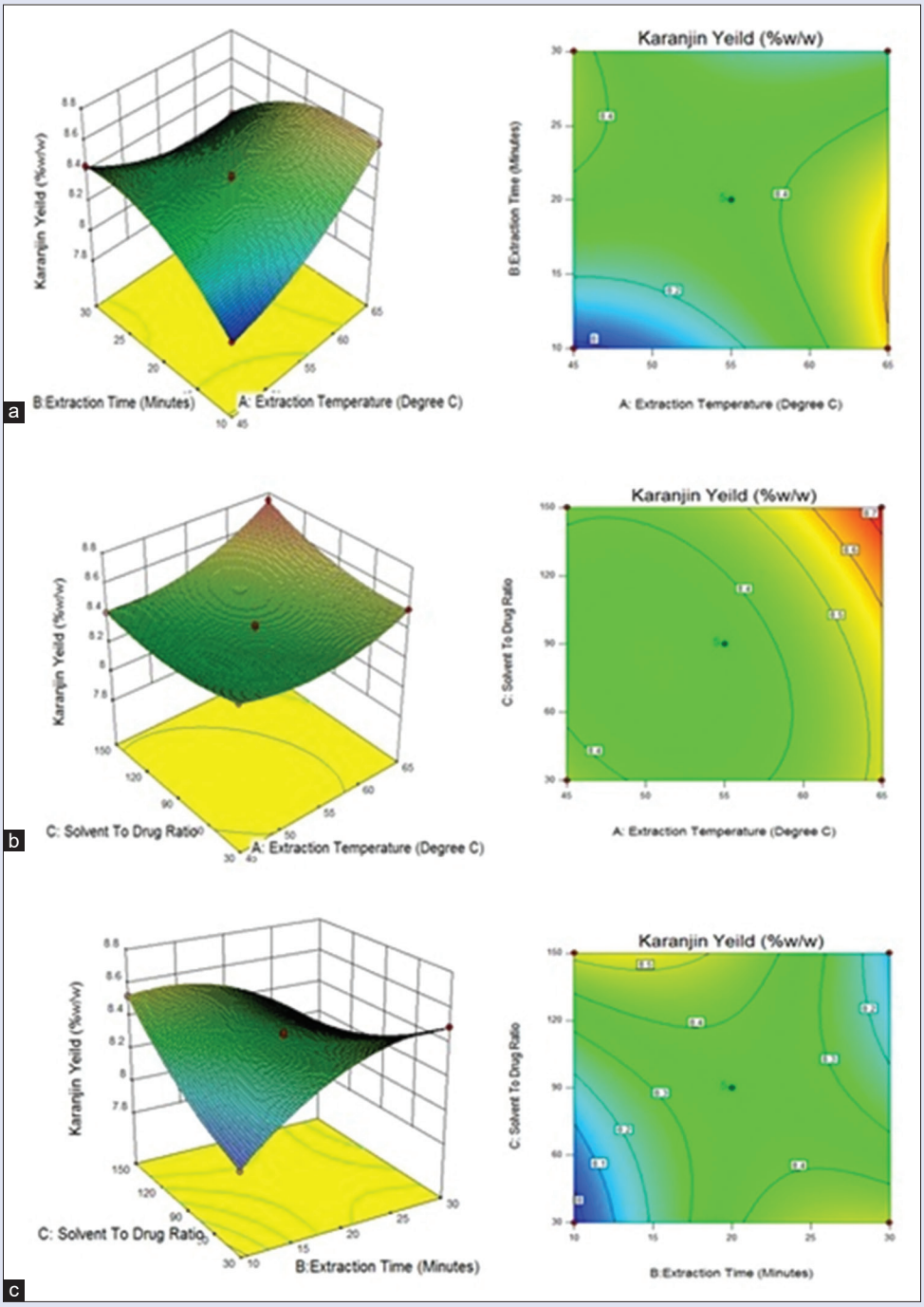

Figure 6: (a) Three-dimensional response surface and contour plot for extraction temperature and extraction time. (b) Three-dimensional response surface and contour plot for extraction temperature and solvent-to-drug ratio. (c) Three-dimensional response surface and contour plot for extraction time and solvent-to-drug ratio

To validate the suitability of the model equation for predicting the optimum response value, an experimental rechecking was performed using the inferred optimal conditions. Under the determined conditions, a mean value of Karanjin yield of 8.22% w/w (n = 3) was obtained from the real experiments, slightly lower than the predicted maximum value (8.33%w/w) with 99.25% validation of the model. However, no significant difference was observed between the predicted yield and experimental one when the student t-test was conducted, indicating that the model was satisfactory and adequate for reflecting the expected optimization.

DISCUSSION

Some studies suggest that biological activity of any extract is affected by extraction technique employed. Thus, it is important to select a suitable solvent as well as the extraction method, which is least influenced by the presence of interfering substances, based on the chemical and physical properties of the sample matrix.[23]

Karanjin is an important herbal compound possessing plethora of therapeutic activities. Thus, it becomes important to extract it out in maximum amount and optimize its extraction process. HPLC technique was used for its quantification, which provided rapid separations are than classical methods and provide high resolution and sensitivity.[24]

In the present study, we took advantage of RSM to optimize the extraction process of karanjin from *P. pinnata* seeds. BBD was used in this regard because of certain advantages over other designs of RSM.

The outcome of our research will help the other researchers to take advantage of the conditions given by RSM, BBD to isolate the maximum amount of karanjin from *P. pinnata* seeds through a non-thermal method. Non-thermal method provides the additional benefit of avoiding thermal degradation of the phytocompound as well as being environment friendly.

CONCLUSION

From our research work, it can be concluded that non-thermal method of extraction, namely UAE is the most efficient mode for extraction of karanjin, also, polar solvents are the most promising solvent for extraction of karanjin.

Acknowledgements

The authors are grateful to the Head, Department of Pharmacognosy and Phytochemistry, School of Pharmaceutical Education and Research, Jamia Hamdard, New Delhi, India, for providing necessary research facilities to carry out this project.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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