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ORIGINAL ARTICLE

Computer-aided Box–Behnken Outlook Toward Optimization of Extraction of Baicalin from *Oroxylum indicum* L. Stem Barks

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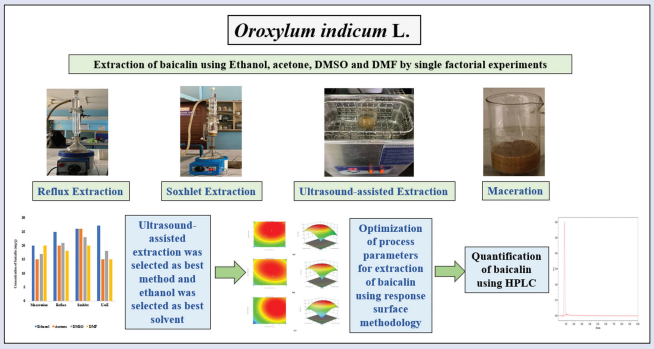
ABSTRACT

Background: Baicalin, a flavone glucuronide exhibiting profuse pharmacological activities present in *Oroxylum indicum* stem barks. Our research highlights the process parameter at which a high yield of baicalin can be extracted from *O. indicum* stem barks in "one run." **Objectives:** The study compares the efficacy of different modern and traditional methods for baicalin extraction. Box–Behnken design (BBD) was availed for optimization of process parameters for the extraction of baicalin from *O. indicum* stem barks. **Materials and Methods:** Extraction conditions (extraction time, solvent-to-drug ratio, and extraction temperature) were optimized by response surface methodology (RSM), specifically BBD. Quantification analysis of baicalin in different extracts was done using high-performance liquid chromatography. **Results:** Ultrasound-assisted extraction (UAE) method conferred the highest yield of baicalin, and ethanol was found to be the most efficient extractive solvent. Through the use of BBD, the optimal conditions for baicalin extraction were established as extraction time—29.058 min, solvent-to-drug ratio—21.124 ml/g, and extraction temperature—67.963°C. Under such conditions, baicalin was yielded as 26.572 mg/g, which was nearly close to the predicted value of 27.16 mg/g. **Conclusion:** The UAE method stood out to be the best among all the other thermal and non-thermal modes of extraction used, and ethanol proves to be the most efficient extracting solvent. Additionally, baicalin extraction was significantly affected by all three different variables. Our study highlights the use of RSM, a modern-day statistical technique in the extraction field of therapeutically potent phytocompounds, which makes the optimization method cheap and less laborious than the traditional optimization method.

Key words: Baicalin, extraction, optimization, *Oroxylum indicum*, response surface methodology

SUMMARY

- Baicalin, a flavone glucuronide present in *O. indicum* stem barks, exhibits profuse pharmacological activities. Our research compares the efficacy of different modern and traditional methods for baicalin extraction and highlights the process parameter at which a high yield of baicalin can be extracted from *O. indicum* stem barks in "one run." In the present study, BBD was availed for the optimization of process parameters for the extraction of baicalin from *O. indicum* stem barks. Extraction conditions (extraction time, solvent-to-drug ratio, and extraction temperature) were optimized by RSM, specifically BBD. Quantification analysis of baicalin in different extracts was done using HPLC. The experimental results revealed that the UAE method stood out to be the best among all the other thermal and non-thermal modes of extraction used, and ethanol proves to be the most efficient extracting solvent. Through the use of BBD, the optimal conditions for baicalin extraction were established as extraction time—29.058 min, solvent-to-drug ratio—21.124 ml/g, and extraction temperature—67.963°C. Under such conditions, baicalin was yielded as 26.572 mg/g, which was nearly close to the predicted value of 27.16 mg/g. Additionally, baicalin extraction was significantly affected by all three different variables. Our study highlights the use of RSM, a modern-day statistical technique in the extraction field of therapeutically potent phytocompounds, which makes the optimization method cheap and less laborious than the traditional optimization method.



Abbreviations used: 3D: Three-dimensional; °C: Degree Celsius; ANOVA: Analysis of variance; BBD: Box–Behnken design; G: Gram; HPLC: High-performance liquid chromatography; RSM: Response surface methodology; UAE: Ultrasound-assisted extraction.

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INTRODUCTION

Oroxylum indicum Linn., commonly kenned as the Tree of Damocles, belongs to the Bignoniaceae family.[1] It is a prime herb in indigenous and ayurvedic medical systems. It has been used as a prime ingredient in various ayurvedic medicinal preparations such as *Dasamula, Brahma Rasayana*, *Dantyadyarista, Dhanawantara Ghrita, Amartarista, Chyawanprash Awaleha,* and *Narayana Taila*.[2,3] As per Ayurveda, diversified medicinal properties are ascribed to various parts of the

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Indian trumpet tree.[4] The plant exhibits antioxidant, anti-inflammatory, analgesic, antimicrobial, anticancer, photocytotoxic, antimutagenic, antiarthritic, immunostimulant, and antiproliferative activities. Various other effects like antiallergic, antiasthmatic, antihelmintic, antidiabetic, cardioprotective, gastroprotective, hepatoprotective, antiobesity, and wound healing have also been explored. The potential against SARS CoV-2 has also been reported.[5-20]

The *O. indicum* contains a broad variety of phytochemicals such as tannins, alkaloids, saponins, sterols, flavonoids, lignins, glycosides, phenols, fats, and oils. The active constituent of the *O. indicum* is baicalin.[4]

Baicalin, a bioactive natural glycosyloxyflavone having formula 5,6-Dihydroxy-4-oxygen-2-phenyl-4H-1-benzopyran-7-β-D-gl ucopyranose acid, has been copiously explored both *in vivo* and *in vitro*.[21] Baicalin exhibits profuse pharmacological activities including cardioprotective, nephroprotective, hepatoprotective, and neuroprotective activity. It exhibits anti-viral, anti-tumor, anti-inflammatory, antibacterial, antioxidant, antiarthritic, and antipsoriatic effects.[22] Baicalin is also the main ingredient of flavocoxid, which is an approved medical food and is classified under the generally recognized as safe category by the US FDA.[23] Owing to its health-endorsing properties ergo can be availed as a propitious candidate in research and development areas.

In these circumstances, where the phytocompounds manifest vast therapeutic potential, optimizing their extraction parameters becomes significant. The maximum amount of therapeutically active constituents can be extracted from the plants in a single run.

Traditionally, optimization is carried out using "one-factor-at-a-time" method, wherein one factor is varied at a time. Howbeit, this method is laborious, time-consuming, and requires more amount of solvent. Moreover, it fails to study the interaction of different variables. Therefore, other techniques like response surface methodology (RSM) have come into play, a statistical, mathematical technique introduced by Box and Wilson in the year 1951 for the purpose of analyzing and modeling any process wherein the response of interacting variable is dependent on different variables. RSM can be effectively operated wherein different combinations of the input variables (for example, extraction time, extraction temperature, and pH) are specified, and their effect on the response (quantity of phytocompound) is determined.[24]

RSM is time-saving and less laborious and helps in studying the interactive effects of different variables and then overcomes the drawbacks associated with traditional optimization methods.[25] The multivariate technique has been copiously exploited by the researchers for the purpose of optimization of extraction parameters of various phytocompounds like atropine from *Atropa belladonna,*[26] carthamin from *Carthamus tinctorious*,[27] embelin from *Emblica ribes*,[28] flavonoids from *Vitis vinifera*,[29] glycyrrhizic acid from *Glycyrrhiza glabra*,[30] karanjin from *Pongamia pinnata*,[31] lupeol from *Ficus racemosa*,[32] and quercitrin from Herba *Polygoni capitati.*[33]

Our current study utilizes RSM for optimization of the extraction parameters (a drug-to-solvent ratio, extraction temperature, extraction time) of baicalin from *O. indicum* stem barks, and quantification of the phytoactive constituent is done with the help of the high-performance liquid chromatography (HPLC). Several studies have reported the extraction of baicalin from *Scutellaria baicalensis*, *Scutellaria galericulata,* and *Scutellaria lateriflora.*[34,35] However, none of the researchers have developed the extraction process for the isolation and quantification of baicalin from *O. indicum*. Therefore, our study employs different techniques like Soxhlet, maceration, reflux technique, and ultrasound-assisted extraction (UAE) for the extraction of baicalin, which has not been reported by any other researcher yet.

MATERIALS AND METHODS

Collection and authentication of the Plant Material

The stem barks of *O. indicum* L. were procured from the medicinal plant market, in Delhi. Identification and authentication were done at the National Institute of Science Communication and Policy Research (NIScPR), New Delhi, with authentication number NIScPR/RHMD/Consult/2021/3880-81.

Chemicals

Standard baicalin was acquired from Sigma Aldrich. HPLC grade water and methanol were purchased from S.D. Fine Chem Limited, India. All other analytical grade chemicals were obtained from S.D. Fine Chem Limited, India.

Preparation of plant material

The stem barks were thoroughly cleaned to eliminate all the cling foreign material and dust particles and washed under running water. Further, they were dried at room temperature, powdered, and passed through 40 mesh sieves, and stowed in an air-lock container.

Extraction of baicalin

Different solvents—acetone, ethanol, dimethylformamide (DMF), and dimethyl sulfoxide (DMSO) of varying polarity were employed to extract baicalin from *O. indicum* using four different modes of extraction, namely, reflux, Soxhelation, maceration, and UAE.

Reflux extraction

Extraction was carried out using a reflux apparatus at a temperature of 50°C for 1 h with a solvent-to-drug ratio of 10:1 ml/g. After extraction, the solid residue was separated and removed by filtration, and the filtrate was concentrated using a rotary evaporator.

Soxhlet extraction

Extraction was carried out using a Soxhlet apparatus at a temperature of 50°C for 1 h, with a solvent-to-drug ratio of 10:1 ml/g. After extraction, the solid residue was separated and removed by filtration, and the filtrate was concentrated using a rotary evaporator.

Extraction by maceration

Three grams of the powdered drug were taken in a beaker and immersed in 30 ml of the solvent, having a solvent-to-drug ratio of 10:1 ml/g at ambient temperature for 3 days. The menstruum was filtered, and the filtrate was evaporated. The extract was then collected and stored for further analysis.

Ultrasound-assisted extraction

UAE was done in an ultrasonic device at a temperature of 50°C for 1 h, with a solvent-to-drug ratio of 10:1 ml/g. After extraction, the solid residue was separated and removed by filtration, and the filtrate was concentrated using a rotary evaporator.

Comparison of different extraction techniques for extraction of baicalin

Quantification of baicalin in different extracts of *O. indicum* was done by HPLC using Shimadzu HPLC Quaternary System (Japan) attached with a C_{18} reverse-phase Lichrospher column (Merck, Germany) of 5 µm particle size and 25 x 4.6 mm length. A stock solution of standard baicalin and a sample solution of different extracts of *O. indicum* were prepared in HPLC grade methanol. The dilutions

of standard baicalin ranged from 20 ug/ml to 100 ug/ml were also prepared in HPLC grade methanol. All the solutions were filtered through a 0.2 µm membrane filter (Axiva) before subjecting to the HPLC system. Water: methanol (15:85) was used as mobile phase at a 1 ml/minute flow rate in isocratic mode,[16] and detection was done at a wavelength of 316 nm. The calibration curve was made between concentration for standard baicalin and peak area. Baicalin content in different extracts was then calculated from the linear equation of the calibration curve.

Single factorial experiments

After establishing the most efficient extraction mode and the best solvent, single factorial experiments were run on three parameters such as extraction temperature, solvent-to-drug ratio, and extraction time. To study the influence of a particular parameter on the yield of baicalin, two parameters were kept constant, and one was varied during the experimental trial. The ranges evaluated for different parameters are shown in Table 1. Baicalin content in each extract was quantified using HPLC.

Extraction parameters optimization by Box–Behnken design (BBD) for baicalin

The BBD, RSM, was availed to optimize the parameters of extraction for baicalin from *O. indicum* employing Design-expert software (Version 13), Stat-Ease, Inc. USA. The experimental design comprises five replicates of the center point and 12 factorial experiments. The three chosen factors selected were encrypted as *Y*1, *Y*2, and *Y*3 and were designed into three levels ciphered as −1, 0, and +1 for low, intermediate, and high levels, respectively, coding of the test variables was done in accordance with the equation mentioned below:

$y_{i=}(Y_i - Y_0)/\Delta Y$

Where

y_i represents coded value of an independent variable,

Y_i represents actual value of an independent variable,

Y_0 represents actual value of an independent variable at the center point, and

ΔY represents step-change value of an independent variable.

The actual and the coded values of three variables are mentioned in Table 1, and the 17 runs of BBD experiments are mentioned in Table 2.

Quantification of baicalin in various extracts by HPLC

Different extracts for BBD experiments were analyzed using HPLC for the quantification of baicalin content in *O. indicum*.

RESULTS AND DISCUSSION

Comparison of different extraction techniques for extraction of baicalin

Four different modes of extraction were used to extract baicalin from *O. indicum* employing four different solvents of varying polarity divulging that ethanol stood out to be the most efficient solvent, and the UAE method proved to be the elite extraction mode for the extraction of baicalin [Figure 1]. Quantitative analysis of baicalin in each extract was done via HPLC, and the ensuing chromatogram of standard baicalin and sample baicalin in ethanolic extract of *O. indicum* showing baicalin peak at retention time 0.849 are shown in Figures 2 and 3. The retention time, area under the curve, and quantification values of baicalin in different extracts of *O. indicum* from different extraction methods are shown in Table 3.

Table 1: Ranges of different parameters assessed in a single factorial experiment along with their coded and actual values

Independent variables	Lower range	Higher range	Coded levels		
			−1	1	+1
Extraction temperature	50	70	50	60	70
Extraction time	20	30	20	25	30
Drug-to-solvent ratio	1:10	1:30	10	20	30

Single factorial experiments

Holding to the elite extraction mode and the most efficient solvent for baicalin extraction, single factorial runs were performed—the results from single runs assisted in selecting the ranges of different parameters of BBD [Figure 4].

Optimization of Extraction parameters by BBD

Different experimental runs were performed in accordance with the design of BBD. A polynomial equation of second order was obtained through the multiple regression analysis, which defines the relationship between tested variables and response variable (baicalin content) stated below:

$Y = +27.16 + 1.05A + 4.13B + 2.34C + 0.7450AB - 1.44AC - 0.5750BC - 3.39A^2 - 4.75B^2 - 4.53C^2$

Where Y—Baicalin content,

A—Extraction temperature (°C),

B—Extraction time (minutes), and

C—solvent-to-drug ratio (ml/g)

To determine the goodness of the model, analysis of variance (ANOVA) was employed [Table 4].

The regression coefficient (R^2) was found to be 0.9999, which apprises the closeness of the data with fitted regression. A difference of <0.2 between predicted R^2 and adjusted R^2 signifies an excellent fit for the model. Meanwhile, a low value of the coefficient of variance (% CV of 0.3367) implies better dependability of the experimental values. Signal to noise ratio called adequate precision is expected to be more than four was 298.2156, which shows the model's goodness. The lack of fit test provides data variation around the fitted model. The *P* value and *F*-value for the lack of fit were found to be 0.1231 and 3.62, insinuating it to be non-significant, making it a good model. The *P* values for each coefficient were checked for their significance, and all the values were found to be <0.1, making them significant and thus implying that the model can be utilized to predict the responses.

The retention time, area under the curve, and quantification values of baicalin in 17 runs are shown in Table 5.

Figure 5 shows contour plots and three-dimensional (3D) response surface plots, which aid in understanding the interactions between the responses and variables more clearly. It is noticeable from the 3D graph that baicalin yield increases as the extraction time are increased from 25 min to 29.058 min and drug-to-solvent ratio from 1:20 g/ml to 1:21.124 g/ml. However, a further increase in both shows a decrease in baicalin yield. This implies that both factors are significant for baicalin extraction. Similarly, the yield of baicalin increases as extraction temperature increases from 60°C to 67.963°C and the drug-to-solvent ratio from 1:20 g/ml to 1:21.124 g/ml. Further increase of both factors shows the decrease in baicalin yield. Similarly, baicalin yield increases as extraction temperature increases from 60°C to 67.963°C and extraction time from 25 min to 29.058 min.

The point prediction analysis revealed that the optimal conditions for baicalin extraction from *O. indicum* stem barks are extraction time—29.058 min, solvent-to-drug ratio—21.124 ml/g, and

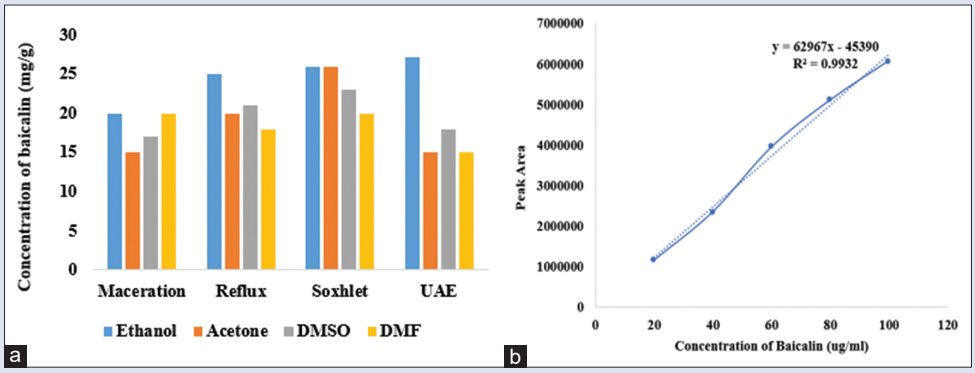

Figure 1: (a) Results of prefatory experiments and (b) Standard plot of baicalin

Table 2: Experimental design, BBD

Run	Extraction temperature Z_1	Extraction time (min) Z_2	Drug-to-solvent ratio (g/ml) Z_3	Response (Y) Baicalin content (mg/g)		
				Experimental value, Y_e	Predicted value, Y_i	Y_e–Y_i
1	60	20	30	16.60	16.66	−0.0625
2	60	25	20	27.21	27.16	0.0500
3	50	30	20	21.32	21.35	−0.0300
4	60	30	10	20.30	20.24	0.0625
5	60	25	20	27.21	27.16	0.0500
6	60	25	20	27.13	27.16	−0.0300
7	60	20	10	10.88	10.84	0.0425
8	60	30	30	23.72	23.76	−0.0425
9	60	25	20	27.10	27.16	−0.0600
10	70	25	10	19.32	19.39	−0.0725
11	60	25	20	27.15	27.16	−0.0100
12	70	25	30	21.23	21.20	0.0325
13	50	25	10	14.39	14.42	−0.0325
14	50	25	30	22.04	21.97	0.0725
15	70	20	20	15.23	15.20	0.0300
16	70	30	20	24.95	24.94	0.0100
17	50	20	20	14.58	14.59	−0.0100

Table 3: The retention time, area under the curve, and quantification values of baicalin in different extracts of *O. indicum* from different extraction methods

Extraction method	Solvent used	Retention time (min)	Area under curve	Baicalin content (mg/g)
Maceration	Ethanol	0.849	1,213,950	20.02
	Acetone	0.889	899,115	15.03
	DMSO	0.901	1,025,049	17.06
	DMF	0.903	1,213,948	20.01
Reflux	Ethanol	0.849	1,528,785	25.01
	Acetone	0.889	1,213,942	20.00
	DMSO	0.901	1,276,917	21.02
	DMF	0.903	1,088,016	18.03
Soxhlet	Ethanol	0.849	1,339,884	22.06
	Acetone	0.889	1,591,752	26.01
	DMSO	0.901	1,402,851	23.02
	DMF	0.903	1,213,942	20.00
UAE	Ethanol	0.849	1,667,942	27.21
	Acetone	0.889	899,108	15.02
	DMSO	0.901	1,088,008	18.01
	DMF	0.903	899,105	15.01

extraction temperature—67.963°C. Also, the maximum baicalin yield at these optimal conditions was found to be 26.572 mg/g of *O. indicum*.

Model validation

To validate the model equation adequacy, the optimal extraction conditions for baicalin extraction from *O. indicum* were modified, and experiments were performed in triplicate to re-evaluate the run. Moreover, the baicalin content was found to be 27.16 mg/g using an extraction time—60 min, solvent-to-drug ratio—20 ml/g, and extraction temperature—60°C.

However, there was no significant difference between the experimental and predicted yield, which infer that the response model was adequate and satisfactory for optimization.

CONCLUSION

In our present study, different extraction techniques, namely, Soxhelation, reflux, maceration, and UAE, were employed to extract therapeutically potent flavanoid, baicalin from *O. indicum* stem barks. BBD, a modern-day statistical technique, was being exploited for optimizing the extraction parameters of baicalin. This method is superior as it is time-saving, economical, and less laborious, and it holds supremacy over other traditional methods, and the interaction between different independent variables can be studied. In our work, BBD serves as a valuable tool for optimizing the extraction parameters of baicalin from *O. indicum* stem barks. Quantitative analysis of baicalin was done using HPLC on a C_{18} reverse-phase column with UV

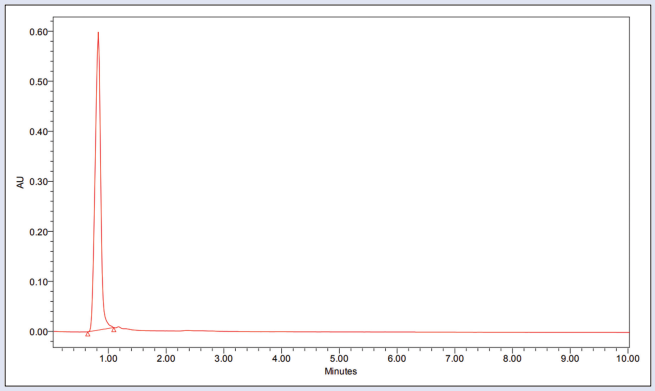

Figure 2: HPLC chromatogram of standard baicalin

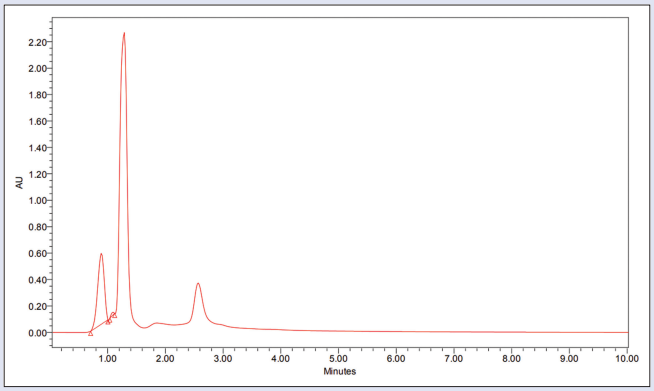

Figure 3: HPLC chromatogram of ethanolic extract of *O. indicum* stem barks

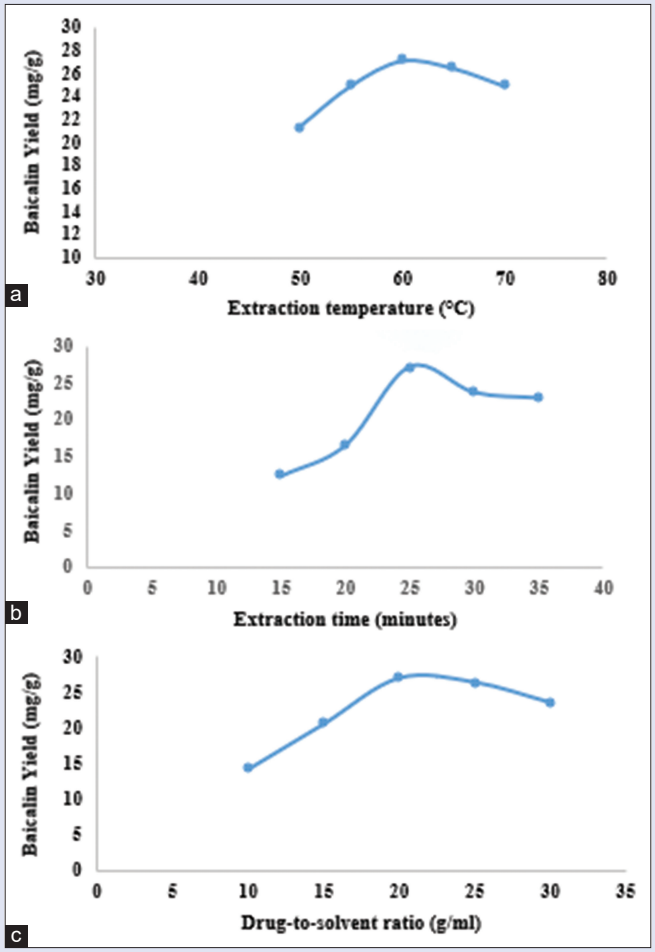

Figure 4: Results of single factorial experiments (a) Effect of extraction temperature, (b) Effect of extraction time, and (c) Effect of drug-to-solvent ratio

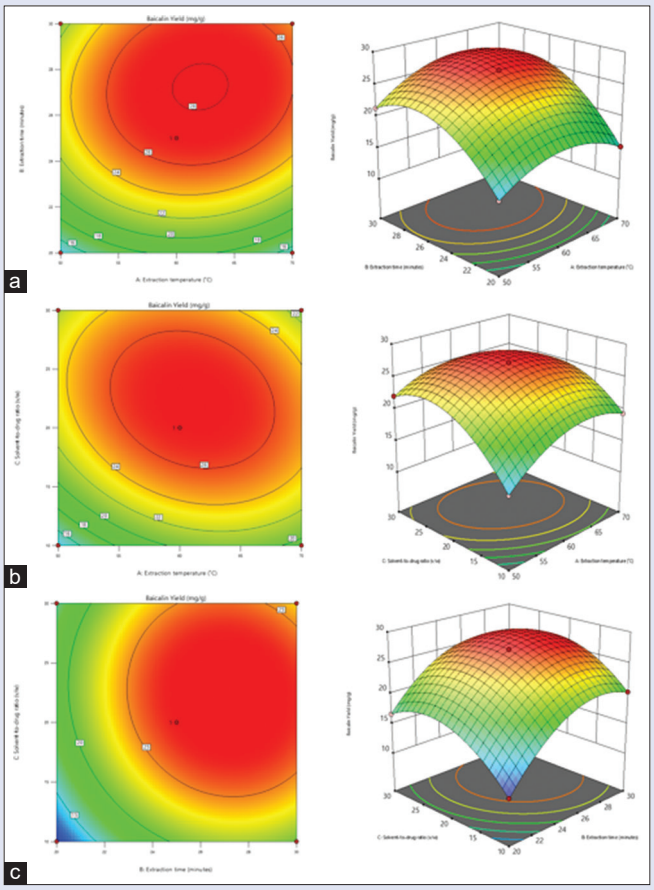

Figure 5: 3D response surface graphs and contour plots of (a) A and B, (b) A and C, and (c) B and C

(Ultraviolet visible) detection at 316 nm. An eluting solution consisting of water: methanol (15:85 *v/v*) was used as a mobile phase at a 1 ml/min flow rate in isocratic mode.

However, none of the researchers has developed the extraction process for the isolation and quantification of baicalin from *O. indicum*. Therefore, our study employs different techniques like Soxhlet, maceration, reflux technique, and UAE for the extraction of baicalin, which has not been reported by any other researcher yet.

Prior to using BBD, single factorial experiments were performed, and the results attained were used in BBD. Our study concluded that ethanol is the efficient extracting solvent, and the UAE method gives a better yield of baicalin than other thermal and non-thermal techniques opted.

By employing multiple regression analysis, the experimental data were fitted in the polynomial equation of second order, and optimal conditions

Table 4: ANOVA for response surface quadratic model

Variables	Sum of squares	Degree of Freedom (DF)	Mean square	*F*	*P*	Remarks
Model	456.56	9	50.73	9960.88	<0.0001	Significant
A	8.82	1	8.82	1731.84	<0.0001	
B	136.13	1	136.13	26728.61	<0.0001	
C	43.71	1	43.71	8582.85	<0.0001	
AB	2.22	1	2.22	435.92	<0.0001	
AC	8.24	1	8.24	1617.34	<0.0001	
BC	1.32	1	1.32	259.68	<0.0001	
A^2	48.25	1	48.25	9473.10	<0.0001	
B^2	95.20	1	95.20	18692.87	<0.0001	
C^2	86.40	1	86.40	16965.68	<0.0001	
Residual	0.0356	7	0.0051			
Lack of fit	0.0260	3	0.0087	3.62	0.1231	Non-significant
Pure error	0.0096	4	0.0024			
Cor total	456.60	16				

Table 5: The retention time, area under the curve, and quantification values of baicalin in ethanolic extract of *O. indicum* for 17 runs

Runs	Retention time (min)	Area under curve	Baicalin content (mg/g)
1	0.849	999,862	16.60
2	0.849	1,667,942	27.21
3	0.849	1,297,066	21.32
4	0.849	1,232,840	20.30
5	0.849	1,667,942	27.21
6	0.849	1,662,904	27.13
7	0.849	639,690	10.88
8	0.849	1,448,187	23.72
9	0.849	1,661,015	27.10
10	0.849	1,171,132	19.32
11	0.849	1,664,164	27.15
12	0.849	1,291,399	21.23
13	0.849	860,705	14.39
14	0.849	1,342,402	22.04
15	0.849	913,597	15.23
16	0.849	1,525,636	24.95
17	0.849	872,668	14.58

for baicalin extraction from *O. indicum* stem barks were estimated using the model equation extraction time—60 min, solvent-to-drug ratio—20 ml/g, and extraction temperature—60°C. Under these conditions, baicalin content was found 27.16 mg/g, which coincides with the predicted value.

Our research will be fruitful for the pharmaceutical industries and the upcoming researchers who wish to extract baicalin in a maximum amount from *O. indicum* stem bark.

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Conflicts of interest

There are no conflicts of interest.

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