

Development and extraction optimization of baicalein and pinostrobin from *Scutellaria violacea* through response surface methodology

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ABSTRACT

Objective: To develop a process that involves optimization of the amount of baicalein and pinostrobin from the hydro-methanolic extract of the leaves of *Scutellaria violacea* by response surface methodology (RSM). **Materials and Methods:** The combinatorial influence of various extraction parameters on the extraction yield was investigated by adopting Box–Behnken experimental design. Preliminary experiments carried out based on the traditional one variable at a time optimization revealed four such operational parameters to play a crucial role by influencing the yield. These four process parameters at three levels were considered to obtain the Box–Behnken experimental design. **Results:** RSM based model fitted to the resulting experimental data suggested that 52.3% methanol/water, 12.46:1 solvent-solid ratio, 285 rpm agitation and 6.07 h of extraction time are the optimal conditions which yielded a maximized amount of baicalein and pinostrobin of 2.9 and 4.05 mg/g DM. Analysis of variance revealed a high correlation coefficient ($R^2 = 0.999$ for baicalein and 0.994 for pinostrobin), signifying a good fit between the regression model (second order) and the experimental observations. **Conclusion:** The present study signifies that both the metabolites have been extracted from *S. violacea* for the first time. Further, this study developed an optimized extraction procedure to obtain maximum yield of the metabolites, which is unique and better than conventional extraction methodology. The operational parameters under optimized conditions accounts for the lowest cost in extraction process thus, providing an efficient, rapid and cost-effective method for isolation and scale up of these commercially vital flavonoids.

Key words: Baicalein, flavonoids, pinostrobin, response surface methodology, *Scutellaria violacea*

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INTRODUCTION

The quest for various pharmaceutically active compounds from natural sources has led to the development of distinctive processes in extraction and purification of metabolites. However, the recovery and yield of these metabolites in addition to process economics becomes vital, especially for molecules having low recovery, but high market demand. The active extraction of phytochemicals from pervious plant material is a key step in process development and manufacturing of therapeutic phytochemical molecules. The major focus for such molecules lies with separation of polyphenols, a broad class of biologically active

phyto-molecules known for their abundance in the plant kingdom.^[1] Flavonoids are an enormous class of polyphenol phytochemicals found in most fruits and vegetables, and have been proved to have various lucrative effects on human health, such as antioxidant, anti-inflammatory, antiallergic, antiviral, and anticarcinogenic activities.^[2,3]

Scutellaria (Lamiaceae) is a genus, which includes about 350 species commonly known as skullcaps.^[4] Phenolics and terpenoids are the two major phytochemical groups present in this genus, besides alkaloids, phytosterols, and polysaccharides. The medicinal potential of this genus is largely due to the flavonoids and their glycosides such as the baicalein, wogonin, baicalin, and wogonoside, etc., present in the genus.^[5,6] Such molecules are usually isolated through conventional extraction and chromatography procedures.^[7-12] Even though, baicalein has been previously reported from genus *Scutellaria*, pinostrobin has been

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reported to be extracted for the first time in the present study, from *Scutellaria violacea*.

Baicalein forms a major component in *S. baicalensis* and is known for its efficient cytotoxic activity against cancer cells.^[13] Treatment with baicalein attenuates endothelium intimal hyperplasia and radiation-induced inflammation process.^[14,15] Baicalein also has an inhibitory effect on colorectal cancer and enacts anticancer activity in prostate cancer.^[16,17] Pinostrobin has been previously reported from honey, thainginger (*Boesenbergia pandurata*), *Polygonum limbatum*, Propolis, etc.^[18-20] It is a potent anticancer compound^[21] which also possesses anti-microbial activity.^[19] Recent pharmacological investigates have shown diverse bio-activities of pinostrobin, which includes suppression of spontaneous contractions of intestinal smooth muscle,^[22] inhibition of aromatase, anti-proliferation on estrogen-induced cells,^[23] anti-inflammatory effects by inhibiting cyclooxygenases,^[24] and reduction of sodium channel-activated depolarization of mouse brain synaptoneurosome.^[25] In addition, pinostrobin has been reported to have strong antioxidant effect *in vitro*.^[18]

Extraction of a target compound is a very vital level in the recovery of bioactive molecules from natural samples such as plants, where extraction processes must be versatile, fairly simple, and inexpensive along with ability to both preserve and extract most of the active compounds present in a plant matrix. However, the lack of availability of a generalized protocol makes it essential to develop distinct processes for a particular compound from a particular plant matrix. This is due to the fact that plant materials have diverse chemical compounds based on polarity and solubility, and extraction processes can interact with other modules of the plant matrix.^[26,27] In addition, the extractable level of phenolic compounds is also affected by other factors including solvent composition, extraction time, pH, extraction temperature, solvent to solid ratio and the number of extraction steps.^[28-34] Thus, the extraction process for the flavonoid compounds from *Scutellaria violacea* must also be optimized in order to obtain high yield and recovery of two major compounds baicalein and pinostrobin.

Classical optimization protocols use the “one-factor-at-a-time” methodology, in which a single factor is varied at any given time keeping others constant thus a time-consuming and expensive approach. In addition, evaluation of possible interaction effects arising between factors is difficult and misleading inferences may occur. The response surface methodology (RSM) can however, overcome these difficulties, by allowing the study for possible interaction effects between variables.^[21,35] If adequately used, this potent tool can provide the best

optimal conditions that might significantly improve a separation process.^[36]

To the best of our knowledge and literature search, no previous reports are available for the isolation of baicalein and pinostrobin from *S. violacea*. Since, both the metabolites are medicinally important, single standard process was developed in the present study to optimize the yield and recovery of both the metabolites through Box–Behnken experimental design (RSM). This process with further scale up advancements can significantly increase the utility of these bioactive compounds in commercial therapeutic market for management of diseases.

MATERIALS AND METHODS

Plant material and isolation of active molecules

Leaves of *S. violacea* were obtained from the dense tropical forests along Western Ghats, Nilgiris, India. The plant was authenticated by Dr. Jayendran, Department of Botany, Government Arts College, Ootacamund, India. A voucher specimen (JDB1435) was deposited in Government Arts College, Ootacamund, India. The leaves were shade dried and ground to a fine powder. Extracts were prepared by soaking plant material (10 g) in 100 ml of suitable solvents at room temperature (RT) for 24 h and repeated thrice with the residue. The extract was filtered through Whatman No. 1 filter paper, and then all the filtrates were pooled up successively and concentrated under vacuum by a Rotary evaporator (Buchi® Rotavap R-210). Based on thin layer chromatography (TLC) profiling, one of the extracts were advanced to silica gel column chromatography and the subsequent fractions were collected for isolation of metabolites.

Selection of extraction solvent

Extraction was initially performed using different solvents based on polarity. Plant material (10 g) was soaked in 100 ml of respective solvents. Extract obtained was analyzed through high performance thin layer chromatography (HPTLC). The solvent, which yielded maximum amount of desired metabolites was considered for further studies.

Selection of variables and experimental ranges

Preliminary set of tests were performed by following the classical “one variable at a time” approach to roughly select the applicable factors and the range of these factors in hydro-methanolic extraction. Firstly, the effect of % methanol/water on extraction was investigated, where six sets of plant material (1 g) containing 50 ml (30–80% methanol in water) was kept for incubation at RT for 6 h. Secondly, we investigated the influence of solvent-to-solid ratio in extraction process by considering six ratios

(6:1–18:1): 6, 4.5, 3.6, 3, 2.6, 2.25, 2 g of plant material in 36 ml of 40% methanol/water as solvent at RT for 6 h. The influence of agitation was then studied where, six sets containing 3 g of plant material in 36 ml of 40% MeOH/water was kept for stirring at 100–600 rpm at RT for 6 h. Finally, the impact of extraction time on yield of the metabolites from the plant material was studied by keeping eight sets (each containing 3 g plant material in 36 ml of 40% methanol/water at 300 rpm) for 3–24 h. Observations on yield were analyzed by HPTLC and were further considered for systematic experimental design to find the optimum parameter, set through RSM procedure.

Experimental design for response surface methodology

The influential parameters identified based on the preliminary experiments namely, % methanol/water, solvent-to-solid ratio, agitation and extraction time (four factor) and three levels (–1, 0, +1) from their scanned range were considered for Box–Behnken method based experimental design which generated 27 set of experiments carried out with two replicates and the average is depicted in Table 1. Experimental data thus obtained were fitted in

second-order polynomial model and regression coefficients were determined as in Equation 1.

$$y = \beta_0 + \sum_{i=1}^k \beta_j X_i + \sum_{i=1}^k \beta_{ii} X_i^2 + \sum_{i=1}^{k-1} \sum_{j=2}^k \beta_{ij} X_i X_j \quad (1)$$

Where, Y is the predicted response factor, β_0 is the intercept and β_i , β_{ii} , β_{ij} are regression coefficients for linear effects, regression coefficients for squared effects, regression coefficients for interaction effects and X_i and X_j are the parameters, respectively.

Analysis of response variables

Chromatography

Commercial grade of baicalein and pinostrobin (Sigma-Aldrich®) were used as standards. The yield obtained was the response variable. The amount was quantified by HPTLC densitometric analysis, which was performed on aluminum-backed plates (20 cm × 20 cm) coated with 0.2 mm layer of Silica gel 60 F₂₅₄ (E-Merck, Germany). Sample application was done as 6 mm bands using CAMAG automatic TLC sampler 4 applicator (Switzerland) fitted with

Table 1: Box-Behnken design of the independent variables and experimental results for the response variables, amount of baicalein and pinostrobin

Experiments	Factors				Baicalein (mg/g DM) (Y ₁)	Pinostrobin (mg/g DM) (Y ₂)
	Methanol-water ratio (X ₁)	Solvent-solid ratio (X ₂)	Agitation (X ₃)	Extraction time (X ₄)		
1	60 (1)	12 (0)	300 (0)	3 (–1)	1.8	2.6
2	50 (0)	12 (0)	200 (–1)	9 (1)	1.7	2.45
3	50 (0)	12 (0)	300 (0)	6 (0)	2.8	3.9
4	50 (0)	14 (1)	300 (0)	9 (1)	1.4	2.5
5	50 (0)	10 (–1)	300 (0)	9 (1)	1.05	1.5
6	40 (–1)	12 (0)	300 (0)	3 (–1)	0.58	0.9
7	60 (1)	12 (0)	200 (–1)	6 (0)	2.2	2.9
8	50 (0)	12 (0)	200 (–1)	3 (–1)	1.26	1.95
9	50 (0)	14 (1)	300 (0)	3 (–1)	1.35	2
10	40 (–1)	12 (0)	300 (0)	9 (1)	1.1	1.4
11	50 (0)	10 (–1)	300 (0)	3 (–1)	0.97	1.5
12	40 (–1)	10 (–1)	300 (0)	6 (0)	0.45	0.7
13	50 (0)	14 (1)	400 (1)	6 (0)	1.57	2.43
14	60 (1)	10 (–1)	300 (0)	6 (0)	1.7	2.2
15	50 (0)	14 (1)	200 (–1)	6 (0)	1.69	2.6
16	50 (0)	10 (–1)	400 (1)	6 (0)	1.23	1.9
17	40 (–1)	10 (0)	200 (–1)	6 (0)	0.8	1.1
18	50 (0)	10 (0)	400 (1)	9 (1)	1.2	2.05
19	50 (0)	12 (–1)	200 (–1)	6 (0)	1.3	1.68
20	50 (0)	10 (0)	300 (0)	6 (0)	2.85	3.92
21	60 (1)	10 (0)	300 (0)	9 (1)	1.43	2.2
22	40 (–1)	10 (0)	400 (1)	6 (0)	1.4	1.7
23	50 (0)	10 (0)	400 (1)	3 (–1)	1.5	2
24	50 (0)	10 (0)	300 (0)	6 (0)	2.88	3.87
25	60 (1)	10 (0)	400 (1)	6 (0)	1.52	2.25
26	40 (–1)	14 (1)	300 (0)	6 (0)	1.3	2
27	60 (1)	14 (1)	300 (0)	6 (0)	1.64	2.51

Values in parentheses are coded form of variables. DM: Dry material

a CAMAG microliter syringe. Constant application rate of 150 nL/s was maintained. Linear ascending technique was used to develop the plates to a distance of 80 mm with solvent system (hexane-ethyl acetate 1:1) as mobile phase in CAMAG automatic developing chamber 2, which was previously saturated with mobile phase vapor for 30 min at 25°C. Developed plates were scanned using CAMAG TLC scanner 4, and visualized under ultraviolet light at 254 nm and 365 nm. The scanned images were later processed for densitometric analysis using Vision CATS v1.4.0 (CAMAG, Switzerland). Validation of the proposed HPTLC method was done based on guidelines of the international conference on harmonization.^[37] Concentration of 100–800 ng/spot was checked for linearity of both the compounds and concentration was plotted against peak area and concentration curve was obtained. Specificity of this method was confirmed by analyzing the R_f values of spots of baicalein and pinostrobin and comparing those with standards.

The HPTLC analysis for samples obtained from different RSM runs and final optimized run was done using the similar conditions used for standards. The area of the peak which corresponded to the same R_f value as that of standards was taken, and the amount of metabolite present was calculated using a regression equation obtained from the calibration plot.

Statistical analysis

Results obtained were expressed as the mean \pm standard deviation of the replications. Results obtained in the experimental run (RSM) were expressed as mean of replicates. RSM based model fitting and statistical analysis was performed using Design Expert (release 9.0.3.1; State-Ease, Inc., Minneapolis, MN, USA). An analysis of variance (ANOVA) was performed to determine the significant levels defined at $P < 0.05$, $P < 0.01$ and $P < 0.001$.

The corresponding extracts from RSM experiments were analyzed for the dependent variables (responses): Amount of baicalein (Y_1), amount of pinostrobin (Y_2). Mean values were analyzed using least-square regression and fitted to the generalized second-order polynomial model (Equation 1) to all of the dependent Y response variables.^[38] Response surfaces plots were plotted using reduced fitted polynomial models, which allow the relationship between the experimental levels and the response of each factor to be examined and the optimum conditions to be recognized.

RESULTS AND DISCUSSION

Selection of extraction solvent

Leaves of *S. violacea* were used for extraction studies based on preliminary screening tests which showed possible availability of flavonoids in leaves compared to other plant

parts. Extraction then was initially performed using different solvents based on polarity. Pure solvents [Figure 1] in the increasing order of their polarity were selected for study, as solubility of metabolites varies with polarity. Binary solvents were also tried such as methanol/water and ethanol/water since the amount of metabolites increased as polarity increased. However, pure water extract had no appreciable yield of desired metabolites and hence was excluded from further studies. Ten gram of dried leaves were soaked in 100 ml of respective solvents and the extract obtained was analyzed for target metabolites through HPTLC. The solvents used and the amount of extract, baicalein and pinostrobin obtained from each solvent is depicted in Figure 1. 70% methanol/water produced the maximum yield and was selected for conventional isolation of metabolites and RSM. This experimental result corresponded with the previous studies suggesting the use of binary solvent system than to a mono-solvent system (water or pure methanol) with regards to their polarity.^[29,39] The relatively low yield of metabolites in pure solvent(s) suggests their inability to extract the flavonoid compounds effectively.^[40]

Isolation of molecules

Initially, hydro-methanolic extract due to its high yield and better TLC profile was subjected to silica gel column chromatography according to conventional methodology.^[10] The fractions collected lead to the isolation of two molecules identified as baicalein and pinostrobin respectively as characterized by nuclear magnetic resonance spectroscopy [Figures S1-S5]. The spectral data were in comparison with previous studies.^[41,42] As the opted extraction process produced low yield of baicalein and pinostrobin, further studies were done to optimize the extraction efficiency and recovery of these metabolites (RSM).

Selection of variables and experimental ranges

Methanol/water ratio

Firstly, the effect of % methanol in water on extraction was investigated. The extract obtained was analyzed by

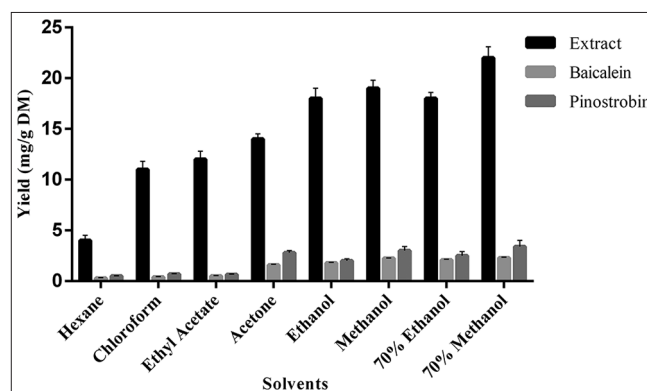


Figure 1: Effect of different solvents on extraction of baicalein and pinostrobin from *Scutellaria violacea*. Size: Column width

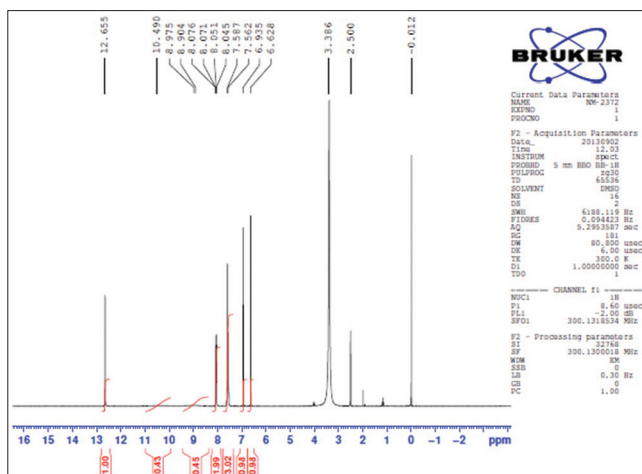


Figure S1: Proton nuclear magnetic resonance of baicalein

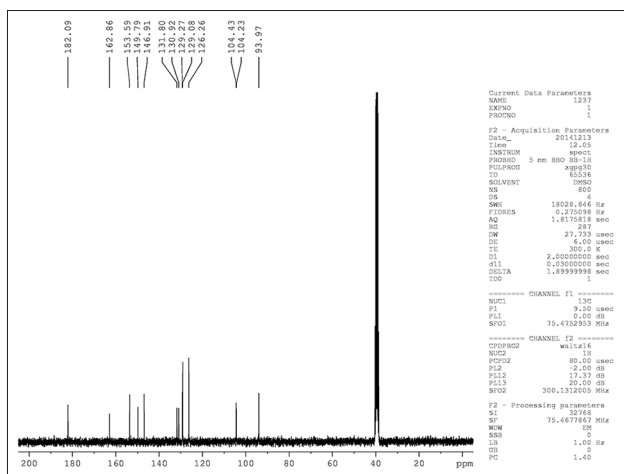


Figure S2: Carbon nuclear magnetic resonance of baicalein

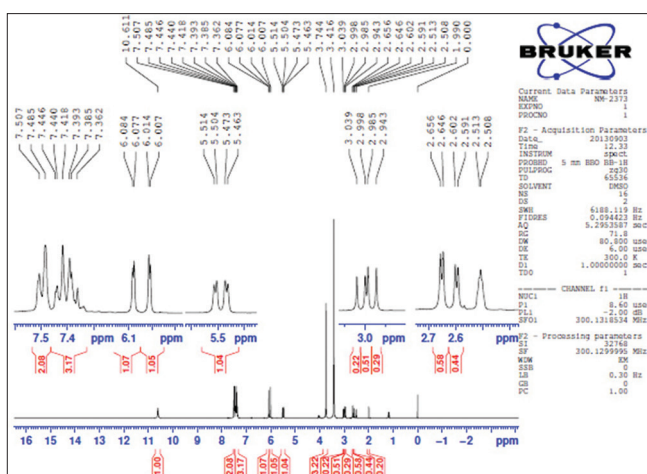


Figure S3: Proton nuclear magnetic resonance of pinostrobin

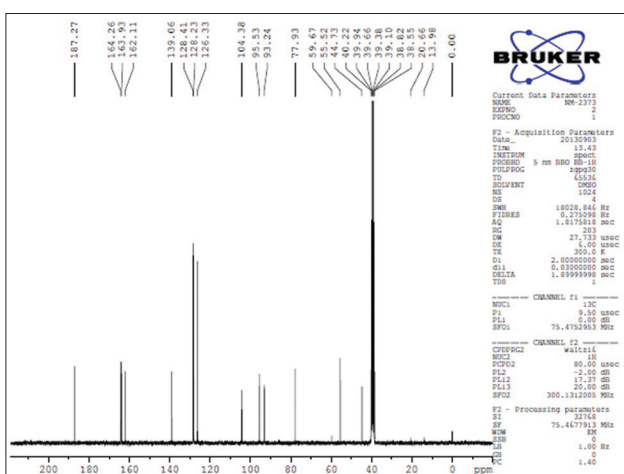


Figure S4: Carbon nuclear magnetic resonance of pinostrobin

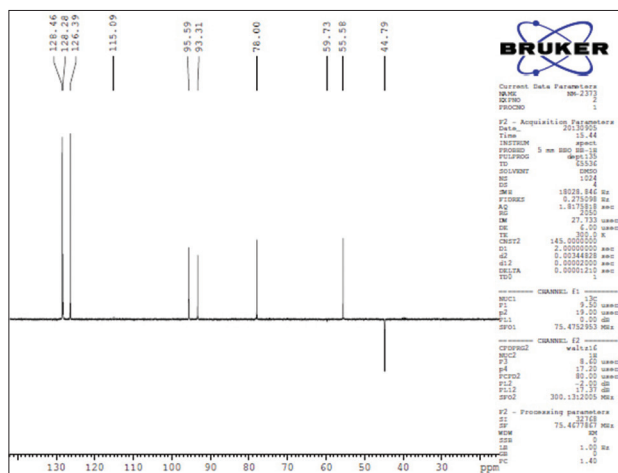


Figure S5: Distortionless enhancement by polarization transfer-13C nuclear magnetic resonance of pinostrobin

HPITLC. The maximum yield obtained was 2.2 mg/g DM and 3.6 mg/g DM for baicalein and pinostrobin respectively at 40% methanol/water composition [Figure 2]. The extraction of compounds from a material is directly related

to the solvent compatibility according to the principle “like dissolves like”.^[38] Change in methanol content in water changes polarity which affects the solubility of a specific class of compounds.

Solvent-solid ratio

The influence of solvent-to-solid ratio in the extraction process was studied by considering six ratios (6:1–18:1). The maximum yield obtained was 2.3 and 3.3 mg/g DM at 12:1 solvent-solid ratio for baicalein and pinostrobin respectively [Figure 2]. An increase in yield corresponded with an increase in solvent amount with highest obtained at 12:1 due to the fact that it increased concentration gradient, which might be due to increase in diffusion rate allowing maximum extraction.^[43-45] A previous report suggested an increase in extraction yield from dry peel powders of pomegranate in increasing solvent–solid ratios.^[46]

Agitation

In comparison with nonagitated experiments, experiments, which involved agitation, always yielded more metabolites.

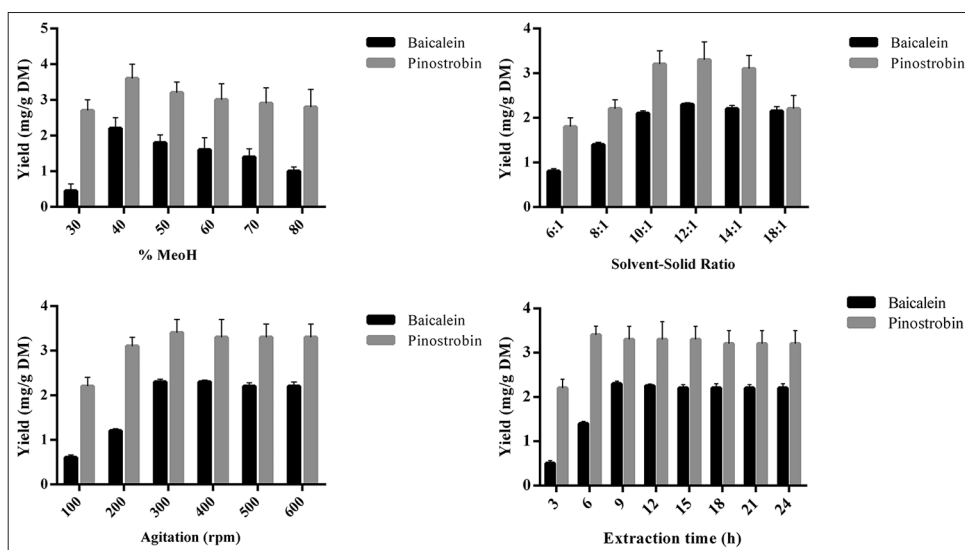


Figure 2: Influence of operational parameters on extraction process of baicalein and pinostrobin. Size: Column width

This might be due to the fact that stirring favors the convective movement in the solvent bulk and compensates the gradient decrease created by increasing solute concentration. High speed stirring creates more turbulence resulting in high mass transfer rate of solute. It is clear from Figure 2 that the maximum amount of metabolites is obtained at 300 rpm. Above, 300 rpm the extractive value remains moderately constant. This shows that the speed of agitation above 300 rpm has no significant effect on the yield of extraction which clearly shows that external mass transfer resistance is negligible at 300 rpm.

Extraction time

The amount of metabolites increased along with time, but became constant over a time limit. This might be due to the fact that the maximum extractive value is reached over the time duration and hence no further mass transfer of solute from plant material is possible. The maximum time required to reach maximum yield of metabolites is 6 h, which is relatively sooner than any conventional extraction process which involves a minimum of 12–24 h.

Extraction optimization by response surface methodology

High performance thin layer chromatography analysis

Amount of metabolites in each optimization step and experimental runs (RSM) was analyzed and quantified by HPTLC. The HPTLC profiles and the resulting chromatogram are given in Figure 3. The mobile phase was Hexane: Ethyl acetate, 1:1 (% v/v), which resulted in a sharp significantly resolved peak at R_f values of 0.3 and 0.4 for baicalein and pinostrobin respectively. The calibration plot of peak area was obtained against area of

baicalein and pinostrobin and was linear in the range of 100–800 ng/spot. The linear regression equations were $Y = 12.3x + 1437$ and $Y = 12.35x + 864$ respectively, where Y is the response (area) and x is the concentration of metabolite. The correlation coefficient (R^2) was 0.98 and 0.987 respectively and was highly significant ($P < 0.05$).

Peaks of both the metabolites from different process samples were identified by comparing their spots at their respective $R_f = 0.3, 0.4$ values with those obtained by chromatography of the standards under the same conditions as given in Figure 3a and b. The content of metabolites was later quantified using regression equations.

The chromatogram obtained is given in Figure 3c. The comparison of lane 1 and 2 depicts that the amount of target metabolites has been significantly increased as illustrated by the peaks. Lanes 3–4 show the R_f values of both the metabolites which corresponded with the values of those samples from experiments.

Statistical analysis

Using the Box–Behnken experimental design, the second-order polynomial quadratic response equation (Equation 1) was utilized to institute a mutual link between the considered parameters.^[47] The equation based on the coded factors was established as below.

$$\begin{aligned}
 Y_1 = & 2.84 + 0.39X_1 + 0.19X_2 - 0.044X_3 + \\
 & 0.035X_4 - 0.23X_1X_2 - 0.32X_1X_3 \\
 & - 0.22X_1X_4 - 0.012X_2X_3 - 0.373X_2X_4 \\
 & - 0.19X_3X_4 - 0.77X_1^2 - 0.8X_2^2 \\
 & - 0.59X_3^2 - 0.84X_4^2
 \end{aligned} \quad (2)$$

$$\begin{aligned}
 Y_2 = & 3.9 + 0.57X_1 + 0.38 X_2 - 0.029 X_3 + 0.096 X_4 \\
 & - 0.25 X_1X_2 - 0.31 X_1X_3 - 0.23 X_1X_4 \\
 & - 0.097 X_2X_3 + 0.13 X_2X_4 - 0.11 X_3X_4 \\
 & - 1.10 X_1^2 - 0.97 X_2^2 - 0.78 X_3^2 - 1.03 X_4^2
 \end{aligned}
 \quad (3)$$

The Box–Behnken matrix and experimental results for recovery of baicalein and pinostrobin from hydro-methanolic extraction process are summarized in Table 1. ANOVA was used to assess the statistical significance of the quadratic model.^[48,49] It is suggested that for a good statistical model, the correlation coefficient (R^2) value should be in the range of 0–1.0, which explains the observed variability in obtained data from the model. Whereas, R^2 adj modifies the R^2 by considering

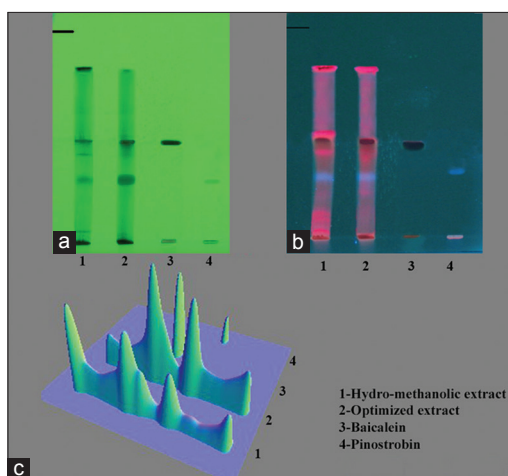


Figure 3: High performance thin layer chromatography (HPTLC) profiles at (a) ultraviolet (UV) 254 nm (b) UV 365 nm. And (c) the three-dimensional chromatogram of HPTLC analysis. Size: Column width

the number of predictors in the given model. Statistical observations from ANOVA in Tables 2 and 3 demonstrates that the regression model has a high coefficient of determination ($R^2 = 0.999$ for baicalein and 0.994 for pinostrobin), indicating that 99.9% and 99.4% of the variations in extraction process of metabolites could be explained by the independent factors. Also, the R^2 adj (0.998 and 0.987) value explains significance of the model.^[50] There seems no significant difference between R^2 and R^2 adj values which is desirable for the model. “Adeq precision” measures the difference between signals to noise ratio (>4.0),^[51] which for this model are 117.133 and 48.258 for baicalein and pinostrobin respectively. In addition, coefficient of variation is low (4.18 and 1.81) indicating good reliability of experiments. ANOVA in Table 4 demonstrates the results of the lack of fit test for the models describing the variation in the data around the fitted model. In present case, the F-values are 0.35 and 15.43 (not significant) and imply that the models sufficiently describe the obtained data.

The coefficients and standard error are depicted in Table 3. The corresponding F-values for coefficients indicate that the % methanol in water (X_1) produces the largest effect in extracting baicalein and pinostrobin in the process (F-value: 2437.67 and 475.47, $P < 0.0001$). Further it implies that other factors are relatively less important. Also, the coefficients of main effects (X_1 – X_4) were significant compared to the interaction effects. Also, the square effects of all the main effects are significant ($P < 0.0001$) for both the responses (Y_1 and Y_2). In the interaction effects, all the coefficients were significant except, solvent-solid ratio: Agitation and solvent-solid ratio: Extraction time for response variable (Baicalein).

Table 2: ANOVA for response surface quadratic model

Source	Baicalein				Pinostrobin			
	df	SS	MS	F	df	SS	MS	F
Model	14	9.56	0.68	919.66	14	17.22	1.23	149.13
A	1	1.81	1.81	2437.67	1	3.92	3.92	475.47
B	1	0.42	0.42	568.29	1	1.73	1.73	210.09
C	1	0.023	0.023	31.53	1	0.01	0.01	1.24**
D	1	0.015	0.015	19.8	1	0.11	0.11	13.36
A ²	1	3.17	3.17	4268.79	1	6.45	6.45	782.42
B ²	1	3.45	3.45	4650.78	1	4.99	4.99	605.28
C ²	1	1.85	1.85	2497.32	1	3.26	3.26	394.67
D ²	1	3.79	3.79	5109.54	1	5.62	5.62	681.02
AB	1	0.21	0.21	278.87	1	0.25	0.25	29.71
AC	1	0.41	0.41	551.75	1	0.39	0.39	47.36
AD	1	0.2	0.2	266.75	1	0.2	0.2	24.55
BC	1	0.11	0.11	0.84*	1	0.038	0.038	4.61**
BD	1	0.04	0.04	0.3*	1	0.063	0.063	7.58
CD	1	0.14	0.14	184.41	1	0.051	0.051	6.14**

A: Percent methanol/water; B: Solvent-solid ratio; C: Agitation; D: Extraction time; ANOVA: Analysis of variance; df: Degrees of freedom; SS: Sum of squares; MS: Mean square. All values are significant at 1%. *Not significant; **Significant at 5%

Table 3: Regression coefficients of the predicted second-order model for the response variables, baicalein and pinostrobin

Model parameters	Baicalein		Pinostrobin	
	Regression coefficient	SE	Regression coefficient	SE
Intercept	2.84	0.016	3.9	0.052
A	0.39	0.391	0.57	0.026
B	0.19	0.391	0.38	0.026
C	-0.044	0.391	-0.029**	0.026
D	0.035	0.391	0.096	0.026
A ²	-0.77	0.012	-1.1	0.039
B ²	-0.8	0.012	-0.97	0.039
C ²	-0.59	0.012	-0.78	0.039
D ²	-0.84	0.012	-1.03	0.039
AB	-0.23	0.014	-0.25	0.045
AC	-0.32	0.014	-0.31	0.045
AD	-0.22	0.014	-0.23	0.045
BC	-0.013*	0.014	-0.098**	0.045
BD	-0.373*	0.014	0.12	0.045
CD	-0.18	0.014	-0.11**	0.045
SE	0.09		0.027	
R ²	0.999		0.994	
Adjusted-R ²	0.998		0.987	
CV %	4.18		1.81	
Adeq precision	117.133		48.25	

A: Percent methanol/water; B: Solvent-solid ratio; C: Agitation; D: Extraction time; SE: Standard error; R²: Coefficient of multiple determinations; CV: Coefficient of variance. All values are significant at 1%. *Not significant; **Significant at 5%

Table 4: ANOVA for the lack of fit testing for baicalein and pinostrobin

Source	Baicalein				Pinostrobin			
	df	SS	MS	F	df	SS	MS	F
Lack of fit	10	0.28	0.135	0.35*	10	0.098	0.486	15.43*
Pure error	2	0.162	0.104		2	0.062	0.115	
Total error	12	0.44	0.135		12	0.099	0.41	

ANOVA: Analysis of variance; df: Degrees of freedom; SS: Sum of squares; MS: Mean square. *Not significant

Adequacy of the applied model is to be checked in any experimental analysis to ensure an excellent estimation of real conditions.^[52] This was depicted by Figure 4, showing comparison between experimental and predicted data. It demonstrates good agreement between the axes ($R^2 = 0.999$ for baicalein and 0.994 for pinostrobin).

The interactive effect of operational factors

A three-dimensional response surface and contour plots were drawn based on obtained model equation to assess the interaction among the operational factors and to determine the optimal values of each parameter.^[48,53] The effects of % methanol/water, solvent-solid ratio, agitation and extraction time on recovery of baicalein and pinostrobin are shown in Figures 5 and 6. These plots show how low and high values of operational factors influence extraction

Table 5: Optimum conditions obtained from response surface modeling and one variable at a time methods

Variable name	Optimum values obtained		
	Response surface modelling	One variable at a time	
X ₁	Methanol-water ratio	52.3	50
X ₂	Solvent-solid ratio	12.46	12
X ₃	Agitation	285 rpm	300 rpm
X ₄	Extraction time	6.07 h	6 h
Predicted values*			
	Baicalein	2.89±0.05	-
	Pinostrobin	3.99±0.11	-
Observed values**			
	Baicalein	2.90±0.03	2.1-2.3
	Pinostrobin	4.05±0.04	3.2-3.4

*Mean±95% CI; **Mean±SD (n=3). CI: Confidence interval; SD: Standard deviation

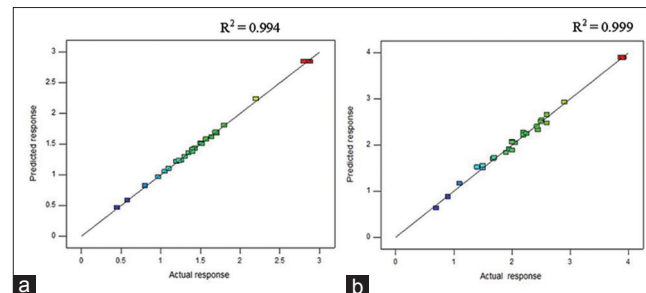


Figure 4: Plot of predicted response versus the calculated response for (a) baicalein and (b) pinostrobin. Size: Column width

response variables. In both Figures 4 and 5, the yield of metabolites increases with increase in factor levels up to moderate level (0) and then decrease. For example, in Figures 4a and 5a, interaction between % methanol/water (A) and Solvent-solid ratio (B) is plotted where, yield of metabolites increases as A increases from 40 to 50 and then decreases when it extends to 60. Similarly, as solvent-solid ratio (B) reaches 12:1, the yield reaches maximum and then decreases. This phenomenon is seen all the surfaces drawn based on interaction effects of different factors.

It is vital to discuss that in the present study, response contours and surfaces behave the same way for both the response variables, and thus emphasizing the fact that single optimized process model is enough for efficient recovery of both the metabolites. Also, the conditions imply use of fewer solvents, moderate agitation and less duration for maximum recovery of metabolites.

Optimization of operational factors

Usually numerical optimization method is used for optimization in which a desirable value for each input

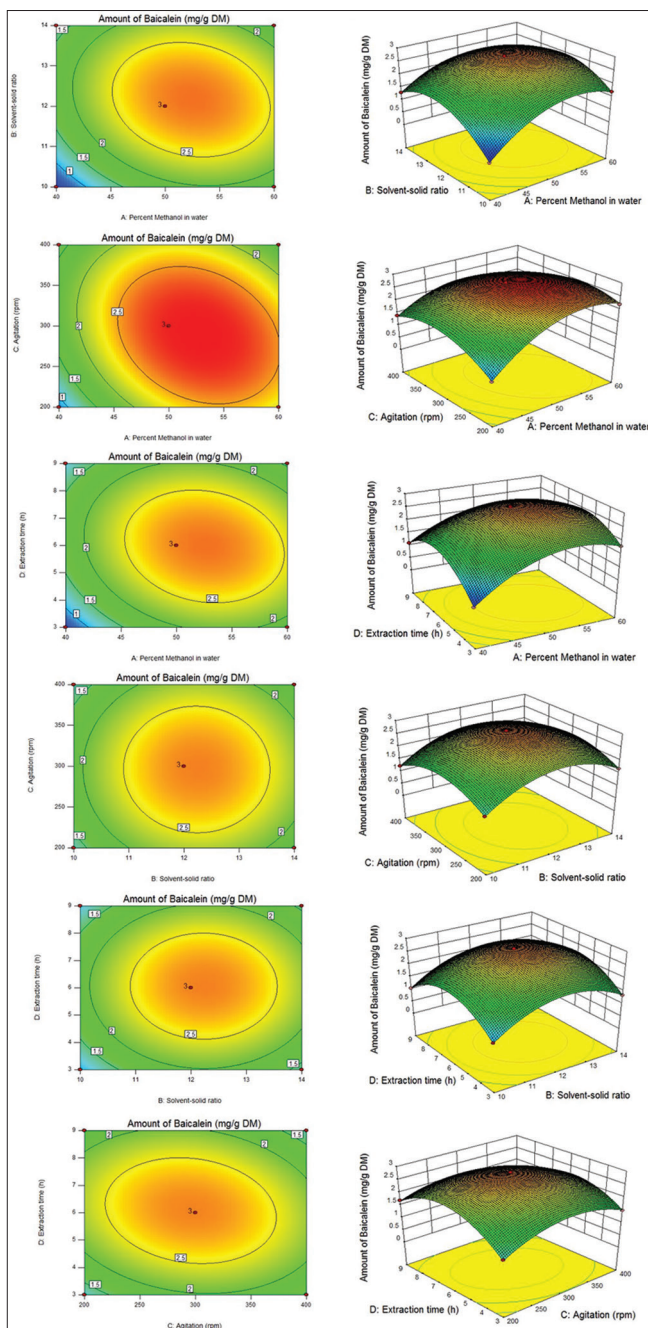


Figure 5: Response surface plots (contour and surface) for yield of baicalein showing effect of operational parameters. Size: Full page width

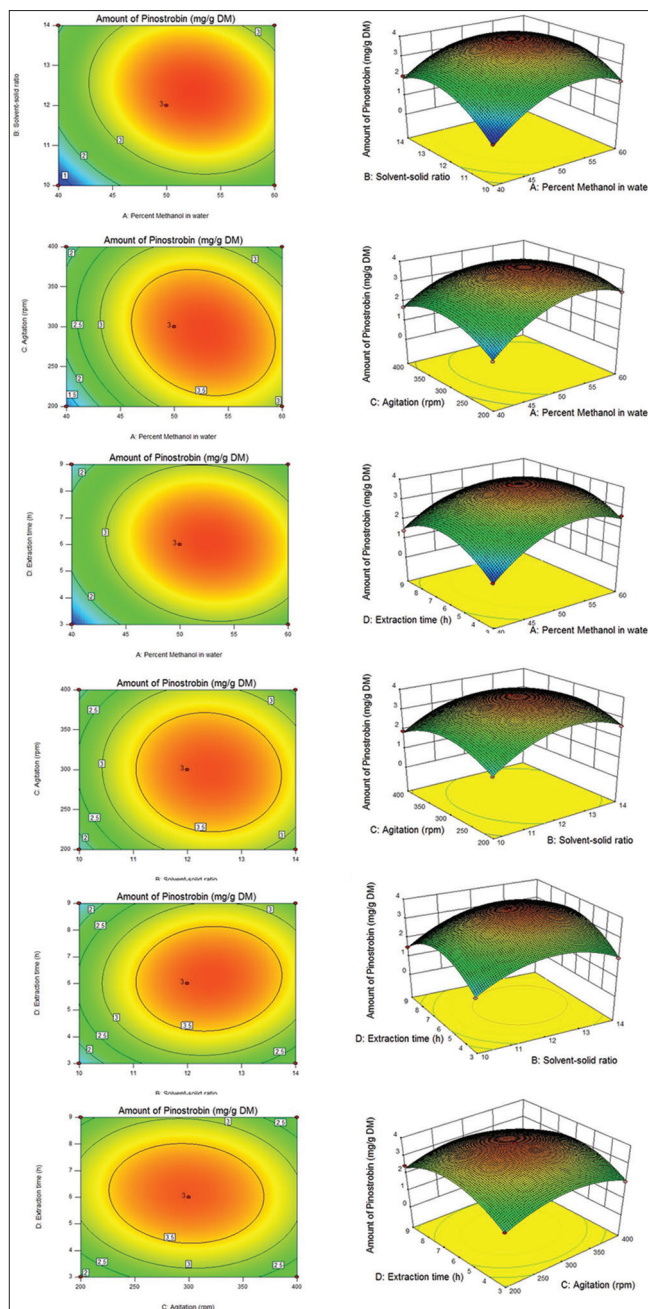


Figure 6: Response surface plots (contour and surface) for yield of pinostrobin showing effect of operational parameters. Size: Full page width

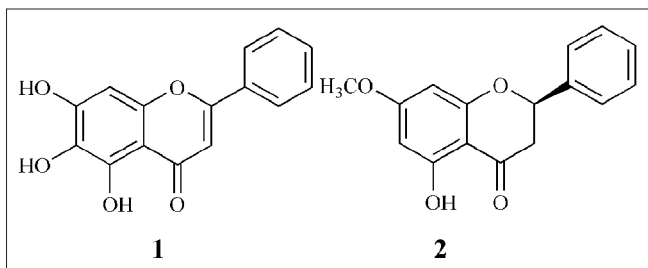


Figure 7: Structure of extracted compounds

element and response can be selected.^[50] Optimizations can be set to establish an output value for a given set of conditions by selecting possible input optimizations including range, maximum, minimum, target or none (for responses). The input parameters were entered for specific range values, whereas the response was designed to achieve a maximum value. Using these conditions, the maximum achieved amount of baicalein and pinostrobin was 2.89 and 3.99 mg/g DM at 52.3% methanol in water,

Table 6: Recovery of metabolites from response surface modeling and conventional extraction methods

Methodology	Yield (%)		Increase in yield (%)	
	Baicalein	Pinostrobin	Baicalein	Pinostrobin
Conventional extraction	0.19	0.3	-	-
Optimized through one variable at a time	0.21-0.23	0.32-0.34	10-11	6-13
Optimized through RSM	0.29	0.41	52.6	36

RSM: Response surface methodology

12.46:1 solvent-solid ratio, 285 rpm agitation and 6.07 h of extraction. This result indicates an acceptable fit among the obtained data and the desirability of the model at all points. An additional experiment confirmed the amount of metabolites yielded at optimized conditions. The values obtained were 2.9 and 4.05 mg/g DM for baicalein and pinostrobin respectively. This was in accordance to predicted values as in Table 5. The final yield of metabolites was 0.29% (baicalein) and 0.41% (pinostrobin) which clearly substantiates a significant increase compared to initial yields of (0.19% and 0.3%) as given in Table 6. The optimized yields depict an increase of 52.6% and 36% in total recovery of baicalein and pinostrobin respectively [Figure 7], further emphasizing the need for optimization of extraction procedure taking into account the process economics.

CONCLUSION

In the present study, the amount of baicalein and pinostrobin from hydro-methanolic extract of leaves of *S. violacea* was optimized by Box–Behnken experimental design and RSM based model fitting and optimization in a batch mode extraction process. Analyses of the response surfaces were carried out as a function of the percent methanol/water, solvent-solid ratio, agitation and extraction time. ANOVA demonstrated a high correlation coefficient ($R^2 = 0.999$ and 0.994), indicating a good fit between the regression model and the experimental observations. Optimal conditions obtained through RSM, which yielded a maximized amount of baicalein and pinostrobin of 2.9 and 4.05 mg/g DM included 52.3% methanol/water, 12.46:1 solvent-solid ratio, 285 rpm agitation and 6.07 h of extraction time. It is thus suggested that using this standardized process, *S. violacea* leaves could be efficiently used to extract medicinally important flavonoids baicalein and pinostrobin in better yields. Moreover, standard experimental design and RSM was an efficient strategy for optimizing the operational parameters towards maximizing the recovery of flavonoids

depicting an increase of 52.6% and 36% respectively. The process developed could be easily advanced for scale up for large extraction of these bio-actives. This would lead to further research concerned with use of these bioactive molecules in efficient management of diseases.

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