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 violacaea.*

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, thaiginger (*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 synaptoneurosomes.[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 F_{254} (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 ± 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

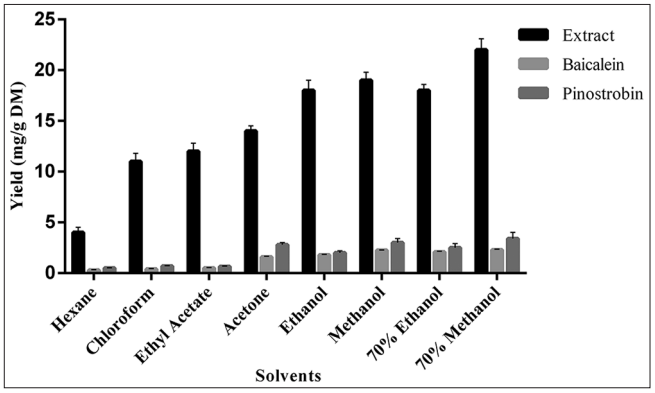

Figure 1: Effect of different solvents on extraction of baicalein and pinostrobin from *Scutellaria violacea*. Size: Column width

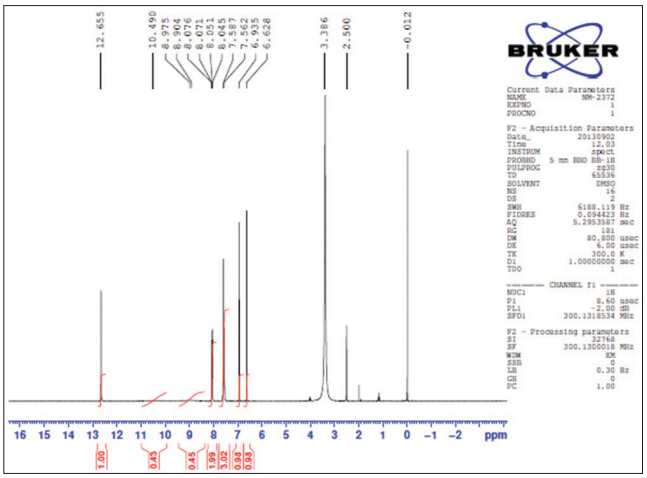

Figure S1: Proton nuclear magnetic resonance of baicalein

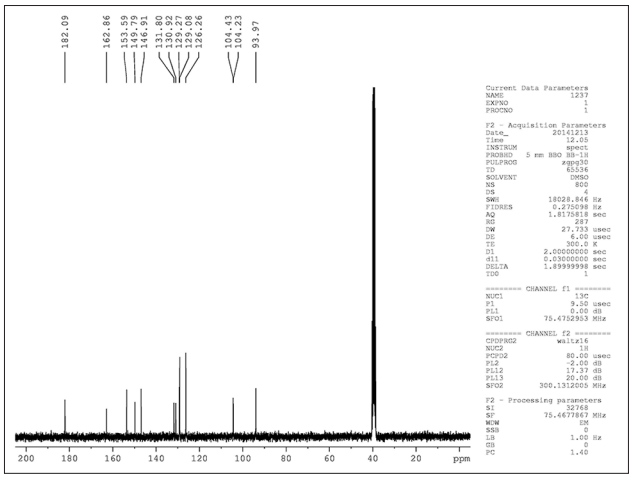

Figure S2: Carbon nuclear magnetic resonance of baicalein

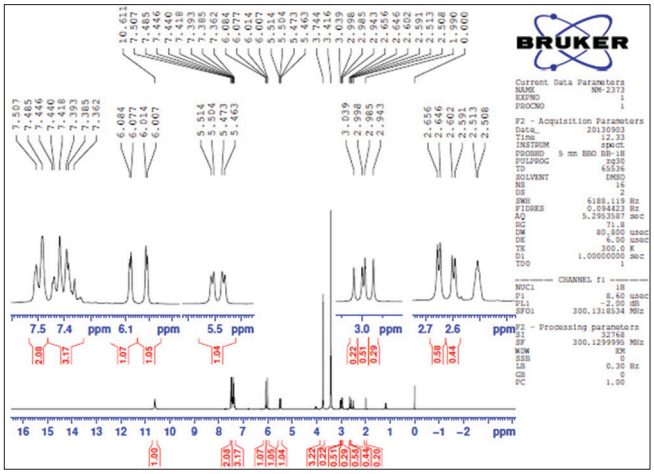

Figure S3: Proton nuclear magnetic resonance of pinostrobin

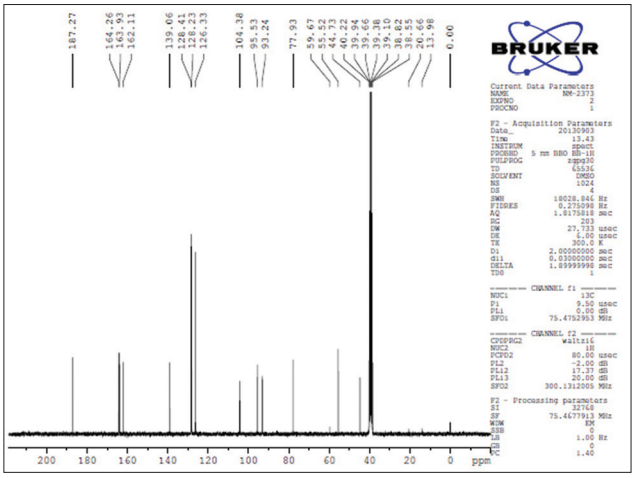

Figure S4: Carbon nuclear magnetic resonance of pinostrobin

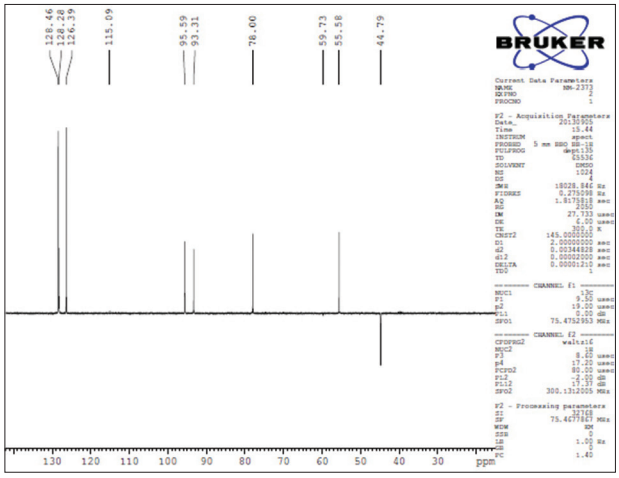

Figure S5: Distortionless enhancement by polarization transfer-135 nuclear magnetic resonance of pinostrobin

HPTLC. 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.

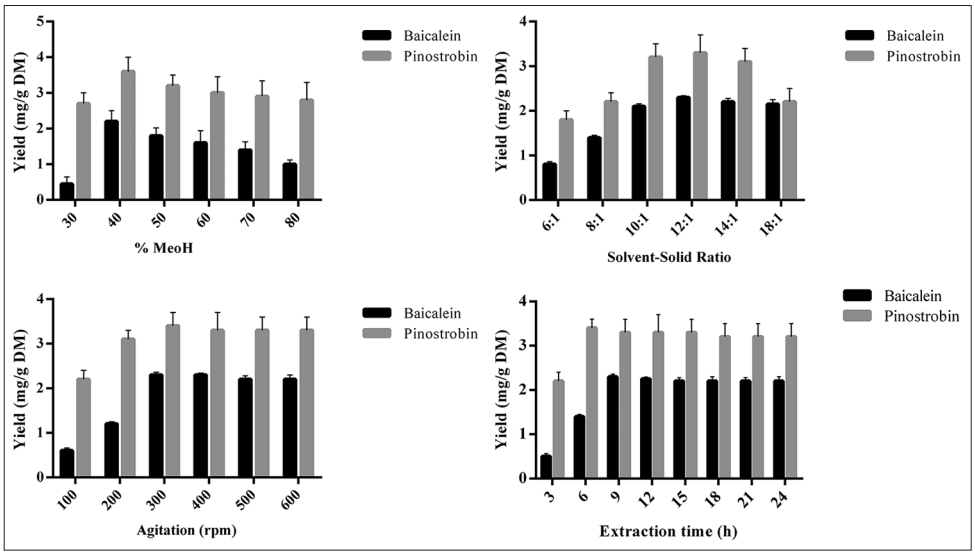

Figure 2: Influence of operational parameters on extraction process of baicalein and pinostrobin. Size: Column width

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.

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

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

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 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.

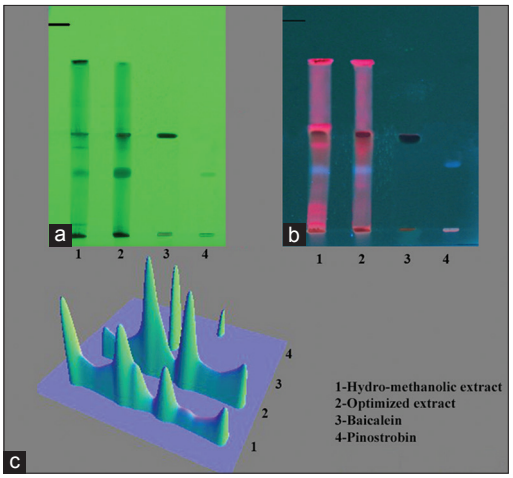

Figure 3: High performance thin layer chromatography (HPTLC) profiles at (a) ultraviolet (UV) 254 nm (b) UV 365 nm. And (c) the three-dimensional chromatogram of HPTLC analysis. Size: Column width

The 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; R^2: 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

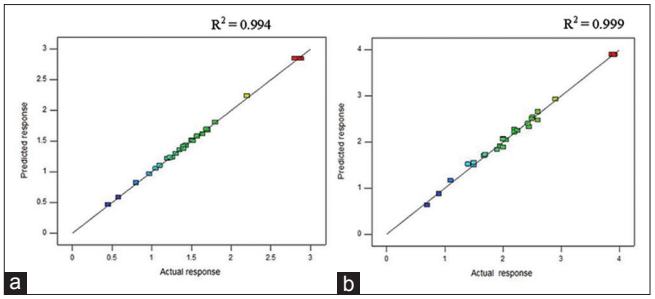

Figure 4: Plot of predicted response versus the calculated response for (a) baicalein and (b) pinostrobin. Size: Column width

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

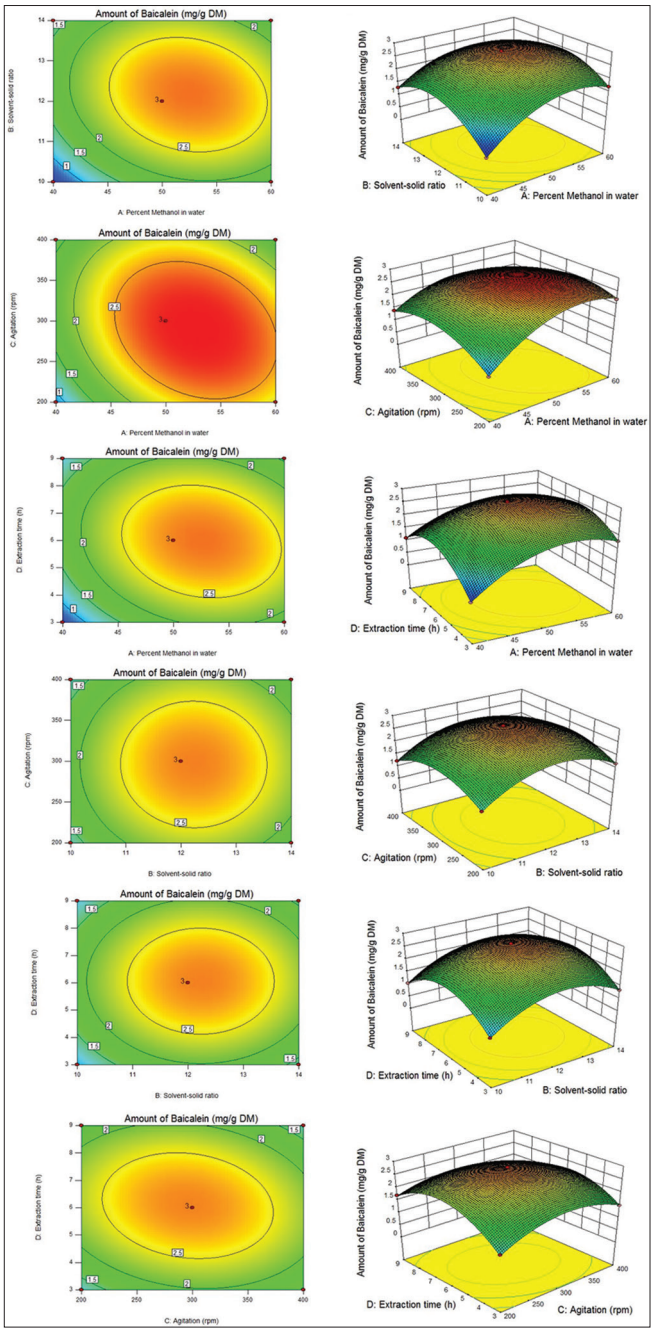

Figure 5: Response surface plots (contour and surface) for yield of baicalein showing effect of operational parameters. Size: Full page width

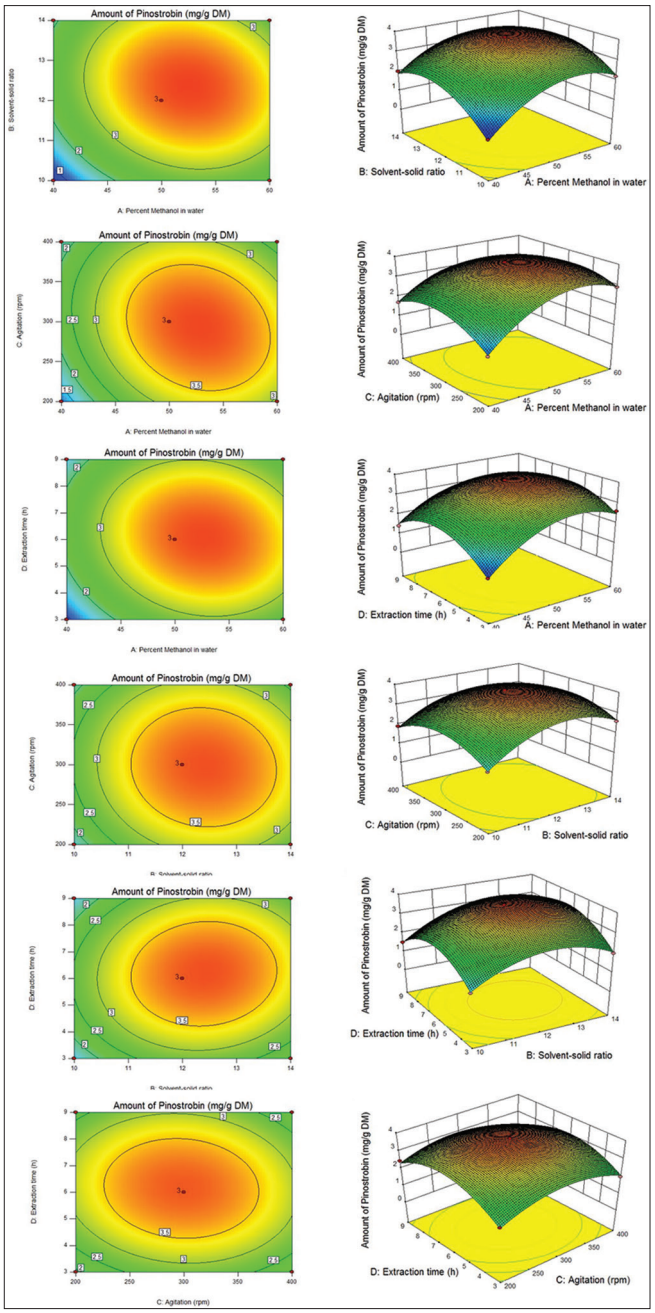

Figure 6: Response surface plots (contour and surface) for yield of pinostrobin showing effect of operational parameters. Size: Full page width

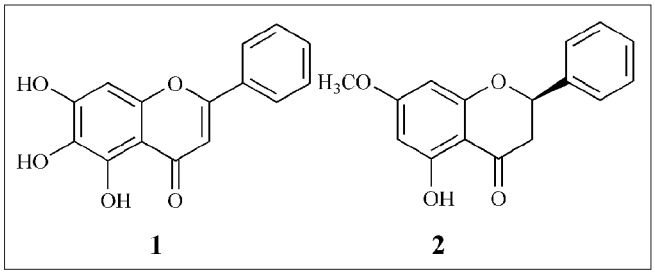

Figure 7: Structure of extracted compounds

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 pinostrobinof 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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REFERENCES

1. Rice-Evans CA, Miller J, Paganga G. Antioxidant properties of phenolic compounds. Trends Plant Sci 1997;2:152-9.
2. Beecher GR. Flavonoids in foods. In: Packer L, Hiramatsu M, Yoshikawa T, editor. Antioxidant Food Supplements in Human Health. New York: Academic Press; 1999. p. 269-81.
3. Nijveldt RJ, van Nood E, van Hoorn DE, Boelens PG, van Norren K, van Leeuwen PA. Flavonoids: A review of probable mechanisms of action and potential applications. Am J Clin Nutr 2001;74:418-25.
4. Willis JC. A Dictionary of the Flowering Plants and Ferns. 7th ed. UK: Cambridge University Press; 1966.
5. Awad R, Arnason JT, Trudeau V, Bergeron C, Budzinski JW, Foster BC, *et al*. Phytochemical and biological analysis of skullcap (*Scutellaria lateriflora* L.): A medicinal plant with anxiolytic properties. Phytomedicine 2003;10:640-9.
6. Makino T, Hishida A, Goda Y, Mizukami H. Comparison of the major flavonoid content of *S. baicalensis*, *S. lateriflora*, and their commercial products. J Nat Med 2008;62:294-9.
7. Soundarya Devi S, Malathi R, Rajan SS, Aravind S, Krishnakumari GN, Ravikumar K. A new clerodane diterpene with antifeedant activity from *Teucrium tomentosum*. Acta Crystallogr C 2003;59:o530-2.
8. Subramaniam S, Keerthiraja M, Sivasubramanian A. Synergistic antibacterial action of β-sitosterol-D-glucopyranoside isolated from *Desmostachya bipinnata* leaves with antibiotics against common human pathogens. Rev Bras Farm 2014;24:44-50.
9. Satyan RS, Sakthivadivel M, Shankar S, Dinesh MG. Mosquito larvicidal activity of linear alkane hydrocarbons from *Excoecaria agallocha* L. against *Culex quinquefasciatus* Say. Nat Prod Res 2012;26:2232-4.
10. Moorthy G, Subramaniam S, Murali S, Muralidharan R, Sivasubramanian A. Acetyl alkannin from *Alkanna tinctoria* works synergistically along with commercial antibiotics against common human pathogens. Pharm Chem 2014;6:129-34.
11. Behlol AA, Shankar S, Ganapathy V, Natarajan H, Aravind S, Anbazhagan V. β-Sitosterol-D-glucopyranoside isolated from *Desmostachya bipinnata* mediates photoinduced rapid green synthesis of silver nanoparticles. RSC Adv 2014;4:59130-6.
12. Shankar S, Anbumathi P, Aravind S. A unique solvent assisted 'green' hydrotropic precipitation and response surface optimization for isolation of the dietary micronutrient β-Sitosterol-D-glucopyranoside from *Desmostachya bipinnata*. RSC Adv 2014;5:7479-85.
13. Chou CC, Pan SL, Teng CM, Guh JH. Pharmacological evaluation

of several major ingredients of Chinese herbal medicines in human hepatoma Hep3B cells. Eur J Pharm Sci 2003;19:403-12.

14. Peng CY, Pan SL, Huang YW, Guh JH, Chang YL, Teng CM. Baicalein attenuates intimal hyperplasia after rat carotid balloon injury through arresting cell-cycle progression and inhibiting ERK, Akt, and NF-kappaB activity in vascular smooth-muscle cells. Naunyn Schmiedebergs Arch Pharmacol 2008;378:579-88.
15. Lee EK, Kim JM, Choi J, Jung KJ, Kim DH, Chung SW, *et al*. Modulation of NF-κB and FOXOs by baicalein attenuates the radiation-induced inflammatory process in mouse kidney. Free Radic Res 2011;45:507-17.
16. Lea MA, Ibeh C, Deutsch JK, Hamid I, desBordes C. Inhibition of growth and induction of alkaline phosphatase in colon cancer cells by flavonols and flavonol glycosides. Anticancer Res 2010;30:3629-35.
17. Pidgeon GP, Kandouz M, Meram A, Honn KV. Mechanisms controlling cell cycle arrest and induction of apoptosis after 12-lipoxygenase inhibition in prostate cancer cells. Cancer Res 2002;62:2721-7.
18. Fahey JW, Stephenson KK. Pinostrobin from honey and Thai ginger (*Boesenbergia pandurata*): A potent flavonoid inducer of mammalian phase 2 chemoprotective and antioxidant enzymes. J Agric Food Chem 2002;50:7472-6.
19. Dzoyem JP, Nkuete AH, Kuete V, Tala MF, Wabo HK, Guru SK, *et al.* Cytotoxicity and antimicrobial activity of the methanol extract and compounds from *Polygonum limbatum*. Planta Med 2012;78:787-92.
20. Li F, Awale S, Tezuka Y, Esumi H, Kadota S. Study on the constituents of Mexican propolis and their cytotoxic activity against PANC-1 human pancreatic cancer cells. J Nat Prod 2010;73:623-7.
21. Poerwono H, Sasaki S, Hattori Y, Higashiyama K. Efficient microwave-assisted prenylation of pinostrobin and biological evaluation of its derivatives as antitumor agents. Bioorg Med Chem Lett 2010;20:2086-9.
22. Meckes M, Paz D, Acosta J, Mata R. The effects of chrysin and pinostrobin, two flavonoids isolated from Teloxys graveolens leaves, on isolated guinea-pig ileum. Phytomedicine 1998;5:459-63.
23. Le Bail JC, Aubourg L, Habrioux G. Effects of pinostrobin on estrogen metabolism and estrogen receptor transactivation. Cancer Lett 2000;156:37-44.
24. Wu D, Nair MG, DeWitt DL. Novel compounds from Piper methysticum Forst (Kava Kava) roots and their effect on cyclooxygenase enzyme. J Agric Food Chem 2002;50:701-5.
25. Nicholson RA, David LS, Pan RL, Liu XM. Pinostrobin from *Cajanus cajan* (L.) Millsp. inhibits sodium channel-activated depolarization of mouse brain synaptoneurosomes. Fitoterapia 2010;81:826-9.
26. Serrano J, Goni I, Saura-Calixto F. Food antioxidant capacity determined by chemical methods may underestimate the physiological antioxidant capacity. Food Res Int 2007;40:15-21.
27. Sun-Waterhouse D, Wen I, Wibisono R, Melton LD, Wadhwa S. Evaluation of the extraction efficiency for polyphenol extracts from by-products of green kiwifruit juicing. Int J Food Sci Technol 2009;44:2644-52.
28. Liyana-Pathirana C, Shahidi F. Optimization of extraction of phenolic compounds from wheat using response surface methodology. Food Chem 2005;93:47-56.
29. Wang J, Sun B, Cao Y, Tian Y, Li X. Optimization of ultrasound-assisted extraction of phenolic compounds from wheat bran. Food Chem 2008;106:804-10.
30. Karacabey E, Mazza G. Optimization of solid-liquid extraction of resveratrol and other phenolic compounds from milled grape canes (*Vitis vinifera*). J Agric Food Chem 2008;56:6318-25.
31. Khuri AI, Cornell JA. Response Surfaces: Designs and Analyses. 2nd ed. New York: Marcel Dekker; 1996.
32. Montgomery DC, Runger GC. Applied Statistics and Probability for Engineers. 3rd ed. New York: Wiley; 2003.
33. Peng LX, Zou L, Zhao JL, Xiang DB, Zhu P, Zhao G. Response surface modeling and optimization of ultrasound-assisted extraction of three flavonoids from tartary buckwheat (*Fagopyrum tataricum*). Pharmacogn Mag 2013;9:210-5.
34. Yang L, Qu H, Mao G, Zhao T, Li F, Zhu B, *et al.* Optimization of subcritical water extraction of polysaccharides from *Grifola frondosa* using response surface methodology. Pharmacogn Mag 2013;9:120-9.
35. Tang X, Yan L, Gao J, Ge H, Yang H, Lin N. Optimization of extraction process and investigation of antioxidant effect of polysaccharides from the root of *Limonium sinense* Kuntze. Pharmacogn Mag 2011;7:186-92.
36. Bas D, Boyaci HI. Modeling and optimization I: Usability of response surface methodology. J Food Eng 2007;78:836-45.
37. International Conference on Harmonisation (ICH). ICH Harmonized Triplicate Guideline on Validation of Analytical Procedures. In: International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human use. Switzerland: ICH; 2010.
38. Ho CH, Cacace JE, Mazza G. Extraction of lignans, proteins and carbohydrates from flaxseed meal with pressurized low polarity water. LWT Food Sci Technol 2007;40:1637-47.
39. Zhang ZS, Li D, Wang LJ, Ozkan N, Chen XD, Mao ZH, *et al*. Optimization of ethanol –water extraction of lignans from flaxseed. Sep Purif Technol 2007;57:17-24.
40. Chirinos R, Rogez H, Campos D, Pedreschi R, Larondelle Y. Optimization of extraction conditions of antioxidant phenolic compounds from mashua (*Trapaeolum tuberosum* Ruiz and Pavon) tubers. Sep Purif Technol 2007;55:217-25.
41. Ronald B, Pegg RA, Oszmianski J. Confirming the chemical structure of antioxidative trihydroxy flavones from *Scutellaria baicalensis* using modern spectroscopic methods. Pol J Food Nutr Sci 2005;14/55:43-50.
42. Ching AY, Wah TS, Sukari MA, Lian GE, Rahmani M, Khalid K. Characterization of flavonoid derivatives from *Boesenbergia rotunda* (L.). Malays J Anal Sci 2007;11:154-9.
43. Cacace JE, Mazza G. Mass transfer process during extraction of phenolic compounds from milled berries. J Food Eng 2003;59:379-89.
44. Al-Farsi MA, ChangYL. Optimization of phenolics and dietary fibre extraction from date seeds. Food Chem 2007;108:977-85.
45. Chalermchat Y, Fincan M, Dejmek P. Pulsed electric field treatment for solid–liquid extraction of red beetroot pigment: Mathematical modelling of mass transfer. J Food Eng 2004;64:229-36.
46. Wenjuvan P, Zhongli MH. Extraction modeling and activities of antioxidants from pomegranate marc. J Food Eng 2010;99:16-23.
47. Fathinia M, Khataee AR, Zarei M, Aber S. Comparative photocatalytic degradation of two dyes on immobilized TiO_2 nanoparticles: Effect of dye molecular structure and response surface approach. J Mol Catal Chem 2010;333:73-84.
48. Soltani RD, Rezaee A, Godini H, Khataee AR, Hasanbeiki A. Photoelectro chemical treatment of ammonium using seawater as a natural supporting electrolyte. Chem Ecol 2012;29:72-85.
49. Campos JL, Garrido-Fernandez JM, Mendez R, Lema JM. Nitrification at high ammonia loading rates in an activated sludge unit. Bioresour Technol 1999;68:141-8.
50. Amini M, Younesi H, Bahramifar N, Lorestani AA, Ghorbani F, Daneshi A, *et al.* Application of response surface methodology for optimization of lead biosorption in an aqueous solution by *Aspergillus niger*. J Hazard Mater 2008;154:694-702.

51. Parthiban R, Iyer PV, Sekaran G. Anaerobic tapered fluidized bed reactor for starch wastewater treatment and modeling using multilayer perceptron neural network. J Environ Sci (China) 2007;19:1416-23.
52. Zhang Z, Zheng H. Optimization for decolorization of azo dye acid green 20 by ultrasound and H2O2 using response surface methodology. J Hazard Mater 2009;172:1388-93.
53. Senthilkumar SR, Dempsey M, Krishnan C, Gunasekaran P. Optimization of biobleaching of paper pulp in an expanded bed bioreactor with immobilized alkali stable xylanase by using response surface methodology. Bioresour Technol 2008;99:7781-7.

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