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Screening and optimizing media constituents for enhanced production of medicinal N-alkylamide Deca-2*E*,6*Z*,8*E*-trienoic acid isobutylamide from dedifferentiated *in vitro* cell lines of *Spilanthes paniculata*

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ABSTRACT

Spilanthes paniculata is a rich source of therapeutic polyunsaturated fatty acid amides. This report established the cell cultures from leaf-disc explants of *Spilanthes paniculata* as an alternative method of cell biomass utilization and conservation of natural plant resources. Furthermore, a methodology to maximize the production of a prime *N*-alkylamide, Deca-2*E*,6*Z*,8*E*-trienoic acid isobutylamide (spilanthol), from *in vitro* cell cultures was optimized using statistical tools. Placket-Burman (PB) design was applied to identify the significant nutrient media constituents. Among the five variables tested, Murashige and Skoog (MS) major salts, sucrose, 2,4-dichlorophenoxyacetic acid (2,4-D) and N6-benzylaminopurine (BAP), were found to have significant effect on spilanthol production. The optimal concentrations of the four variables were determined using central composite design (CCD), which is widely used response surface methodology (RSM) design. The most suitable concentration of variables for spilanthol production were, 1½ MS, 5% sucrose, 4.82 µM BAP and 1.8 µM 2,4-D. At these optimal parameters, the maximum yield of spilanthol was obtained experimentally as 2.81 mg/g DW, which was found to be very close to its predicted value of 3.72 mg/g DW. The developed mathematical model was found to fit well with the experimental data by which the higher production of metabolite was achieved as compared to non-optimized media constituents. Before optimization, the callus cultures and leaves from parental plant (control) yielded 1.75 mg/g DW and 0.26 mg/g DW spilanthol, respectively. Thus, by using statistical model, it is possible to understand the existence of interactions between variables and their effect on maximizing the production of therapeutic compounds.

1. Introduction

The genus *Spilanthes paniculata* L. (Family: Asteraceae) is a rich source of various bioactive constituents possessing numerous properties. The spilanthol (Deca-2*E*,6*Z*,8*E*-trienoic acid isobutylamide), is one of the most important therapeutic phytochemicals present in the genus and is universally known as *N*-alkylamide of polyunsaturated fatty acid (Ghokale and Bhide, 1945; Khadir et al., 1989). It possesses several significant biological activities, such as stimulation of immune system, attenuation of the inflammatory responses, diuretic, larvicidal, bioinsecticidal and anthelminthic activities (Ramsewak et al., 1999; Saraf and Dixit, 2002; Ratnasooriya et al., 2004; Pandey et al., 2007; Wu et al., 2008; De Spiegeleer et al., 2013; Singh et al., 2014). It also possesses transdermal behaviour on mycotoxins, as the plant is frequently infected by the toxic environmental substances (Demarne and Passaro, 2008). This metabolite offers key benefits of beauty care cosmetics by providing anti-age applications by reducing muscle tensions and thus, decreases facial wrinkles (Tiwari et al., 2011). The plant is extensively used as spices, as fresh vegetable for culinary purpose and a folklore remedy of throat complaints, mouth ailments, stomatitis, stammering and toothache (Jondiko, 1986). It is laden with antioxidant, analgesic, antiseptic and cytotoxic properties (Saritha et al., 2002; Rai et al., 2004; Prachayasittikul et al., 2009). The World Health Organization's (WHO) estimated that five million people are infected due to malarial disease worldwide (WHO, 2008). Due to economics, medicinal plant preparations seem to be the well utilized options for the treatment of malaria. The spilanthol, a key active principle present in the *Spilanthes* has attracted the attention for its use in the treatment of malaria as well (Spelman et al., 2011).

Owing to the tremendous relevant ethanopharmocological significance and demand of the genus, an alternate strategy is explored in the present study to maximize the cell biomass utilization and also restoration of natural plant resources. The plant tissue culture technique offers to generate the cell biomass in a shorter duration, through-

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out the year, irrespective of the seasons and regions with a provision of tunability of the cells to increase the metabolite production. In the current work, cell biomass was induced and established from leaf-disc explants for increased production of spilanthol. Further, to maximize the spilanthol yield from *in vitro* cell cultures mathematical model was developed for optimization of media constituents.

Statistical strategies of the current work include the execution of Placket-Burman (PB) design to identify the significant factors followed by Response surface methodology (RSM) which can be used to ensure the possible interactions effects between variables by employing central composite design (CCD). These statistical tools help to overcome the limitations of classical methods consisting of one dimensional approach by varying distinct factors, one at a time, and, hence may not guarantee the optimal conditions. Classical methods are also considered as laborious, tedious, expensive and also devoid of information on interactions between optimized factors (Ratnam et al., 2005; Haider et al., 2007; Hanchinal et al., 2008). In contrast to this, the statistical techniques contributes to increased product yield, reduced process variability by regression analysis and closer validation of output response to insignificant target requirements, reduced development, overall cost of production and also with limiting number of experiments (Rao et al., 2000). To the best of the knowledge, there are no such reports documented in *Spilanthes*.

2. Materials and methods

2.1. Plant material and its surface sterilization

Fresh, healthy and young leaves growing along the tropical and subtropical regions were collected from the campus of Indian Institute of technology Guwahati, Assam, India. The collected plant materials were washed with distilled water and placed on a blotting paper to remove excess water. The leaves were then washed with 1% (v/v) savlon (Johnson and Johnson, India) for 15 min followed by rinsing thrice with sterile distilled water (SDW). Using 0.1% mercuric chloride ($HgCl_2$) solution, surface sterilization of leaves were carried out for 5 min followed by SDW rinsing for atleast three times in the laminar-air-flow chamber (Saveer Biotech, India).

2.2. Establishment of in vitro plant material

After punching the leaves in a sterile condition to a size of around 10 mm using sized cork-borer, leaf-disc cultures were obtained. The sterile leaf-disc cultures were inoculated abaxially on Murashige and Skoog (1962) (MS) medium. The medium was fortified with various combinations of plant growth regulators, such as α-naphthalene acetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D) and N_6-benzylaminopurine (BAP) at a concentration range of 1–10 μM for the induction and multiplication of unorganized cells. All media combinations consisted of sucrose (30 gL^{-1}) as carbon source and were solidified using 0.8% agar. The pH of the medium was adjusted to 5.8 using base and acid solution, such as (0.1N) NaOH or (0.1N) HCL, respectively. Consequently, rimless 150 mm×25 mm glass tubes were dispensed with 20 mL medium. Before autoclaving at 15 psi for 15 min, the culture tubes were plugged with (non-absorbent) cotton wrapped in cheese cloth. All the cultures were maintained at a temperature of 25 ± 2 °C with relative humidity (RH) 50–60% and provided with diffuse light (1000–2000 lx) for 16 h of photoperiod and 8 h of dark period. Each experiment was repeated thrice and at least twenty four cultures were raised for each treatment. After callus induction, the biomass of cells was frequently subcultured on fresh medium. The cultures were observed after every 4 weeks.

2.3. Extract preparation from in vitro cell lines

A month old calli differentiated from *in vitro* leaf cultures were harvested for extraction. Leaves from the field grown parent plants as control were also utilized. Both the samples were oven dried separately at 30 ± 2 °C to remove the moisture. Thereafter, the dried mass of cells and the leaves from parent plants were powdered separately using porcelain pestle and mortar. The samples were immersed in methanol for 12 h. Then, the extract was centrifuged at 5000 rpm using high speed refrigerated centrifuge (Sigma 4 K 15C, Osterode Am Harz, Germany) for 10 min. The aliquots were collected separately and the left over residue was re-dissolved using 10 mL methanol and the experiment was repeated three times. Subsequently, the overall collected methanolic solution was filtered and evaporated using rotary evaporator (Buchi Rotavapor R-200, Japan) at 40 °C. Furthermore, the residue obtained was redissolved in methanol (HPLC grade) and filtered using (0.22 μm) nylon membrane. The filtered aliquot (20 μl) of sample solution was manually injected and analyzed using HPLC.

2.4. Standard solution preparation

As standard of spilanthol is not available commercially, a structurally similar N-alkylamide, Dodeca-2*E*,4*E*-dienoic acid isobutylamide, was taken as a reference standard for spilanthol quantification. Both these compounds consist of long chain carbon and isobutylamide group. The standard solution of concentration 1 mg mL^{-1} was prepared using HPLC grade methanol. Different levels of external standards at the range of 15–250 μg mL^{-1} were attained by serial dilutions. Before HPLC analysis, each concentration of standards were filtered using 0.22 μm nylon filter membrane (Millipore, USA) and then, the same was stored at −20 °C.

2.5. Identification and quantification of secondary metabolite

Analytical separation of spilanthol was carried out on HPLC system (Prostar Varian Prostar, USA) with C18 column (BDS Hypersil RP-18 column (4.6 mm×250 mm, Thermo, USA). MilliQ water (18 Ω) from Millipore was used for experimental purpose. Spilanthol, an *N*-alkylamide, was identified and quantified using HPLC by loading (20 μl) methanolic solutions into injection loop connected to C18 analytical column. HPLC separations were performed using isocratic conditions acetonitrile-water (93:7) at a flow rate (0.5 mL min^{-1}). The sample was controlled with a flow rate (0.5 mL min^{-1}). The eluted sample was monitored at 237 nm and the fractions were collected manually. Subsequently, the eluted spilanthol peak from HPLC was confirmed by ESI-MS Q-TOF premier mass spectrometry (Waters model, USA). Based on our reported protocol (Singh et al., 2012), spilanthol was quantified accordingly.

2.6. Plackett–Burman design (PB)

The PB design criterion evaluates the significance of various nutrients on spilanthol production. The PB design is assessed based on first-order polynomial model (3):

$$Y = ß_0 + \Sigma ß_i X_i \quad (1)$$

where, Y is probability of target response, β_i is linear coefficient, β_o is scaling constant and, X_i is level of five independent variables, such as MS major salts, sucrose as a carbon source and three plant growth regulators BAP, 2,4-D and NAA. These factors were tested at two different levels, a higher (+1) and lower (−1) level with twelve different media combinations (Table 1). This design is used to scrutinize the important factors, but it is devoid of interaction among the selected factors. The higher and lower level of each variable was set in such a way to categorize significant media constituents for elevated response. All experiments and the average value of response towards spilanthol production were conducted and considered for statistical analysis. The variables which were significant at 95% level ($p < 0.05$) were considered to have higher influence on spilanthol production. These

Table 1
Quantification of spilanthol, before optimization, in the samples.

S. No	Sample type	Total amount (mg/g DW)	Total amount (μg/g DW)
1.	Field grown leaves (Control)	0.26	263
2.	In vitro callus cultures from Leaf-disc	1.75	1751

significant variables were further optimized using CCD by neglecting insignificant factors. The design was developed using Minitab 16.1.1 version statistical software package.

2.7. Response surface methodology (RSM)

RSM is a multivariate statistical method which allows the interaction between variables. The optimal concentrations of the significant variables (also called factors) obtained from PB design was obtained using central composite design (CCD) which is falling under the RSM. The developed experimental design and statistical data analysis was executed with the statistical software package Minitab (16.1.1. version). The CCD, a response surface method, was implemented to determine the level of four significant factors screened from the PB design criterion for maximum response. The five different coded values $-\alpha$, −1, 0, +1, $+\alpha$ were calculated for MS major salts, sucrose, BAP and 2,4-D by the following Eq. (2) (Paul et al., 1992)

$$\text{Coded value} = \frac{\text{actual level}-(\text{high level} + \text{low level})/2}{(\text{High level} - \text{low level})/2} \quad (2)$$

where, $\alpha=2^{(n/4)}$, here n is the number of factors and 0 is considered to be central point. The influenced factors from PB were fed to 2^n factorial resulted in 31 numbers of experiments. This method correlates the relationship between response value (spilanthol content) and the response of variables *via* second-order polynomial model. The data was fitted into the multiple regression procedure to obtain model of polynomial Eq. (3) for analysis is given below:

$$Y = ß_0 + \Sigma ß_i X_i + \Sigma ß_{ii} X_i + \Sigma\Sigma ß_{ij} X_i X_j \quad (3)$$

where, Y refers predicted response, β_i stand for linear coefficient, β_{ii} denotes quadratic coefficient, β_{ij} symbolizes interaction coefficient, β_o indicates scaling constant, and X_i, X_j are coded independent variables. The experimental design protocol for RSM which includes the analysis of variance (ANOVA), the effect and regression coefficients of individual linear, quadratic, and interaction terms was developed using statistical software package. The analysis of variance can be calculated by the following standard equation to validate the effect and to evaluate the statistical significance of model (4),

$$\text{Effect} = \frac{2\{\Sigma R(H) - \Sigma R(L)\}}{N} \quad (4)$$

where, R(H) refers all components response at high levels, R(L) refers all components response at low levels and N is the number of factors. The significance of all terms in the polynomial was judged statistically by computing F value at a probability ($p < 0.05$). From the regression models, the regression coefficients were used to make statistical calculations to generate response surface curves. To test the model accuracy, R^2, adjusted R^2 (R_{adj}^2) and predicted R^2 (R_{pred}^2) were estimated. The expansion of second-order polynomial for the response of significant variables from CCD was determined using Minitab response optimizer under a global solution of desirability (equal to 1) to achieve the optimal levels of individual variables. The accuracy of values was corroborated by correlating the predicted values from the mathematical model and the measured values from the given experiment.

3. Results and discussion

3.1. Establishment of dedifferentiated mass of cells from leaf-disc explant

An aseptic leaf-disc explant of 10 mm size was excised and inoculated on MS basal medium supplemented with auxin, like α-naphthalene acetic acid (NAA); 2,4-dichlorophenoxyacetic acid (2,4-D) and/or cytokinin, like N6-benzylaminopurine (Fig. 1A). The three growth regulators were tested selectively for callus induction based on the previous reports of Singh and Chaturvedi (2012). Callus induction was observed on all the media, MS+BAP (7 μM), MS +2,4-D (5 μM) and MS+NAA (5 μM). However, the calli did not show sustained growth beyond first subculture of two weeks duration on the parent medium. Therefore, these calli were transferred to a set of growth regulator combination, MS+BAP (5 μM)+2, 4-D (1 μM)+NAA (1 μM), where brown, friable callus was obtained after 8 weeks (Fig. 1B). After 15 weeks of culture, fresh, healthy, friable, green profusely growing calli were obtained (Fig. 1C). The calli were maintained and multiplied on this medium by frequent subculture, after every 8 weeks, for their efficient utilization for quantification of spilanthol.

3.2. Identification and quantification of spilanthol

Spilanthol was quantified using HPLC from methanolic extracts prepared from undifferentiated *in vitro* calli and leaves from field grown parent plant. Due to the non-availability of commercial standard of spilanthol, it was screened and quantified with the tentative,

Fig. 1. Establishment of leaf-disc cultures from *S. paniculata*. (A) One-day-old leaf-disc culture on callus induction medium (×0.15 cm), (B) An 8-week-old brown, friable, undifferentiated calli on MS+BAP (5 μM)+2, 4-D (1 μM)+NAA (1 μM) (×0.16 cm), (C) Same as B, 15-week-old healthy, fresh, friable massively growing calli (×0.15 cm).

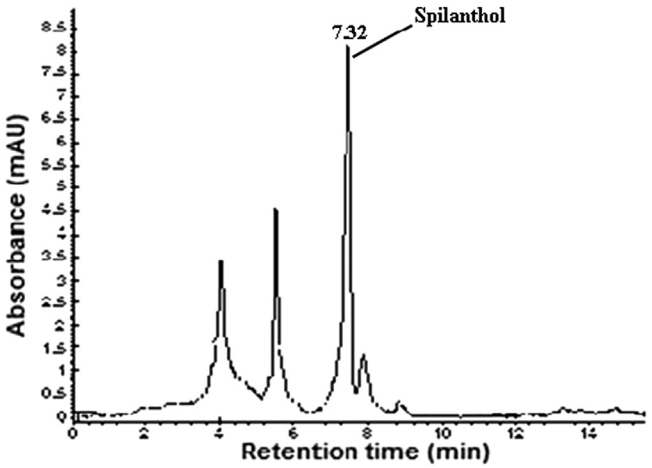

Fig. 2. HPLC chromatogram from extracts of leaf-disc callus cultures, established from *S. paniculata*, showing presence of spilanthol peak at a retention time of 7.32.

structurally similar compound, dodeca-2*E*,4*E*-dienoic acid isobutylamide, which contains similar amine substituents and longer polyunsaturated acid chain.

The sample (20 µl) was injected to flow isocratically at 0.5 mL min^{-1} with acetonitrile (93%): water (7%). A characteristic chromatogram of spilanthol was obtained at retention time 7.32 ± 0.36 (Fig. 2). The amount of spilanthol was quantified by the calibration curve generated from standard (Table 1). The eluted peaks were collected and the presence of spilanthol was evidentially reconfirmed based on characteristic collision-induced dissociation of fragmentation patterns by mass spectrometry in positive mode due to its reproducibility (Fig. 3). The $[M+H]^+$= 222 *m/z* is a fragmented spilanthol with two stable fragmentations $[M-72]^+$=149 *m/z* (fragment represents the dissociation of C-N bond from the isobutylamide to lose the entire amine functional group) and $[M-55]^+$=166 *m/z*, (represents the dissociation C–N bond from the isobutylamide to lose the alkyl group directly attached with the amine group (Bae et al., 2010; Boonen et al., 2010). Moreover, the characteristic fragment ion obtained greater than the base peak of 222 *m/z* clearly indicates the sodium ion adduct formation [M+Na]+ with 244 *m/z* (Singh and Chaturvedi, 2012).

Table 2
Screening the levels of media constituents for spilanthol production using Plackett-Burman design criterion.

Run order	MS major salts Low-0.75 High-1.25	Sucrose (%) Low-2 High-4	BAP (µM) Low-3 High-7	2,4-D (µM) Low-0.6 High-1.4	NAA (µM) Low-0.6 High-1.4	Spilanthol amount (mg/g DW)
1	1.25	2.00	7.00	0.60	0.60	0.05
2	1.25	4.00	3.00	1.40	0.60	2.13
3	0.75	4.00	7.00	0.60	1.40	0.26
4	1.25	2.00	3.00	1.40	0.60	0.49
5	1.25	4.00	7.00	1.40	1.40	1.54
6	1.25	4.00	7.00	0.60	1.40	0.30
7	0.75	4.00	7.00	1.40	0.60	0.46
8	0.75	2.00	3.00	1.40	1.40	0.30
9	0.75	2.00	3.00	1.40	1.40	0.26
10	1.25	2.00	3.00	0.60	1.40	0.31
11	0.75	4.00	3.00	0.60	0.60	0.41
12	0.75	2.00	3.00	0.60	0.60	0.06

3.3. Media optimization using RSM

Two-step approach was implemented for optimizing the media constituents to induce and obtain high spilanthol yielding lines of callus from leaf-disc explants of *S. paniculata*. Firstly, the effect of five individual medium constituents on spilanthol production was tested in a 12-run Placket-Burman (PB) (Table 2). The production of spilanthol obtained from the PB design ranged from 0.05 to 2.13 mg/g DW and clearly indicated the influence of medium constituents. The significance of the variables and the adequacy of the model were scrutinized by regression analysis in terms of variable effects, p-value and t-value. In general, larger t-value and lesser p-value indicate the greater significance of model term. Amongst five different variables chosen for the study, MS major salts (X_1), sucrose (X_2), BAP (X_3), 2,4-D (X_4) and NAA (X_5), four variables, MS major salts (X_1), sucrose (X_2), BAP (X_3)and 2,4-D (X_4) were found to have significant effect on spilanthol production, which was evidently proved by p-values ($p < 0.05$) *via* regression analysis (Table 3).

Pareto chart highlights the degree of effect of factors where the bars

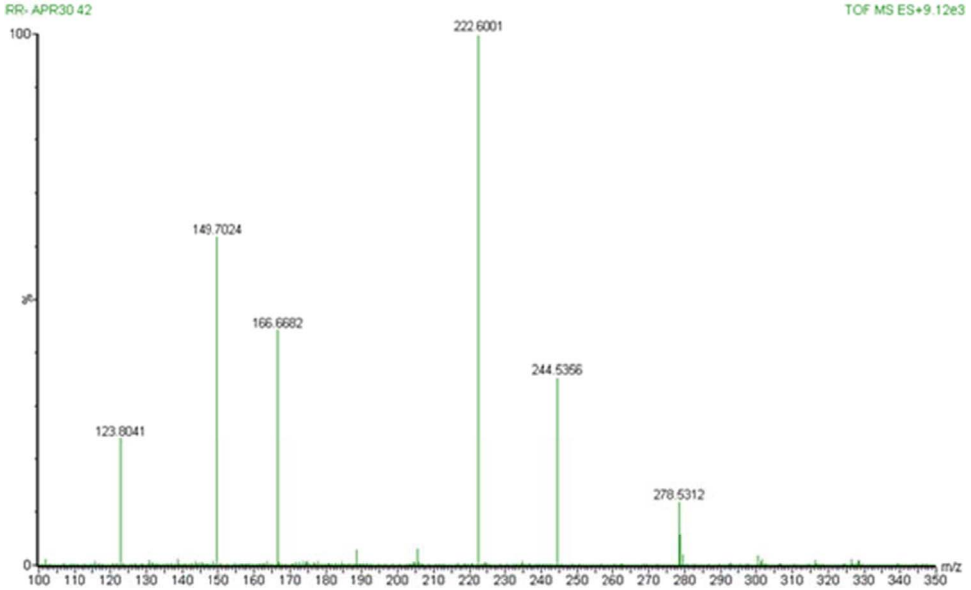

Fig. 3. Mass spectrum of protonated molecular ion of spilanthol, purified from HPLC of callus extracts, showing the base peak at *m/z* 222 and its corresponding fragments.

Table 3
Estimated effects and coefficients for spilanthol content.

Term	Effect	Coefficient	T	P
Constant	0.000	5.250	6.710	0.001
MS	5.567	2.783	3.560	0.012
Sucrose	6.500	3.250	4.150	0.006
BAP	−5.200	−2.600	−3.320	0.016
2,4-D	5.867	2.933	3.750	0.010
NAA	−1.500	−0.750	−0.960	0.375

R^2=90.31%; R_{pred}^2=61.25%; R_{adj}^2=82.24%

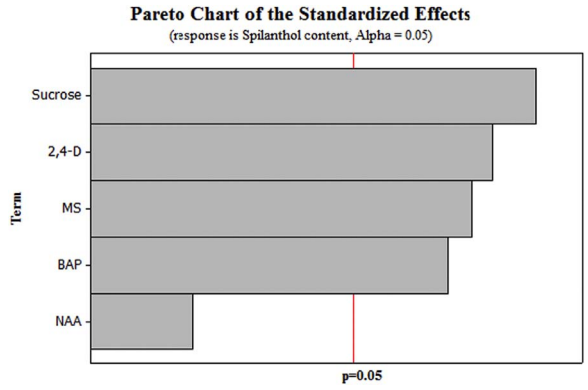

Fig. 4. Pareto chart of five prominent variables influencing spilanthol production.

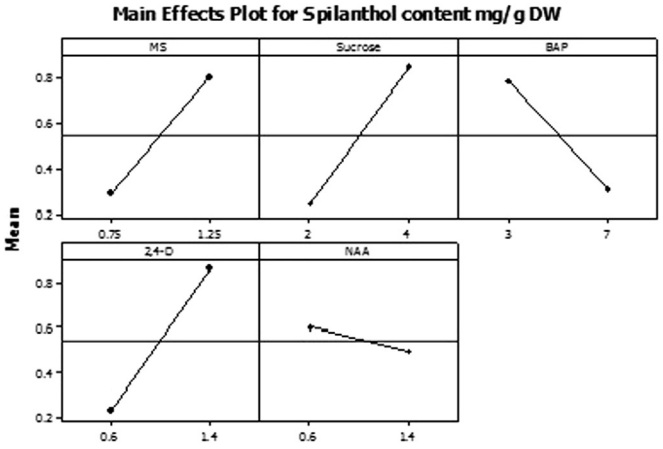

Fig. 5. Main effect plot indicating the effect of concentration levels (high/low) of five variables on spilanthol production in *in vitro* callus cultures.

refer the effects on production of spilanthol in the descending order as shown in Fig. 4. The length of the bar is directly proportional to the absolute t-value. Main effect plot depicts the concentration level (high/low) at which the constituents are effective in increasing the production of response. Hence, the effect of salts and the sucrose was found to be positive at high level concentration, whereas the effect of plant growth regulators, like 2,4-D was positive at higher level of concentration, BAP has inverse significant effect while NAA has no significant influence, at both levels of concentration, on spilanthol production from dedifferentiated calli (Fig. 5). The study confirms that MS salts, sucrose and 2,4-D required at higher concentration than BAP. Therefore, by neglecting insignificant variable, model Eq. (5) of first order can be inscribed as:

$$Y = 5.25 + 2.78 \times X_1 + 3.25 \times X_2 - 2.60 \times X_3 + 2.93 \times X_4 \quad (5)$$

Table 4
The coded and actual values used for optimization of media constituents using CCD.

Significant variables	Coded values				
	−2	−1	0	+1	+2
MS	0.50	0.75	1.00	1.25	1.50
Sucrose	1.00	2.00	3.00	4.00	5.00
BAP	1.00	3.00	5.00	7.00	9.00
2,4-D	0.20	0.60	1.00	1.40	1.80

3.4. RSM based on CCD towards spilanthol production

In the second level of experiment, the coded and uncoded values of significant factors obtained from PB design (Table 4) influencing spilanthol production were used to obtain the combined effect by CCD (Table 5). A total of thirty-one experiments were generated by CCD with 2^4=16 cubic points plus 7 central points and 2×4=8 axial points (α value) with experimental and predicted values.

The Minitab develops a design and a mathematical model, which depicts the quadratic relationship between the spilanthol production and the media constituents by applying multiple regression analysis on the experimental data, which gives the following second order polynomial coefficient equation for each term as:

$$\begin{aligned} Y = {} & 1.7924 + 0.1880X_1(\text{MS major salts}) - 0.1555X_2(\text{sucrose}) \\ & - 0.1019X_3(\text{BAP}) + 0.2542X_4 \\ & (2,4\text{–}D) - 0.3005X_{12}(\text{MS major salts x MS major salts}) \\ & - 0.2473X_{22}(\text{sucrose x sucrose}) - 0.2158 \\ & X_{32}(\text{BAPxBAP}) - 0.0160 \times X_{42}(2,4\text{–}D \text{ x } 2,4\text{–}D) \\ & + 0.2684X_1X_2(\text{MS major salts x sucrose}) \\ & + 0.0148X_1X_3(\text{MS major salts x BAP}) \\ & + 0.3200X_1X_4(\text{MS major salts x } 2,4\text{–}D) + 0.3101X_2X_3 \\ & (\text{sucrose x BAP}) + 0.3101 \times X_2X_4(\text{sucrose x } 2,4\text{–}D) - 0.0008 \times X_3X_4 \\ & (\text{BAPx}2,4\text{–}D) \end{aligned} \quad (6)$$

The linear significance of the media constituents ($p < 0.05$) like MS major salts, sucrose, BAP and 2,4-D obtained from PB design were evaluated *via* CCD based RSM on spilanthol production. Correspondingly, the regression analysis determined that p-value corresponds to the linear effect of MS major salts and 2,4-D had positive coefficients, whereas sucrose and BAP had negative significance on the response.

The plant growth regulator, 2,4-D had greater impact on response with high positive coefficient (+0.25) followed by MS major salts (+0.19), while sucrose and BAP had negative linear effect with (−0.16) and (−0.10), respectively. The quadratic terms of the factors were significant with negative coefficient, nevertheless mutual interaction between 2,4-D was insignificant ($p > 0.05$). Therefore, the impact of the linear effect of sucrose and BAP as well as the quadratic terms of MS major salts, sucrose and BAP towards spilanthol production was increasing as well as decreasing when the level of all these variables were increased at certain levels. Moreover, it was quite evident that the p-value corresponding the interaction between MS major salts *vs* sucrose, MS major salts *vs* 2,4-D and sucrose *vs* 2,4-D were significant with positive coefficient while MS major salts *vs* BAP, sucrose *vs*. BAP and BAP *vs* 2,4-D were insignificant with positive coefficient (Table 6).

The adequate precision of the response surface statistical model can be obtained by the analysis of variance (ANOVA) to validate the regression model as shown in Table 6. The value of *F*-test higher than the critical value determines the adequate variation occurred in the

Table 5
Central composite design (CCD) experimental design matrix of four variables and the resulting observed and predicted values of spilanthol.

Run order	MS major salts	Sucrose	BAP	2,4-D	Spilanthol amount (mg/g DW)		Studentized residuals
					Observed	Predicted	
1	0.75	2	3	0.6	1.66	1.67	−0.03
2	1.25	2	3	0.6	0.81	0.86	−0.86
3	0.75	4	3	0.6	0.21	0.20	0.25
4	1.25	4	3	0.6	0.45	0.47	−0.35
5	0.75	2	7	0.6	1.40	1.46	−1.05
6	1.25	2	7	0.6	0.65	0.66	−0.25
7	0.75	4	7	0.6	0.06	−0.01	1.05
8	1.25	4	7	0.6	0.29	0.27	0.35
9	0.75	2	3	1.4	0.87	0.91	−0.67
10	1.25	2	3	1.4	1.31	1.39	−1.34
11	0.75	4	3	1.4	0.69	0.69	0.1
12	1.25	4	3	1.4	2.17	2.24	−1.12
13	0.75	2	7	1.4	0.66	0.71	−0.85
14	1.25	2	7	1.4	1.10	1.19	−1.48
15	0.75	4	7	1.4	0.45	0.48	−0.52
16	1.25	4	7	1.4	2.08	2.04	0.68
17	0.50	3	5	1.0	0.21	0.20	0.12
18	1.50	3	5	1.0	1.04	0.96	1.69
19	1.00	1	5	1.0	1.25	1.10	2.98
20	1.00	5	5	1.0	0.42	0.48	−1.17
21	1.00	3	1	1.0	0.93	0.86	1.48
22	1.00	3	9	1.0	0.47	0.45	0.32
23	1.00	3	5	0.2	1.19	1.27	−1.17
24	1.00	3	5	1.8	2.34	2.28	0.80
25	1.00	3	5	1.0	1.74	1.78	−0.57
26	1.00	3	5	1.0	1.81	1.78	0.43
27	1.00	3	5	1.0	1.94	1.78	2.25
28	1.00	3	5	1.0	1.81	1.78	0.51
29	1.00	3	5	1.0	1.76	1.78	−0.26
30	1.00	3	5	1.0	1.75	1.78	−0.32
31	1.00	3	5	1.0	1.74	1.78	−0.46

Table 6
Estimated regression coefficients of CCD for spilanthol production.

Term	Coefficient	*T*	*P*
Constant	1.79	59.22	0.000
MS major salts	0.19	11.50	0.000
Sucrose	−0.16	−9.52	0.000
BAP	−0.10	−6.23	0.000
2,4-D	0.25	15.55	0.000
MS major salts×MS major salts	−0.30	−20.07	0.000
Sucrose×Sucrose	−0.25	−16.52	0.000
BAP×BAP	−0.28	−18.80	0.000
2,4-D×2,4-D	−0.02	−1.07	0.302
MS major salts×Sucrose	0.27	13.41	0.000
MS major salts×BAP	0.01	0.74	0.470
MS major salts×2,4-D	0.32	15.99	0.000
Sucrose×BAP	0.01	0.63	0.539
Sucrose×2,4-D	0.31	15.49	0.000
BAP×2,4-D	0.00	−0.04	0.966

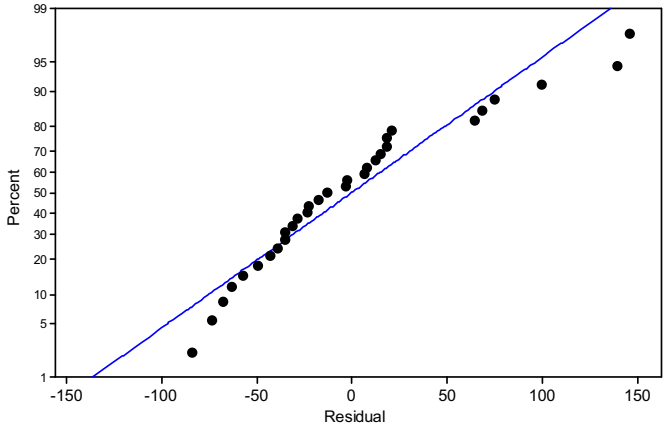

Fig. 6. Normal distribution plot of residual *vs* percent of confidence limit on spilanthol production in *in vitro* callus cultures.

Table 7
Analysis of variance of CCD for spilanthol production in *in vitro* callus cultures.

Source	DF	Sequential SS	Adjusted SS	Adjusted MS	*F*	*P*
Model	14	13.11	13.11	0.94	146.06	0.000
Linear	4	3.23	3.23	0.81	125.9	0.000
Square	4	5.55	5.55	1.39	216.29	0.000
Interaction	6	4.34	4.34	0.72	112.68	0.000
Lack of fit	10	0.07	0.07	0.01	1.43	0.344
Pure error	6	0.03	0.03	0.01		
Total	30	13.22				

R^2=99.22%; R_{pred}^2=96.54%; R_{adj}^2=98.54%. D.F., degree of freedom; sequential SS, sequential sum of square; adjusted SS, adjusted sum of square; Adjusted MS, adjusted mean square; *F*, *F*-statistics test to determine significance and *P*, probability.

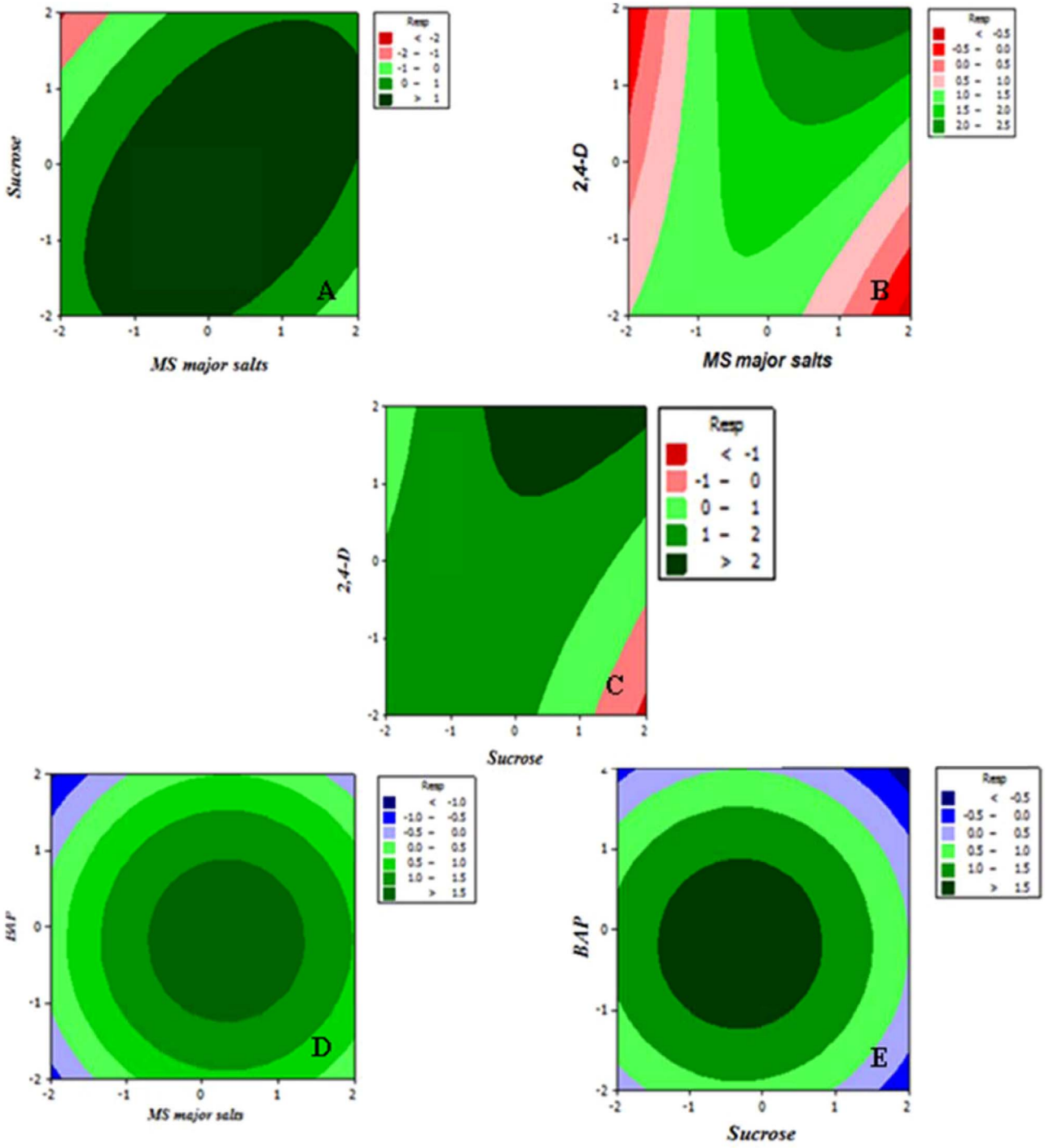

Fig. 7. Response surface plots from quadratic model with respect to two variables to analyse spilanthol production. (A) MS major salts and Sucrose, fixed level: BAP and 2,4-D; Response: Spilanthol content mg/g DW, (B) MS major salts and 2,4-D, fixed level: Sucrose and BAP; Response: Spilanthol content mg/g DW, (C) Sucrose and 2,4-D, fixed level: MS major salts and BAP; Response: Spilanthol content mg/g DW, (D) MS major salts and BAP fixed level: Sucrose and 2,4-D; Response: Spilanthol content mg/g DW, (E) Sucrose and BAP, fixed level: MS major salts and 2,4-D; Response: Spilanthol content mg/g DW.

experimental data at the $p < 0.05$ level of significance. The squared regression static R^2=0.99, whereas $R_{pred}^2$and R_{adj}^2 are 96.54% and 98.54%, respectively, expressed the goodness of fit of the model. Thus, the variation of the model was explainable upto 99.22% and also indicated high significance of the model. When R value is closer to value 1, the observed and predicted values remains highly significant. The p=0.344 corresponding to the lack of fit which precisely explains the fit of model as shown in Table 7 and revealed the effects of media constituents. The normal distribution plot is an important tool to diagnose the homogeneous scatter of the errors and the residuals above and below the X-axis. Thus, confirmed the normality assumption and the independence of the residuals. This plot indicates the residuals are independent of the value of spilanthol and therefore, fits the adequacy of the model (Fig. 6) (Sanjeeviroyar et al., 2010).

The response surface contour plot of mutual interaction between the factors on spilanthol production from *S. paniculata* were predicted based on the nature of shapes of contour whether elliptical, circular or saddle. The interaction between MS major salts *vs* sucrose were found elliptical, indicating high significance of variables as shown in Fig. 7A. When *α*-level of MS major salts *vs* 2,4-D was increasing, the response

was found to be increasing upto 2.5 mg/g DW as can be seen in Fig. 7B followed by sucrose *vs* 2,4-D as shown in Fig. 7C with its regression coefficients 0.32 and 0.31, respectively. Fig. 7D-E shows the circular nature of surface contours depicts the lesser significant of factors on response. This experimental study leads to the maximum production of spilanthol upto 2.34 mg/g DW of plant cells which was found to be higher than the predicted value of about 2.28 mg/g DW of the cells.

3.5. Validation of model

From the experimental data the optimum values of four media constituents obtained by resolving the second order polynomial equation were: $1^1/_2$ MS major salts, sucrose (5%), BAP (4.82 μM) and 2, 4-D (1.8 μM). To validate the model the above mentioned experiment was performed to obtain 2.81 mg/g DW from *Spilanthes paniculata*. The amount of production was slightly far closer to the predicted value of about 3.72 mg/g DW of cells.

4. Conclusion

Placket-Burman (PB) design was applied to identify the significant nutrient media constituents in leaf-disc callus cultures. Among the five variables tested, Murashige and Skoog (MS) major salts, sucrose, 2,4-dichlorophenoxyacetic acid (2,4-D) and N6-benzylaminopurine (BAP), were found to have significant effect on Spilanthol production. The optimal concentrations of the four variables were determined using central composite design (CCD), which is widely used response surface methodology (RSM) design. The most suitable concentration of variables for spilanthol production were, 1½ MS, 5% sucrose, 4.82 μM BAP and 1.8 μM 2,4-D. At these optimal parameters, the maximum yield of Spilanthol was obtained experimentally as 2.81 mg/g DW, which was found to be very close to its predicted value of 3.72 mg/g DW. The developed mathematical model was found to fit well with the experimental data by which the higher production of metabolite was achieved as compared to non-optimized media constituents. Before optimization, the callus cultures and leaves from parental plant (control) yielded 1.75 mg/g DW and 0.26 mg/g DW spilanthol, respectively. The study further confirmed that plant tissue culture technique can be used as an alternative source to obtain lead compounds.

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