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Original Article

OPTIMIZATION OF GROWTH PROMOTERS ON *DESMODIUM GANGETICUM* (L) DC USING RSM-CCD AND ITS ANTIOXIDANTS ACTIVITY

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ABSTRACT

Objective: *Desmodium gangeticum* (Linn.) DC of Fabaceae is the vital plant used in ancient medicinal system of Ayurveda which was originated in India. This plant was reported to be a good result on cardiac system and nervous system. It has an ability to cures burning sensation, fever, cough, difficult breathing, dysentery, thirst and vomiting. *D. gangeticum* has also been reported to contain alkaloids, flavone and isoflavanoid glycosides. The plant has been reported to contain gangetin, a pterocarpnoid shown to possess anti-inflammatory and analgesic activities. The potent in vitro antioxidant activity of the 50% hydroalcoholic (total) extract. Phenolics are reported to be good antioxidant and anti-inflammatory agents. Since the plant under investigation is reported to contain both alkaloids and flavones along with isoflavanoid glycosides. The useful part is the root and it is an important ingredient in more than 50 Ayurvedic formulations like Chyavanaprasam, Dhanuantharam kuzhambu, Dasamularishtam etc. The objectives of the work is to optimize the maximum callus production by *Desmodium ganageticum* using response surface methodology (RSM) by developing an empirical model with different combination of Hormones and Growth promotes (IAA, IBA, BAP, and Kinetin) which were selected as the parameters.

Methods: Different medium were screened for the maximum callus production and Murashige and Skoog was selected in which different range of hormones and growth promotes were added. The model evaluates the effect of each independent variable to a response. The mathematical relationship of the independent variables and the response can be calculated by the quadratic polynomial equation. The anti-oxidant activity was analyzed.

Results: Models were developed by Central composite design (CCD) with the selected parameters. The regression analysis (R2) of RSM showed 97%. The optimal conditions for the maximal callus yield were IAA - 3, IBA - 0.5, BAP - 0.75 and Kinetin - 0.75.The work reported is a novel concept of combining the statistical modeling for an improved yield of callus of *Desmodium gangeticum*. The antioxidant activity of the intact plant compared which callus by superoxide scavenging, Hydroxy radical scavenging activity, Lipid peroxide assay, Nitric oxide radical inhibition activity.

Conclusion: The development of rapid and efficient method for media preparation was established using RSM-CCD.

Keywords: *Desmodium gangeticum*, Response Surface Methodology –Central Composite Design, Anti-oxidant activity, IAA, IBA, BAP, Kinetin.

INTRODUCTION

Plants are remarkable source of natural products which are used as pharmaceuticals, neutraceuticals, agrochemicals flavor and aroma ingredients, food additives and pesticides [1]. *Desmodium gangeticum* (L.) DC. (Fabaceae) is a small shrub of tropical regions that has been used as a bitter tonic, antipyretic, digestive and antiemetic in inflammatory conditions of the chest and other organs. *D. gangeticum* has also been reported to contain alkaloids, flavone and isoflavanoid glycosides. The plant has been reported to contain gangetin, a pterocarpnoid shown to possess anti-inflammatory and analgesic activities.

Studies have shown that many of these antioxidant compounds possess anti-inflammatory, antiatherosclerotic, antitumor, antimutagenic, anticarcinogenic, antibacterial, and antiviral activities[2,3]. The ingestion of natural antioxidants has been associated with reduced risks of cancer, cardiovascular disease, diabetes, and other diseases associated with ageing [4,5], and in recent years, there has been a worldwide trend towards the use of the natural phytochemical present in berry crops, teas, herbs, oilseeds, beans, fruits and vegetables [6]. The antioxidant activity of some of these plants have been also proved in various in vitro or *in vivo* studies, also as a part of screening assays of many medicinal plants [7,8,9,10,11,12]. However, to the best of our knowledge, very few reports are available on antioxidant properties of *Desmodium gangeticum* pant.

MATERIALS AND METHODS

Plant

Healthy young and tender leaves were used as explant. Freshly harvested plant parts were washed thoroughly with tap water and surface cleaned with 5% teepol solution. Followed by surface sterilization using 70% of ethanol for 30 second followed by 0.1% $HgCl_2$ for 5 minutes the explants were rinsed with sterile water at least three time followed by each disinfection treatment. The explants were transferred to Murashige and Skoog's, Gamborg's media and White's basal medium supplemented with 3% sucrose as carbon source and 0.7% agar as gelling agent. After the analysis Murashige and Skoog medium which showed the maximum growth was selected and growth regulators such as IBA, (0 – 1.5 mg /L), kinetin (0.5 – 1.5 mg/l). IAA (0.5 – 1.5 mg/l), BAP (0.5 – 1.5 mg/l) were added. The pH was adjusted to 5.5 -5.8.

Culture conditions

The cultures were incubated at 25 ± 2ºC light under 16/8 hours of photoperiod with 25 µmol / m^2/s light intensity. Explants were inoculated into glass jars of 110 mm × 60 mm with 40 ml medium for all the experiments. Shoot bud response was expressed based on percent of explants responding to shoot bud formation. The callus was initiated and proliferated followed by sub-culturing the call us at regular interval.

Estimation of relative growth rates of calli

Fresh weight of initiated calli in promising culture media was recorded as the time of initial subculture (four weeks after callus initiation). All the experiments were maintained with triplicates. Growth of established calli was estimated as relative growth rate (RGR) over 30 days subculture period.

$$RGR = 3\frac{(W_2 - W_1)}{t_2 - t_1}$$

Where W_1 – initial callus mass at t_1 in grams

W_2 – final callus mass in t_2 in grams

t_2-t_1 – 30 days.

Preparation of plant and callus extract

The in vivo grown plant and the callus after 30 days of growth were air dried, powdered and extracted with 70% methanol. The extracts were evaporated to dryness and difference concentration (10-100µg/ml) were prepared and used for the study.

Antioxidant activity

Superoxide scavenging activity was analyzed as explained by McCord and Fridovich,(1969) [13], Hydroxy radical scavenging activity was analyzed as designated by Elizabeth and Rao(1990) [14] and Lipid peroxide assay as described by Gow-Chin Yen, et al., 1998 [15] Nitric oxide radical inhibition activity was analyzed according to Garratt et al., 1964 [16] procedure.

Statistical analysis

Response Surface Methodology (RSM) was used to investigate the main effects of variables on the growth of callus. IAA, IBA, BAP, KIN were selected as independent variables. Central composite design was used for the experimental data and data were fitted to a second order polynomial model and regression coefficients obtained. Mathematical models were evaluated, for each response by means of multiple regression analysis.

Response = 1.9 + 0.91A + 0.12B + 0.22C + 0.39D - 0.06AB +0.39AC + 0.15AD + 0.04BC - 0.02BD -0.49 CD + 0.09 A^2- 0.23B^2- 0.19C^2- 0.14D^2

The modeling was started with a quadratic model including linear, squared and interaction terms. Significant terms in the model for each response were found by analysis of variance (ANOVA) and significance was judged by the F statistic calculated from the data. Design Expert Ver. 7.1.0 (Stat-Ease) was used to fit response surfaces and optimize the callus formation.

Values were represented as mean + SEM and data were analyzed using IBM SPSS Statistical 20 software for the Windows.

RESULTS

Callus induction

Callus induction was observed in the three basal mediums MS, Whites and Gamborg in the initiation of callus containing different concentration and combinations of IAA, IBA, BAP, KIN. Within 10-15 days of incubation, the explants depending upon the concentration and combination of hormones were inducted calli. There was a wide range variation in callus induction and average fresh weight of callus. Highest callus induction was observed in MS medium containing 0.5 mg /L BAP and 1 mg /L IAA, Gamborg and 1 mg /L IBA produced the least. The combination with White's medium showed less frequency of callus induction.

Table 1: Effect of basal medium on callus induction of *Desmodium gangeticum*

Medium	Fresh weight in gram
Murashige and Skoog	2.129 + 0.005*
Gamborg's B5	1.0311 + 0.007*
White's	0.078 + 0.005*

*Standard error Mean

Response surface methodology

The surface methodology was done by CCD model and it gives a combination or growth promoters which influence the growth of the callus in tissue culture. This predictable model will gives an appropriate condition and combination of growth promoters which can give a maximum result.

Table 2: Effect of different concentrations of phytohormones on % induction and callusing of *D. gangeticum* DC

Run	Factor 1		Factor 2		Factor 3		Factor 4		Response in Milligram	
	A:IAA coded	actual	B:IBA Coded	Actual	C:BAP Coded	Actual	D:Kin coded	actual	Actual	Predicted
1	1	2	1	1	1	1.5	1	1.5	2.92	3.09
2	-1	0	-1	0	1	1.5	1	1.5	0	-0.09
3	-1	0	1	1	1	1.5	1	1.5	0.56	0.31
4	0	1	2	1.5	0	0.75	0	0.75	1.25	1.23
5	2	3	0	0.5	0	0.75	0	0.75	3.825	3.09
6	0	1	0	0.5	0	0.75	0	0.75	2.15	1.91
7	0	1	0	0.5	0	0.75	0	0.75	2.15	1.91
8	-1	0	-1	0	-1	0	1	1.5	1.26	1.33
9	1	2	-1	0	1	1.5	1	1.5	2.96	2.93
10	1	2	-1	0	-1	0	-1	0	0.56	0.66
11	1	2	-1	0	1	1.5	-1	0	3.03	2.80
12	0	1	0	0.5	2	2.25	0	0.75	1.56	1.59
13	0	1	0	0.5	-2	-0.75	0	0.75	0.56	0.70
14	1	2	1	1	-1	0	-1	0	0.67	0.73
15	-1	0	1	1	-1	0	1	1.5	1.36	1.56
16	1	2	-1	0	-1	0	1	1.5	3.08	2.77
17	-1	0	1	1	1	1.5	-1	0	0.56	0.85
18	0	1	-2	-0.5	0	0.75	0	0.75	0.56	0.76
19	-2	-1	0	0.5	0	0.75	0	0.75	0.56	0.46
20	-1	0	1	1	-1	0	-1	0	0.25	0.13
21	0	1	0	0.5	0	0.75	-2	-0.75	0.56	0.56
22	1	2	1	1	1	1.5	-1	0	3.25	3.04
23	0	1	0	0.5	0	0.75	0	0.75	1.64	1.91
24	0	1	0	0.5	0	0.75	0	0.75	1.67	1.91
25	1	2	1	1	-1	0	1	1.5	3.02	2.76
26	0	1	0	0.5	0	0.75	0	0.75	1.96	1.91
27	0	1	0	0.5	0	0.75	2	2.25	1.96	2.13
28	0	1	0	0.5	0	0.75	0	0.75	1.86	1.91
29	-1	0	-1	0	1	1.5	-1	0	0.25	0.37
30	-1	0	-1	0	-1	0	-1	0	0	-0.19

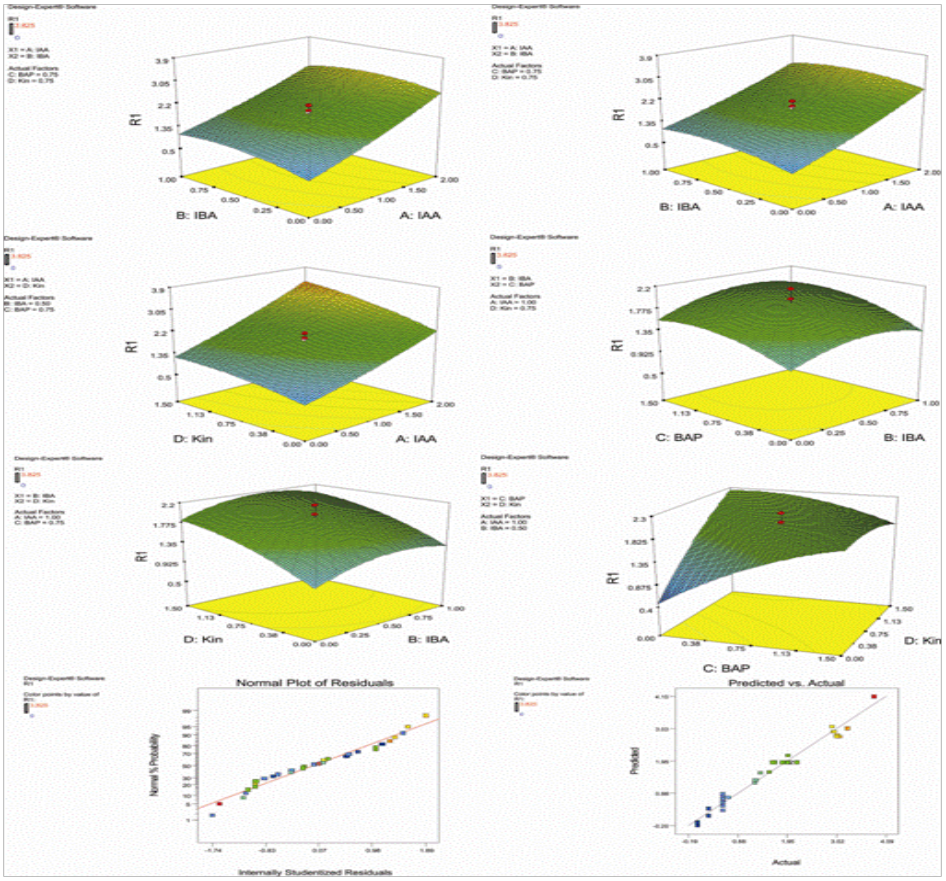

Fig. 1: Interaction of different factor with respect for response

Table 3: ANOVA for Response Surface Quadratic Model

Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F
Model	34.82	14	2.48	37.64	< 0.0001
A-IAA	19.76	1	19.76	299.05	< 0.0001
B-IBA	0.33	1	0.33	5.04	0.0401
C-BAP	1.18	1	1.18	17.90	0.0007
D-Kin	3.67	1	3.67	55.58	< 0.0001
AB	0.06	1	0.06	0.92	0.3510
AC	2.50	1	2.50	37.89	< 0.0001
AD	0.34	1	0.34	5.22	0.0373
BC	0.03	1	0.03	0.40	0.5368
BD	0.01	1	0.01	0.10	0.7527
CD	3.89	1	3.89	58.87	< 0.0001
A^2	0.24	1	0.23	3.62	0.0766
B^2	1.43	1	1.43	21.68	0.0003
C^2	0.99	1	0.98	14.95	0.0015
D^2	0.54	1	0.54	8.11	0.0122
Residual	0.99	15	0.07		
Lack of Fit	0.74	10	0.07	1.48	0.3488
Pure Error	0.25	5	0.05		

Table 4: Summary of ANOVA Table

Summary	Values
Std. Dev.	0.257084
Mean	1.533167
R-Squared	0.972323
Adj R-Squared	0.946491
Pred R-Squared	0.9308
Adeq Precision	23.57
C.V. %	16.77
PRESS	4.63

Antioxidant scavenging properties of *Desmodium gangeticum*

Superoxide scavenging activity of both intact plant and the callus of *Desmodium gangeticum* showed at 66µg/µl. Hydroxyl scavenging showed IC_{50} of 118 µg/µl in callus and 98 µg/µl in the intact plant. The inhibition of lipid peroxides was found to be 100 µg/µl in intact plant and 118 µg/µl in callus. Nitric oxide generated from sodium nitro prusside was found to be 116 µg/µl but the callus produced no activity.

DISCUSSION

Many plants species that provide medicinal herbs have been scientifically evaluated for the possible medical applications [17, 18]. It has also been mentioned that natural habitat for medicinal plant are disappearing fast and together with the environmental and geopolitical instabilities and that is the becoming increasingly difficult to acquire plant-derived compounds[19]. Micropropogation in plants as an alternative conventional method for vegetative propagation attracts more attention and should have provided new means for the commercial processing of even rare or endangered plant chemicals they provide. Krishnasamy Karthikeyan et al., in 2012 explained its antimicrobial activity against many Gram negative bacteria which suggest that positive action of the compound which was separated using methanol [20].

In the present investigation it was observed that when explants were cultured on three different basal mediums induction of callus but with wide range of variation in percentage of callus formation. MS medium produced the maximum percentage of the callus. When the callus were cultured in MS medium with different concentration of IAA in combination with BAP and Kinetin, the formed more concentration of callus. This medium containing BA 0.5 mg/L and IAA 1mg/L induced more calli. It was observed that IBA 1 mg/L produced calli was in lower amount [21, 22, 23]. Differentiation of shoot is achieved when calli were subcultured on MS media with 0.5 Kinetin mg/L and 1.5 mg/L of produced the shoot. But both alone produced to fail any shoot. The explant cultured in IAA inducted or produced roots. Gamborg medium supplemented with 0.5 mg/L of IAA also produced root. In the antioxidant studies of 70% methanol extract of both the calli and intact plant was done. It was found that the in vitro grown plant produced antioxidant property but lesser than the intact plant. The morphological differentiation is apt for the production of secondary metabolites. From this study it shows that the antioxidant activity of the plant will be more in the intact plant when compared with that of the callus [24, 25, 26, 27]. The polynomial equation was derivative and was solved using inverse matrix method to obtain optimum concentration of media content and predicted response in terms of callus formation. The optimal concentrations of media content for callus production using *Desmodium gangeticum* has been given in Table 3. The predicted response using the regression coefficients in coded units (shown in Table 4) was found to be 3.09 whereas the observed response using optimized media was found to be 3.825. Superoxide scavenging activity was more in callus compared with intact plant tissues [28, 29, 30, 31, 32].

CONFLICT OF INTERESTS

Declared None

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