ORIGINAL ARTICLE

Application of Response Surface Methodology for Combination of Herbal Extracts Against Antioxidant and Antipsoriatic Activity

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Abstract

Aim: The present paper modeled combination of three herbal extracts, extract from the seed of *Carica papaya*, lyophilized water of *Cocos nucifera*, and extract from the stem of *Ichnocarpus frutescens* to evaluate their free radical scavenging activity, using Box-Behnken design response surface methodology (BBD-RSM), a statistical technique designed to optimize compositions that we applied in a novel manner to design combinations of herbal extracts for better antioxidant and antipsoriatic activity. **Material and Methods:** A BBD-RSM design using three factors and three levels were selected to optimize the ratio of three herbal extracts. Effect of three independent factors, that is, dry weight of *C. papaya* seed extract (ethanolic), dry weight of lyophilized coconut water, and dry weight of *Ichnocarpus* extract (hydroalcoholic) was studied on two dependent responses, that is, superoxide dismutase and 2,2-diphenyl-1-picrylhydrazyl activity. **Results and Discussion:** Combining herbal extracts in optimum ratio have shown synergistic potential against oxidative stress and for free radical scavenging activity. Combination therapy has been shown to be effective in preventing many disorders as compared to their individual potential for cure. The analysis of variance was performed to evaluate the significant differences between the independent variables. BBD-RSM allows for extrapolation of data from three or more compounds in variable ratio combinations. **Conclusion:** It has been proved that the optimized herbal extracts in a proper amount have significant potential against antioxidant and antipsoriatic activity.

Key words: Antioxidant, antipsoriatic, HaCaT cells, keratinocytes

INTRODUCTION

Skin is the largest exposed organ of human body and is easily affected for various allergic and immunological reactions. Many skin disorders, such as dermatitis, angioedema, urticaria, and psoriasis, are immune-mediated chronic disorders which are inflammatory and proliferative in nature.[1,2] Psoriasis is an immune-mediated skin disorder manifested as focal formation of scaly and inflamed plaques derived from massive proliferation of keratinocytes. Traditional herbal medicines employ many different plants for the folk remedy of various dermatological conditions. In present work, the ratio of three herbal extracts was optimized using Box-Behnken design response surface methodology (BBD-RSM) design to achieve a better antioxidant effect. The BBD is one of the most efficient designs of response surface experimental methodology to study the effect of dependent variables on responses for exploring quadratic response surfaces and the second-order polynomial model.[3] The Box-Behnken model is mainly used to hit the target with reduced variability in experiments, reduced amount of waste, increased production yield, and represents various opportunities for extensive financial gain.[4,5] On the basis on extensive review, the three plants selected for the current study were seeds of *Carica papaya*, lyophilized *Cocos nucifera* water, and stem of *Ichnocarpus frutescens*.[6,7] Thus, the study was planned to access the combined effect of *C. papaya* seed extract, lyophilized water of *C. nucifera*, and *I. frutescens* stem extract on antioxidant and antipsoriatic property.

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MATERIAL AND METHODS

Chemicals

All the chemicals and solvents were purchased from CDS, New Delhi, India. Superoxide dismutase (SOD) assay kit (Cat #19160) and lipopolysaccharides were obtained from Sigma-Aldrich. Dulbecco's Modified Eagle's Medium was purchased from Gibco, Invitrogen BioServices India Pvt. Ltd., Bengaluru, India. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was procured from Sigma-Aldrich, New Delhi, India. Sterile double-distilled water was used throughout the experiment. HaCaT cells were arranged from the Central Drug Research Institute (CDRI), Lucknow, India.

Plant materials and preparation of extracts

The plants were collected from Sikandrabad and Dadri region of Uttar Pradesh, India. These plants were morphologically evaluated and authenticated by the National Institute of Science Communication and Information Resources located at New Delhi. To prepare ethanolic extract of seeds of *C. papaya* air and shade dried, *C. papaya* seeds were macerated. Twenty grams of powdered seeds were accurately weighed which was soaked in 100 mL ethanol for 5 days in closed container at room temperature with occasional shaking. The extract was then strained and the process was repeated for twice with fresh solvent. The last residue of extract was pressed out of the plant particles using a mechanical press and filtered with Whatman filter paper 42.[8] The filtered ethanol extract was then concentrated under vacuum and evaporated to dryness at 45°C. The extract solution was then stored in the refrigerator. The processing of freeze-drying of *C. nucifera* water was done by temperature and time variation with the temperature programming with SPC freeze dryer model. Processing was done by pre-freezing at −20°C for 4 h with first drying step at −20°C for 4 h, second drying step at −10°C for 7 h, and third drying step at 0°C for 6 h followed by heat up to 30°C. Extracts of *I. frutescens* stem were obtained by successive soxhletion. Plant stem was dried and chopped into powder, thoroughly rinsed in deionized water for a few hours and oven-dried at 45°C for 3 days to obtain a constant weight. The dried plant material was then blended into fine powder. Fifty grams of this powder were weighed and extracted with 250 ml of 70% ethanol using Soxhlet extractor. This extraction procedure was done for 24 h for each solvent type.

Optimization of ratios of herbal extracts by BBD-RSM

BBD for optimization of ratios of herbal extracts

Design-Expert® software version 11.0.4.0 was used to develop and study the influence of three independent parameters, namely, dry weight of *C. papaya* seed extract, dry weight of lyophilized coconut water, and dry weight of *Ichnocarpus* extract on two dependent variables, namely, SOD and 2,2-diphenyl-1-picrylhydrazyl (DPPH).[9] The independent factors and the dependent variables are listed in Table 1.

The Box-Behnken three factors and three levels complete design consisted of 17 experimental runs with five central points and were performed in triplicate. The design of experiment (DOE) was applied to maximize the efficiency of these extracts to minimize number of trials and to explore the quadratic response surfaces. The polynomial equation which was generated by the experimental design is as follows:

$$Y= b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^3$$

Where Y is the independent variable, b_0 is the intercept, and b_1, b_2, and b_3 are regression coefficients which were calculated from the experimental values of independent variables and dependent variables. Analysis of variance (ANOVA) identifies the significant independent factors which may affect the dependent factors and fitness of model.[10] All the batches of various herbals were evaluated statistically ($P < 0.05$).

Assessment of antioxidant activity

All the developed runs were assessed for their antioxidant activity by SOD and DPPH methods.

SOD enzymatic assay

The antioxidant activity of the superoxide radicals was assessed by the SOD enzymatic assay. It was evaluated using water-soluble tetrazolium-1 reagent, an indicator dye; superoxide radicals were produced by the xanthine-xanthine oxidase (0.25 U) reaction. The hydrogen peroxide was used in the assay which was 2 μM concentration, which is considered to be relevant at physiological level.[11]

Table 1: Level of variables in Box-Behnken design

Independent variables	Low (–1)	Medium (0)	High (+1)
X_1-Dry weight of *Carica papaya* seed (ethanolic extract) (g)	5	3	1
X_2-Dry weight of lyophilized coconut water (g)	1	0.75	0.5
X_3-Dry weight of *Ichnocarpus* extract (g)	5	3	1
Dependent variables		Constraints	
R_1-SOD (%)		Maximum	
R_2-DPPH (%)		Maximum	

SOD: Superoxide dismutase, DPPH: 2,2-diphenyl-1-picrylhydrazyl

DPPH assay

Extracts (as per designed runs) were taken in separate test tubes. The volume of each test tube was kept constant to 100 µL using methanol. Five milliliters of 0.1 mM methanolic solution of DPPH were then added to each test tubes and shaken vigorously. The test tubes were allowed to stand for 25 min at 25°C. The control was also prepared without extract and methanol was used for the baseline correction. Changes in the absorbance of each sample were measured at a wavelength of 517 nm. The combined concentration of extract essential to decrease the initial concentration of DPPH by 50% (IC_{50}) was estimated.[12]

Antipsoriatic activity

Cell culture of HaCaT human keratinocytes

HaCaT human keratinocyte cell line was obtained from CDRI, Lucknow, India and cells were cultured in a flask having culture medium in 5% CO_2 at 37°C. At 70–80% cell confluency, these culture media were aspirated out and these cells were washed twice with calcium and magnesium free phosphate buffer saline.

Evaluation of antipsoriatic activity of extracts

The antipsoriatic activity of optimized extracts ratio was evaluated by an *in vitro* method using cultured HaCaT cell line and viability of cells was determined by the sulforhodamine B (SRB) assay. HaCaT cell lines were seeded in 96 well microtiter plates at a density of 10,000 cells per well and incubated at 37°C for 24h. Control cells were also cultured in a fresh medium containing no extract. Acitretin and methotrexate anticancer drugs were used as positive controls. HaCaT Cell lines were treated in triplicate with eight concentrations of each of methotrexate ranging from 0 to 60µM, acitretin ranging from 0 to 100µM, and optimized ratio of herbal extract ranging from 0 to 1000µg/mL. SRB colorimetric assay was performed to evaluate the cell viability.[13,14] The cells were incubated for 24 h and then fixed with 100µL of cold 10% (w/v) trichloroacetic acid at 4°C for 1h. These plates having fixed cells were washed 4 times and after drying stained with 100µL of 0.057% (w/v) sulforhodamine dissolved in 1% acetic acid for 30min. These plates were finally rinsed 3 times with 1% (v/v) acetic acid to remove the free dye. The protein-bound dye was then solubilized with 250µL of 10mM Tris base solution at pH 10. The absorbance was measured at 510 nm using a microplate reader. The growth inhibition percent was calculated using the following equation.[15,16]

$$\%\,\text{Cell grwoth} = \frac{\text{Mean OD of sample} - \text{Mean OD at 0 day}}{\text{Mean OD of control} - \text{Mean OD at 0 day}} \times 100$$

$$\%\,\text{Growth inhibition} = 100 - \%\,\text{cell growth.}$$

RESULTS

Model fitting and experimental design

A total 17 runs with five central points, three levels, and three factors were designed using three independent variables which were dry weight of *C. papaya* seed extract, dry weight of lyophilized water, and dry weight of *Ichnocarpus* extract to study their effect on two dependent variables which were SOD and DPPH.[17,18] The replication of central point reflects the result of experimental error. Except the three independent variables, all other process parameters were maintained constant. A total of 17 runs as mentioned in Table 2 were formulated as a gel and analyzed for their response on SOD and DPPH. Response results of all the runs are listed in Table 2. Fitting of model was done with sequential model sum of squares and by model summary statistics.

It was observed that within linear models, interactive models, and quadratic models for SOD scavenging activity and for DPPH scavenging activity, the quadratic model was found to be significant with $P < 0.01$.

The polynomial quadratic equation based on the analysis of Design-Expert 10® was generated by the software and the quantification of the effect of independent variables on responses was done with the help of this equation.

$$R_1 = 18.062 - 1.56 \times X_1 + 81 \times X_2 + 1.12 \times X_3 + 9.5 \times X_1.X_2 + 1.06 \times X_1.X_3 - 3.0 \times X_2.X_3 - 0.93 \times (X_1)^2 - 64 \times (X_2)^2 - 0.25(X_3)^2$$

$$R_2 = 85.87 - 8.5 \times X_1 - 41.00 \times X_2 - 10.06 \times X_3 + 7.0 \times X_1.X_2 + 0.68 \times X_1.X_3 - 3.0 \times X_2.X_3 - 2.3 \times (X_1)^2 - 0.68 \times (X_2)^2 + 3.38(X_3)^2$$

The results obtained were further analyzed using ANOVA available in the Design-Expert software and the model was considered to be significant with F value of 12.81 and 14.88, respectively, for R_1 (SOD) and R_2 (DPPH). The Graphs obtained for the response SOD scavenging activity and DPPH scavenging activity are depicted in Figure 1.

The response surface contours graphs and three-dimensional graphs were also constructed using the software. These graphs were used to analyze the interaction of all independent variables on both responses by placing one of the variables at constant level.

Numerical optimization

Numerical optimizations of the ratios of herbal extracts were performed by Design-Expert 11® software and both

Table 2: Response results of all dependent variable

Std.	Run	Factor 1	Factor 2	Factor 3	Response 1 (R_1)	Response 2 (R_2)
		X_1: Dry weight of *Carica papaya* (g)	X_2: Lyophilized coconut water (g)	X_3: Dry weight of *Ichnocarpus* extract (g)	SOD scavenging activity (%)	DDPH scavenging activity (%)
15	1	3	0.75	3	55	51
8	2	5	0.75	5	62	67
16	3	3	0.75	3	55	51
4	4	5	1	3	61	63
12	5	3	1	5	49	62
5	6	1	0.75	1	47	52
10	7	3	1	1	49	53
2	8	5	0.5	3	45	51
7	9	1	0.75	5	40	49
17	10	3	0.75	3	55	51
13	11	3	0.75	3	55	51
11	12	3	0.5	5	54	52
6	13	5	0.75	1	52	59
3	14	1	1	3	40	50
14	15	3	0.75	3	55	51
9	16	3	0.5	1	48	53
1	17	1	0.5	3	43	52

SOD: Superoxide dismutase, DPPH: 2,2-diphenyl-1-picrylhydrazyl

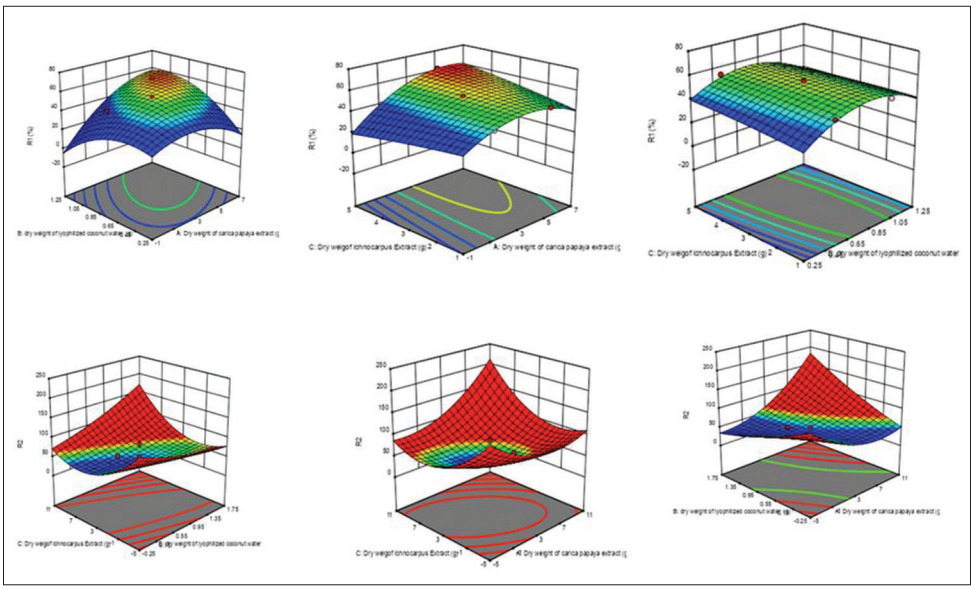

Figure 1: Response surface three-dimensional (3D) graph for superoxide dismutase (R_1) and 2,2-diphenyl-1-picrylhydrazyl (R_2), *(A) response surface 3D plot showing the effect of dry weight of *Carica papaya* seed extract and dry weight of lyophilized coconut water on R_1, (B) response surface 3D plot showing the effect of dry weight of lyophilized coconut water and dry weight of *Ichnocarpus* extract on R_1, (C) response surface 3D plot showing the effect of dry weight of *C. papaya* seed extract and dry weight of *Ichnocarpus* extract on R_2, (D) response surface 3D plot showing effect of dry weight of lyophilized coconut water and dry weight of *Ichnocarpus* extract on R_2

the response variables were optimized using RSM-BBD modeling. The optimized ratio was then selected and formulated as a get with 2.23 g of dry weight of *C. papaya* extract, 0.98 g of lyophilized coconut water, and 2.9 g of dry weight of *Ichnocarpus* extract with desirability 0.77. The optimized ratio of extracts was further evaluated for both the response that is SOD and DPPH % scavenging activity. The observed responses of these variables were again compared

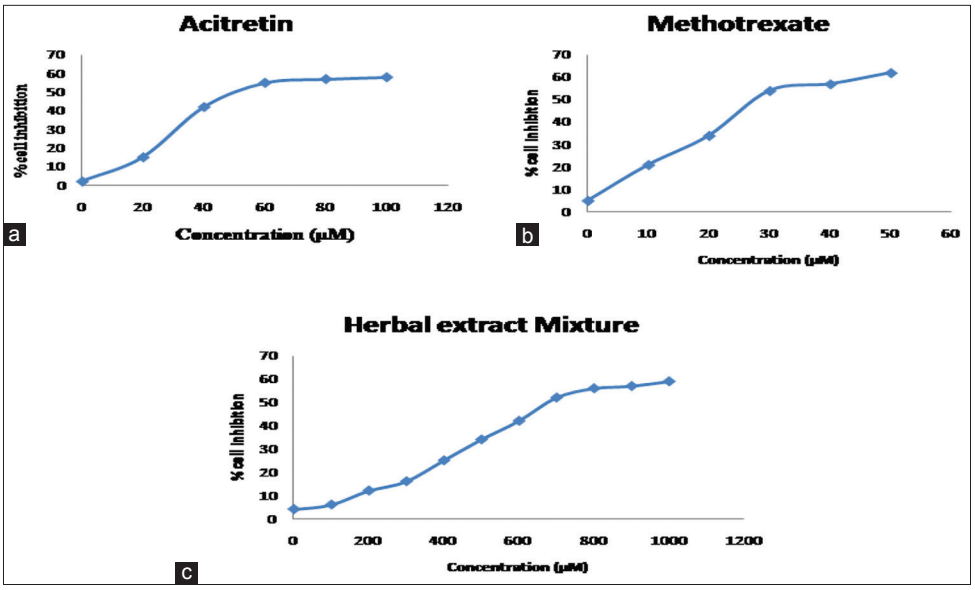

Figure 2: % HaCaT cell growth inhibition, *(a) % cell growth inhibition of acitretin, (b) % cell growth inhibition of methotrexate, (c) % cell growth inhibition of optimized herbal extract

with predicted responses to calculate % error which was found to be within the range.

Antipsoriatic activity on HaCaT cell

The selected ratio of all three herbal extracts was further used to evaluate antipsoriatic activity *in vitro*. HaCaT cells were treated with different concentrations of optimized batch of extract. The cells growth and its viability were evaluated by SRB assay. The results of SRB assay are depicted in Figure 2. The IC_{50} value of extract mixture was found to be 656 ± 45 µM. Acitretin and methotrexate drugs were used as a positive control and they undoubtedly showed effective cell growth inhibition at substantially low concentrations.

DISCUSSION

Seventeen runs were developed using three different ratios of herbal extract as per BBD-RSM by Design-Expert 11® software. Quadratic model was found to be the best fit model among all after model fitting with software. The software predicted R^2 value of 0.084 and signal to noise value of 13.49 for SOD activity and R^2 value of 0.955 and signal to noise ratio of 12.7 for DPPH activity. The value of $F < 0.05$ indicated that the model terms were significant. A good correlation was reflected between predicted and observed values with a higher value of R^2.

Factors with positive values in the above generated quadratic equation indicated that the response increases with an increase in factor value and negative value indicate an inverse relationship.[19,20]

The potential of these herbal extracts with antipsoriatic activity was investigated by HaCaT cell and the findings suggested that these combinations at optimum concentration ratio have significant potential against antipsoriatic activity. Furthermore, studies required and should be performed to provide insights into an understanding of processes at molecular, biochemical, and cellular levels.[21]

CONCLUSION

In this research, combination of three herbal extracts, extract from the seed of *Carica papaya*, lyophilized water of *Cocus nucifera*, and extract from the stem of *Ichnocarpus frutiscens* were optimized by applying RSM. The statistical analysis exhibited a significantly good fit for the model that could be used to navigate the experimental design space. Two different parameters for antioxidant effect i.e., SOD and DPPH were evaluated. The regression model for their preparation was found to be significant and the coefficient of determination (R^2) value of 0.084 and signal to noise value of 13.49 for SOD activity and (R^2) value of 0.955 and signal to noise ratio of 12.7 for DPPH activity respectively. Moreover, the potential antioxidant results were further screened for antipsoriatic activity on HaCat Cells. HaCat cells were treated with different concentrations of optimized batch of extract. The cells growth and its viability were evaluated by SRB assay using Acitretin and methotrexate as standards. The IC_{50} value of extract mixture was found to be 656 ± 45 µM, which was significant when compared with standards. It has been observed that the optimized herbal extracts in specified ratios have significant potential against antioxidant and antipsoriatic activity. This study may provide useful tools to prepare herbal based therapy for psoriasis.

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