Research Article

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STUDIES ON THE ANTIMICROBIAL ACTIVITY OF *EVOLVULUS ALSINOIDES*, *MURRAYA KOENIGII*, *LAWSONIA INERMIS*, *HIBISCUS ROSA-SINENSIS*, *AZADIRACHTA INDICA* AGAINST *MALASSEZIA FURFUR* USING FRACTIONAL FACTORIAL DESIGN

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ABSTRACT

Alopecia is a dermatological disorder with psychosocial implications on patients with hair loss. *Malassezia* sp, sebaceous secretions and individual sensitivity are the major etiological factors that lead to hair loss. *Malassezia* species are normally found in areas rich in sebaceous gland as they are lipid dependent. *Malassezia furfur* is believed to be the causative agent in various dermatological disorders. Medicinal plants have been utilized from ancient times in healthcare systems. The present investigation deals with the optimization of herbal formulation prepared using *Evolvulus alsinoides*, *Murraya koenigii*, *Lawsonia inermis*, *Hibiscus rosa-sinensis*, *Azadirachta indica*. The Disc Diffusion Assay showed promising antifungal activity towards *Malassezia furfur*. Response Surface Methodology studies with a 25 fractional factorial experimental design showed that 0.9 mg of *Evolvulus alsinoides*, 0.41 mg of *Lawsonia inermis*, 1.27 mg of *Hibiscus rosa- sinensis*, 1.61 mg of *Azadirachta indica* and 1.80 mg of *Murraya koenigii* would be an optimum concentration with promising application in its antifungal activity against *Malassezia*.

Keywords: Disc diffusion, Response Surface Methodology, Central Composite Design, Antidandruff activity, Minimum Inhibitory Concentration, Zone of Inhibition

INTRODUCTION

Hair loss (alopecia) is a distressing dermatological disorder common throughout the world. Dandruff is a scalp disorder characterized by visible flakes induced by rapid turnover of scalp cells. The etiological factors that results in dandruff are *Malassezia* fungi, sebaceous secretion and individual sensitivity[1,2]. *Malassezia* a lipophilic, dimorphic fungus is ubiquitous skin resident and an opportunistic pathogen causative of dandruff, Pityriasis Versicolar, Seborrheic dermatitis, etc[3].

India is a repository of medicinal plants. The utility of medicinal herbs is moving from fringe to mainstream since most people endeavour to opt for herbal medicines over allopathic compounds. Moreover, these medicinal plants are devoid of side-effects and cost effective.

Evolvulus alsinoides (dwarf morning glory) has pharmacological activities such as anti-oxidant[4], gastro protective, antibacterial, anti-ulcer, cytoprotective, immunomodulatory, adaptogenic and antiamnesic, and anti-inflammatory activity[5]. All parts of *Azadirachta indica* (Neem) have been used medicinally for centuries. The earliest Sanskrit medical writings refer to the benefits of Neem's fruit, seeds, oil, leaves, roots and bark. In recent studies, utility of neem is being emphasized in pharmaceutical and cosmetic industries[6]. In India the herbal products in the market intended for hair growth include the extract of various parts of *Hibiscus rosa-sinensis*, a glabrous shrub[7]. According to traditional texts it is well accepted that the leaves and flowers of *Hibiscus rosa-sinensis* (shoe flower) have hair growth promoting[8]. Powdered leaves of *Lawsonia inermis* (henna) are used both as a cosmetic dye and as remedy for boils, wounds and some mycotic infections9. The leaves of *Murraya koenigii* (Curry) are used as anti-diabetic[10], antioxidant[11] and antifungal[12].

Response surface Methodology is a collection of statistical tools and techniques for constructing and exploring a functional relationship between a response variable and a set of design variables[13]. The most extensive application of response surface methodology can be found in the industrial world, in situations where several input variables influence some performance measure, called the response, in a way that is difficult or impossible to describe with a rigorous mathematical formulation. In these situations, it might be possible to derive an expression for the performance measure based on the response values obtained from experiments at some particular combination of the input variables[14].

The present work deals with the optimization and development of polyherbal formulation which inhibits the growth of *Malassezia furfur*. The composition of herbs was optimized to obtain maximum antifungal activity.

MATERIALS AND METHODS

Sample Collection and Preparation

Fresh plant materials were collected washed thoroughly under running tap water and shade dried individually. The dried plants were grinded into coarse powder. The leaves of the plants were subjected to ethanol extraction using Soxhlet Apparatus[15]. The filtered extracts were concentrated using Rotary evaporator and freeze dried.

Microorganism

Pure culture of *Malassezia furfur* (MTCC: 1374) was obtained from Institute of Microbial Technology, Chandigarh, India. The culture was maintained in Sabouraud's agar supplemented with olive oil[16]. The plates were incubated at 37°C for 5-7 days.

Evaluation of Antimicrobial Activity of the Plant Extracts

Antimicrobial activity was assessed by the agar well diffusion method[17]. The plates were observed for zones of inhibition (indicated by clear zones) which were measured and recorded in

millimeters using a transparent measuring ruler using the tube-dilution technique[18].

Response Surface Methodology

A 25 fractional factorial experimental design with 32 experiments were employed. The second order polynomial coefficients was calculated and analyzed using the 'Design Expert' software (Version 7, State EaseInc., Minneapolis, USA). The optimal values were calculated using the Second order polynomial equation

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum \sum_{i<j} \beta_{ij} x_i x_j + \varepsilon$$

y is the zone of inhibition, β0 is offset term, βi is the coefficient of individual effect, βij is the coefficient of interaction effect and βii is the coefficient of squared effect[19]. The significance of parameters was determined through statistical method. Three dimensional response surface and contour graphs were plotted on the basis of the model equation to reveal the interactions among variables.

Antidandruff Assay

The Sabouraud's agar plates supplemented with olive oil were inoculated with *Malassezia furfur* using spread plate technique using 50µl of respective concentration as obtained from Central Composite Design. The plates were incubated at 37ºC for 7 days. The radius for the zone of inhibition was measured. Experiments were carried out with three replicates per treatment and the data obtained was statistically analyzed[20].

Table 1: Experimental range and levels of independent variables

Variables	Symbols coded	Levels	
		Low (-) mg	High (+) mg
Evolvulus alsinoides	A	0.2	0.5
Lawsonia inermis	B	0.5	1
Hibiscus rosa-sinensis	C	1	1.5
Azadirachta indica	D	0.5	2
Murraya koenigii	E	0.5	2

Table 2: Experimental Design for Central Composite Design and Corresponding Zone of Inhibition

Run	A	B	C	D	E	Zone of Inhibition(mm)
1	-	-	+	+	+	12.5
2	+	+	+	-	-	3.6
3	-	+	-	+	+	13.2
4	0	0	0	0	0	9.7
5	-	0	0	0	0	9.3
6	-	-	+	-	-	1.5
7	0	0	0	0	0	9.7
8	-	+	+	+	-	3.2
9	0	-	0	0	0	8.3
10	+	-	-	+	+	13.0
11	+	0	0	0	0	11.6
12	+	+	-	+	-	2.8
13	0	0	0	0	0	9.7
14	0	0	0	-	0	7.5
15	-	-	-	+	-	2.9
16	0	0	-	0	0	10.8
17	0	0	0	0	+	13.8
18	-	-	-	-	+	4.3
19	+	+	-	-	+	3.7
20	+	-	-	-	-	2.3
21	+	-	+	-	+	6.2
22	0	+	0	0	0	13.4
23	-	+	-	-	-	2.5
24	+	+	+	+	+	13.1
25	0	0	+	0	0	10.3
26	-	+	+	-	+	6.1
27	0	0	0	0	0	9.7
28	0	0	0	0	0	9.7
29	0	0	0	+	0	13.4
30	+	-	+	+	-	3.8
31	0	0	0	0	0	9.7
32	0	0	0	0	-	7.9

Table 3: Significant sources and corresponding F and P value

Significant Source	Mean Square	F-Value	p-value Prob > F
DD	89.78	35.51945	< 0.0001
EE	170.5089	67.45803	< 0.0001
DE	51.48063	20.36716	0.0009

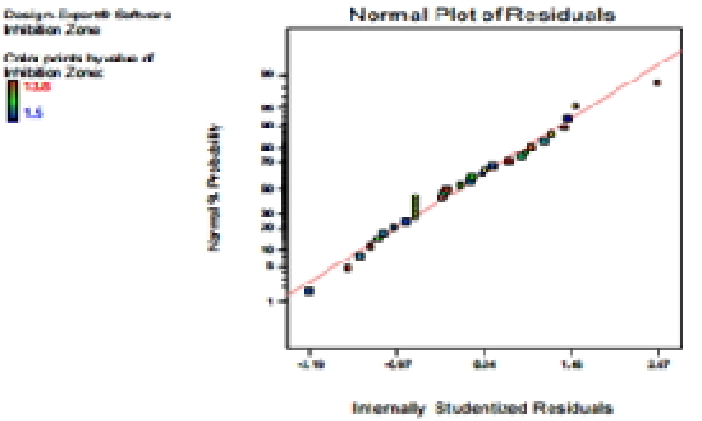

Figure 1: Normal plot of residuals in Response Surface method

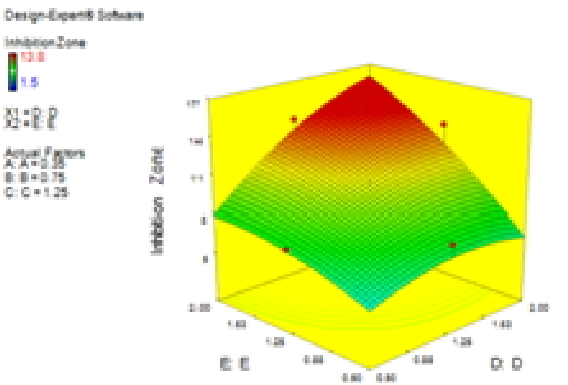

Figure 2: Response Surface plot showing the effect of *Azadirachta indica* and *Murraya koenigii* on *Malassezia furfur*

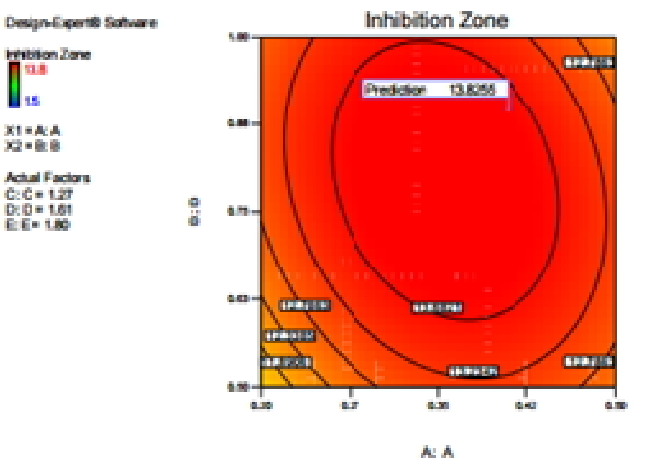

Figure 3: Response and contour plot for *Evolvulus alsinoides* and *Lawsonia inermis* on *Malassezia furfur*

RESULTS AND DISCUSSION

Antidandruff activity studied for ethanolic extracts of *Evolvulus alsinoides*, *Lawsonia inermis*, *Azadirachta indica*, *Hibiscus rosa- sinensis* and *Murraya koenigii*. All the extracts have shown reasonable activity against *M. furfur* [21]. In the present work the experiment was designed with 5 variables viz. *Evolvulus alsinoides*, *Lawsonia inermis*, *Azadirachta indica*, *Hibiscus rosa- sinensis* and *Murraya koenigii* as shown in Table 1. The experiments were planned to obtain a quadratic model consisting on 25 trials. Assigning zone of inhibition as response, experiments were performed using the design obtained from the fractional factorial face-centered Central Composite Design. Table 2 represents the experimental design for Central Composite Design and Corresponding zone of inhibition.

The Statistical analysis of quadratic regression model demonstrates that the model is significant. The regression equation and determination coefficient R^2 were evaluated to test the fit of the model. The model presented a high determination coefficient (R^2=0.9435) explaining 94% of the variability in the response. The adjusted R^2 value (Adj R^2 = 0.840907) is in reasonable agreement with the experimental values. An adequate precision greater than 4 is desirable. An adequate precision of 9.72 indicates an adequate signal for the signal-noise ratio. The value of the adjusted determination coefficient is also high to indicate a high significance of the model[22]. The Model F-value of 9.19 implies that the model is significant. There is only a 0.03% chance that a "Model F-value" this large could occur due to noise. The value of coefficient of variation (CV=19.62%) indicates a better precision and reliability of the experiments carried out.

The coefficients of regression equation were calculated using Design Expert and the following regression equation was obtained.

$$Y = 10.77 + 0.26A + 0.38B + 0.27C + 0.23D + 3.08E - 1.13A2 - 0.73B2 - 1.03C2 - 1.13D2 - 0.73E2 - 0.37AB + 0.28AC - 0.03AD - 0.16AE + 0.14BC -0.094BD - 0.09BE - 0.24CD + 0.13CE + 1.79DE$$

Where Y is the response that is the zone of inhibition and A, B, C, D and E are coded values of the test variables *Evolvulus alsinoides*, *Lawsonia inermis*, *Azadirachta indica*, *Hibiscus rosa- sinensis* and *Murraya koenigii* respectively.

Figure 1 shows the graphical plot of internally studentized residuals against normal % probability. From the figure it is inferred that the experimentally obtained response is in agreement with the predicted response. The significance of each coefficient was determined by f-values and P-values which are listed in Table 3. The larger the magnitude of f-test and smaller the p-value indicates the high significance of the corresponding coefficient [23]. The values of Prob > F less than 0.0500 indicate model terms are significant. It can be seen that D, E, DE are significant model terms. From Table 3 it is inferred that the variable with largest effect was DE. Furthermore, the combined effect of *Azadirachta indica* and *Murraya koenigii* are more significant.

The response and contour plots are the graphical representations of the regression equation. The objective of Response surface plots is to find the optimum values of the significant parameters effectively such that the response is maximized. Each contour curve indicates an infinite number of combinations of two test variables with variables maintained at their respective zero level. The smallest ellipse in the contour diagram indicates the maximum predicted value. Elliptical contours are obtained when there is a perfect interaction between the independent variables.

The contour plots are obtained by plotting the response (Zone of Inhibition) on the Z axis against any two variables while keeping other variables at zero level. These plots are created in order to analyse the Response surface. Figure II shows a response surface plot developed as a function of *Azadirachta indica* and *Murraya koenigii*. It was reported that Response Surface plots provide a method to predict the inhibitory action of the five medicinal herbs mentioned in the work and contour plots help in the identification of type of interactions. The axes of the contour plot indicate the experimental values and the axes is termed the response surface. From the figure it is observed that the inhibition of *Malassezia furfur* was more influenced by *Azadirachta indica* and *Murraya koenigii* and the zone of inhibition was increased from 1.5 mm to 13.8 mm. Figure 3 shows the 2 dimensional contour plot as a function of *Evolvulus alsinoides* and *Lawsonia inermis*.

CONCLUSION

The results of this present investigation have given an optimum herbal formulation for the treatment of *M.furfur*. The optimized formulation for the treatment of Dandruff is 0.9 mg of *Evolvulus alsinoides*, 0.41 mg of *Lawsonia inermis*, 1.27 mg of *H.rosa- sinensis*, 1.61 mg of *Azadirachta indica* and 1.80 mg of *Murraya koenigii*. Experimental value and the predicted value from the model were found to be close, which indicates the second order polynomial equation was in good agreement. The use of an experimental design was to reveal the interaction among the herbs that are used as antidandruff agents.

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