

# Optimization of Anti-Inflammatory Activity of *Rauvolfia tetraphylla* L. Crude Extracts Using Response Surface Methodology

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## ABSTRACT

**Background:** The background of this study revolves around *Rauvolfia tetraphylla* and its potential anti-inflammatory properties. To explore the anti-inflammatory activity of crude extracts obtained from the leaf and fruit of *R. tetraphylla*, with the goal of identifying natural compounds that could serve as safer and more effective alternatives to conventional non-steroidal anti-inflammatory drugs. **Objectives:** Aim of the study is to identify the most potent anti-inflammatory activity from *Rauvolfia tetraphylla* and optimizing the synthesis using Response Surface Methodology (RSM). The RSM analysis is to determine the influence of selected independent variables on dependent variables of the anti-inflammatory properties of *R. tetraphylla*, an endangered medicinal shrub. **Materials and Methods:** The anti-inflammatory activity of various crude extracts (hexane, chloroform, ethyl acetate and methanol) derived from the leaf and fruit of *R. tetraphylla*, was investigated using an *in vitro* assay based on denaturation of protein and utilize Response Surface Methodology to optimize the anti-inflammatory activity of the extracts using bovine serum albumin as the test protein. **Results:** The results showed that the ethyl acetate extract of fruit exhibited greatest inhibitory effect. Furthermore, the 3D response surface plot, perturbation plot, and contour plot were successfully obtained, indicating the interdependence of the four factors on the anti-inflammatory activity. By optimizing the parameters, it may be possible to enhance the anti-inflammatory activity, thereby maximizing the plant's therapeutic potential. **Conclusion:** These findings suggest that *R. tetraphylla* leaf and fruit could serve as a potential source of anti-inflammatory agents with better safety and efficacy profiles compared to Non-Steroidal Anti-inflammatory Drugs.

**Keywords:** *Rauvolfia tetraphylla*, Anti-inflammatory, Protein denaturation, RSM, Protein denaturation.

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## INTRODUCTION

The plant *Rauvolfia tetraphylla* is a perennial evergreen shrub belonging to the family Apocynaceae. It reaches a height up to 6 ft. Apocynaceae, also known as the dogbane family, is classified under the order Gentianales.<sup>1</sup> The plant distributed across Mexico, Central America, the West Indies and parts of South America. It has also been introduced and established in various other regions such as India, Australia, Pakistan, Sri Lanka, China, Bhutan, Bangladesh, Indonesia, and Myanmar. In India, it is commonly found in many states including Tamil Nadu, Andhra Pradesh, Karnataka, Kerala, Bihar, Orissa, West Bengal, Madhya Pradesh, Jammu and Kashmir, and Assam.<sup>2,3</sup> The traditional use

of *Rauvolfia* got attention in the past 300 years ago due to the presence of various pharmacological activities.<sup>4</sup> The paste of whole plant is used to treat a skin disease which was reported in Kancheepuram, Tamil Nadu;<sup>5</sup> Bhadrak, Odisha;<sup>6</sup> Ranga Reddy, Telangana;<sup>7</sup> Coimbatore and Ooty, Tamil Nadu.<sup>8</sup> *R. tetraphylla* has been explored for its biological potential through phytochemical analysis,<sup>9,10</sup> antibacterial activity,<sup>11-13</sup> antioxidant activity,<sup>14,15</sup> cytotoxic activity,<sup>16,17</sup> and cardio-protective activity.<sup>18</sup> It is known to possess important medicinal properties such as snake bite, blood pressure, stomach pain, mental disorder, cholera, headache, eye disease, fever, diarrhea, skin diseases, chronic wound, insect bite and dysentery.<sup>19-21</sup> The phytochemical constituents present in *R. tetraphylla* include indole alkaloids such as ajmalicine, yohimbine, reserpine, demethyl serpentine, mitoridine, sarpagine, serpentine, reserpiline, and yohimbine.<sup>22</sup> The methanol extracts of the root, stem and leaves contain reserpine.<sup>23</sup> The ethanol extract of aerial parts contains five indole alkaloids known as rauvotetraphyllines A-E (1-5).<sup>9</sup> Additionally, there are five hybrid monoterpenoid



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indole alkaloids like rauvotetraphyllines F-H (1, 3, 4), 17-epi-rauvotetraphylline F and 21-epi-rauvotetraphylline H.<sup>24</sup> The crude extracts such as hexane, chloroform, acetone and methanol from the leaves contain curan-17-oic acid, 18,19-Secoyohimban and Reserpiline,<sup>25</sup> furthermore, four antipsychotic indole alkaloids reserpiline,  $\alpha$ -yohimbine, isoreserpiline, and 10-methoxy tetrahydroalstonine are reported in leaves.<sup>26</sup> The methanolic extract of the root contains reserpine and ajmalicine.<sup>27</sup>

Injury, infection, or irritation can cause complex reactions in living tissues leading to inflammation, often accompanied by pain and involving increased vascular permeability, protein denaturation, and membrane alteration.<sup>28</sup> The body's nonspecific internal defence system, inflammation, responds to various types of tissue damage caused by accidents, burns, radiation, bacterial or viral invasion. Acute and chronic are the two types of inflammation, with acute inflammation being a short-term process and chronic inflammation being a prolonged process.<sup>29</sup> Inflammatory responses involve an overactive immune system that releases various inflammatory cells such as macrophages, neutrophils, and lymphocytes, and mediators such as vasoactive amines, peptides, arachidonic acid metabolites, proinflammatory cytokines, chemokines, and proteolytic enzymes.<sup>30</sup>

RSM is a powerful set of statistical and mathematical tools used to optimize and improve processes. It is useful in situations where multiple input variables may influence a particular performance measure or quality characteristic, known as the response. It provides a comprehensive framework for improving and optimizing processes.<sup>31</sup> RSM has been widely applied in various fields to explore the relationship between input variables and response. However, there are only a few studies in the literature that have applied RSM to evaluate the anti-inflammatory activity of natural compounds. For example, RSM was employed by Cho *et al.*,<sup>32</sup> to optimize the antioxidant, anti-diabetic, and anti-inflammatory activities of *Ganoderma lucidum*. Similarly, Kaur<sup>33</sup> investigated the impact of RSM-optimized synthesis parameters on the *in vitro* anti-inflammatory activity of iron nanoparticles synthesized using *Ocimum tenuiflorum*. Despite these studies, there is a lack of literature survey focusing on the denaturation of protein and its relationship with the anti-inflammatory activity of natural compounds using RSM. Therefore, the present study aims to fill this gap by exploring the denaturation of protein as an *in vitro* measure of anti-inflammatory activity of leaf and fruit extracts of *R. tetraphylla* and developing a quadratic model using RSM to optimize the anti-inflammatory activity of natural compounds.

## MATERIALS AND METHODS

### Plant Material

The fresh leaves and fruit of *R. tetraphylla* were collected from Top slip, Western Ghats (Tamil Nadu, India).

### Preparation of Extract

The leaves and fruit of *R. tetraphylla* were collected, shade dried and grounded into a fine powder. Extracts were prepared from various organic solvents such as hexane, chloroform, ethyl acetate and methanol by sequential method. Extracts obtained were allowed to dry till constant weight was achieved.

### Characterization

**Drug:** For the present study, nine different extracts of *R. tetraphylla* were used as the drug types, which included STD, RLH, RLC, RLEA, RLM, RFH, RFC, RFEA, and RFM. These extracts were coded as A in the experimental design using RSM. **Concentration:** It was one of the independent variables which are coded as B and ranging from 31.25 to 1000  $\mu\text{g/mL}$ . **Measurement of pH:** The range of pH values considered for the optimization was between 6.5 and 7.0, which were coded as C in the experimental design. **Temperature:** It was considered as one of the independent variables and varied over a range of 25°C to 30°C, which is coded as D.

### Evaluation of *in vitro* Anti-inflammatory Activity

*In vitro* anti-inflammatory activity was evaluated according to the method described by Chandra *et al.*, (2012).<sup>34</sup> A reaction mixture of 5 mL was prepared, consisting of 0.2 mL of bovine serum albumin, 2.8 mL of Phosphate Buffered Saline (PBS, pH 6.4), and 2 mL of varying concentrations (31.25 to 1000  $\mu\text{g/mL}$ ) of different crude extracts. A similar volume of DMSO was used as a control. The mixtures were then incubated at  $(37 \pm 2)^\circ\text{C}$  in a BOD incubator for 15 min, followed by heating at  $70^\circ\text{C}$  for 5 min. After cooling, the absorbance was measured at 660 nm using a UV spectrophotometer (Labman), with vehicle as the blank. Diclofenac sodium was used as a reference drug, and the same procedure was followed for the determination of absorbance at the same final concentration range as the test samples. The percentage inhibition of protein denaturation was calculated using the formula:

$$\text{Percentage inhibition} = 100 \times (V_t / V_c - 1)$$

Where,  $V_t$  is the absorbance of the test sample and  $V_c$  is the absorbance of the control. The inhibition percentage was used as a response for the experimental design of RSM.

### Screening Design to Select Parameter for Optimization

The experimental design of this study was based on RSM, specifically using a quadratic model<sup>35</sup> in design expert software. The independent variables were drug, concentration, pH, and temperature, while the dependent variable was the inhibition percentage. The dataset contains four variables: A (categorical, representing drug types with nine levels), B (numeric, measuring concentration in  $\mu\text{g/mL}$  with a range from 31.25 to 1000.00, mean

328.12, and standard deviation 341.56), C (numeric, indicating pH levels between 6.50 and 7.00, mean 6.75, and standard deviation 0.1724), and D (numeric, measuring temperature in °C between 25.00 and 30.00, mean 27.50, and standard deviation 1.72). The range inhibition percentage was from 2.30% to 306.52%, with an average inhibition rate of 51.40% and a standard deviation of 60.47%. To select the variables and their levels for the optimization design, a quadratic model was performed with drug, concentrations, pH, and temperature analysed as independent variables. The values of the factors and the experimental design were determined. The inhibition percentage was regressed with respect to the treatment conditions using the quadratic model. ANOVA was performed using the reduced quadratic model of the statistical package. In this analysis, we employ Sum of Squares, Degree of Freedom (DOF), mean of square, F value and p-value to explore the significant impact of design factors and their interactions on the response variable. The contour plot analysis was conducted by plotting the Two-Dimensional (2D) representation of the response (% inhibition) against combinations of numerical factors (concentration and pH). Moreover, correlating the obtained data from the statistical analysis was verified by specific model graphs such as 2D contour and 3D-response surface plots.

## RESULTS AND DISCUSSION

ANOVA was conducted to assess the implication of the factors in the study as shown in Table 1. The results revealed that the model had a highly significant effect on the outcome variable, with a sum of squares of 1.783E+05 and a corresponding F-value of 341.92 ( $p < 0.0001$ ) with 18 degrees of freedom. This indicates that the model was able to explain a substantial amount of variance in the data. Further, examination of the individual factors revealed that both A-Drug and AB (interaction between A-Drug and B-Concentration) were highly substantial with  $p$ -values less than 0.0001. B-Concentration and B<sup>2</sup> (squared value of B-Concentration) also showed significant effects on the outcome variable, with  $p$ -values less than 0.0001. In contrast, the Residual, which represents the inexplicable variation in the data, had a sum of squares of 1014.12 and 35 degrees of freedom, resulting in a mean square of 28.97. The ANOVA results provide strong indications for the significance of the model and its individual factors in influencing the outcome variable. This suggests that the

factors considered in the study, namely A-Drug, B-Concentration, AB, and B<sup>2</sup>, play a significant role in the observed results.

The fit statistics of the model were assessed to evaluate its goodness of fit and predictive accuracy. The standard deviation of the model was found to be 5.38, indicating that the observed data points were relatively close to the mean. The R<sup>2</sup> value, which measures the proportion of the variance in the dependent variable explained by the model, was determined to be 0.9943. This suggests that the model was able to account for approximately 99.43% of the variability in the data, indicating a high degree.

The adjusted R<sup>2</sup> value, which takes into accounts the quantity of interpreters in the model, was calculated to be 0.9914. The adjusted R<sup>2</sup> value addresses the potential issue of overfitting in the model and offers a more conservative assessment of its ability to explain the variation in the data. The predicted R<sup>2</sup> value, which estimates the model's ability to accurately predict future data, was found to be 0.9706. This indicates that the model has a high predictive accuracy and can likely be used to make reliable predictions in similar scenarios. The adequacy precision, which balances the signal-to-noise ratio in the model, was determined to be 95.4751. This indicates that the model is intelligent to reliably predicting the response variable with a high level of precision. The fit statistics suggest that the model is highly reliable; exhibiting a high degree of goodness of fit, predictive accuracy, and precision, indicating that it is a valid and robust for the analysed data.

The coefficients in terms of coded factors (sum contrasts) for the given model, in which the intercept is 83.34 with a standard error of 1.50 and a 95% Confidence Interval (CI) ranging from 80.30 to 86.38. The coefficients for coded factors A[1] through A[8] are -16.37, 15.32, 2.20, -26.27, -35.20, -31.04, 36.08, and 86.07, respectively. The concentration for factor B- is 64.72 with a standard error of 1.05 and a 95% CI ranging from 62.59 to 66.85, and a Variance Inflation Factor (VIF) of 1.00. The coefficients for the interaction terms A[1]B through A[8]B are -17.34, 13.89, -3.99, -25.76, -28.39, -22.77, 38.82, and 81.06, respectively. The coefficient for the squared term B<sup>2</sup> is -12.70 with a standard error of 1.96 and a 95% CI ranging from -16.68 to -8.72, with a VIF of 1.00.

The report provides information on various metrics for each data point in the dataset such as actual values, predicted values,

**Table 1: ANOVA for Reduced Quadratic model.**

| Source   | Sum of Squares | DOF       | Mean Square    | F-value | p-value         | Significant |
|----------|----------------|-----------|----------------|---------|-----------------|-------------|
| Model    | 1.783E+05      | 18        | 9907.13        | 341.92  | <0.0001         | A-Drug      |
| 31663.00 | 8              | 3957.87   | 136.60         | <0.0001 | B-Concentration | 1.108E+05   |
| 1        | 1.108E+05      | 3823.50   | <0.0001        | AB      | 34665.04        | 8           |
| 4333.13  | 149.55         | <0.0001   | B <sup>2</sup> | 1215.26 | 1               | 1215.26     |
| 41.94    | <0.0001        | Residual  | 1014.12        | 35      | 28.97           |             |
|          | Cor Total      | 1.793E+05 | 53             |         |                 |             |

**Table 2: Report of Response Surface Methodology.**

| Run Order | Actual Value | Predicted Value | Residual | Leverage             | Internally Studentized Residuals | Externally Studentized Residuals | Cook's Distance      | Influence on Fitted Value DFFIT S | Standard Order |
|-----------|--------------|-----------------|----------|----------------------|----------------------------------|----------------------------------|----------------------|-----------------------------------|----------------|
| 1         | 4.54         | 6.89            | -2.35    | 0.312                | -0.526                           | -0.521                           | 0.007                | -0.350                            | 1              |
| 2         | 9.09         | 11.53           | -2.44    | 0.277                | -0.533                           | -0.528                           | 0.006                | -0.326                            | 2              |
| 3         | 21.59        | 20.50           | 1.09     | 0.227                | 0.231                            | 0.227                            | 0.001                | 0.123                             | 3              |
| 4         | 40.90        | 37.16           | 3.74     | 0.191                | 0.771                            | 0.767                            | 0.007                | 0.372                             | 4              |
| 5         | 67.04        | 65.42           | 1.62     | 0.263                | 0.350                            | 0.345                            | 0.002                | 0.206                             | 5              |
| 6         | 100.00       | 101.65          | -1.65    | 0.842 <sup>(1)</sup> | -0.773                           | -0.768                           | 0.168                | -1.776                            | 6              |
| 7         | 7.21         | 7.35            | -0.1438  | 0.312                | -0.032                           | -0.032                           | 0.000                | -0.021                            | 7              |
| 8         | 11.34        | 14.01           | -2.67    | 0.277                | -0.583                           | -0.578                           | 0.007                | -0.357                            | 8              |
| 9         | 30.92        | 27.01           | 3.91     | 0.227                | 0.827                            | 0.823                            | 0.011                | 0.445                             | 9              |
| 10        | 49.48        | 51.73           | -2.25    | 0.191                | -0.465                           | -0.460                           | 0.003                | -0.223                            | 10             |
| 11        | 97.93        | 96.11           | 1.82     | 0.263                | 0.394                            | 0.389                            | 0.003                | 0.232                             | 11             |
| 12        | 163.91       | 164.57          | -0.6639  | 0.842 <sup>(1)</sup> | -0.311                           | -0.307                           | 0.027                | -0.709                            | 12             |
| 13        | 6.74         | 12.11           | -5.37    | 0.312                | -1.202                           | -1.210                           | 0.034                | -0.814                            | 13             |
| 14        | 13.48        | 17.61           | -4.13    | 0.277                | -0.902                           | -0.900                           | 0.016                | -0.556                            | 14             |
| 15        | 30.33        | 28.30           | 2.03     | 0.227                | 0.429                            | 0.424                            | 0.003                | 0.229                             | 15             |
| 16        | 53.93        | 48.41           | 5.52     | 0.191                | 1.139                            | 1.144                            | 0.016                | 0.556                             | 16             |
| 17        | 89.88        | 83.56           | 6.32     | 0.263                | 1.367                            | 1.384                            | 0.035                | 0.827                             | 17             |
| 18        | 129.21       | 133.57          | -4.36    | 0.842 <sup>(1)</sup> | -2.042                           | -2.145                           | 1.173 <sup>(2)</sup> | -4.958 <sup>(2)</sup>             | 18             |
| 19        | 5.61         | 5.41            | 0.1983   | 0.312                | 0.044                            | 0.044                            | 0.000                | 0.029                             | 19             |
| 20        | 12.35        | 9.51            | 2.84     | 0.277                | 0.620                            | 0.615                            | 0.008                | 0.380                             | 20             |
| 21        | 16.85        | 17.39           | -0.5414  | 0.227                | -0.114                           | -0.113                           | 0.000                | -0.061                            | 21             |
| 22        | 29.21        | 31.88           | -2.67    | 0.191                | -0.552                           | -0.547                           | 0.004                | -0.265                            | 22             |
| 23        | 55.05        | 55.80           | -0.7484  | 0.263                | -0.162                           | -0.160                           | 0.000                | -0.095                            | 23             |
| 24        | 84.26        | 83.33           | 0.9269   | 0.842 <sup>(1)</sup> | 0.434                            | 0.429                            | 0.053                | 0.991                             | 24             |
| 25        | 3.22         | -0.8864         | 4.11     | 0.312                | 0.920                            | 0.918                            | 0.020                | 0.618                             | 25             |
| 26        | 5.37         | 3.04            | 2.33     | 0.277                | 0.508                            | 0.503                            | 0.005                | 0.311                             | 26             |
| 27        | 10.75        | 10.58           | 0.1651   | 0.227                | 0.035                            | 0.034                            | 0.000                | 0.019                             | 27             |
| 28        | 21.50        | 24.40           | -2.90    | 0.191                | -0.599                           | -0.593                           | 0.004                | -0.288                            | 28             |
| 29        | 38.70        | 46.96           | -8.26    | 0.263                | -1.787                           | -1.847                           | 0.060                | -1.103                            | 29             |
| 30        | 76.34        | 71.78           | 4.56     | 0.842 <sup>(1)</sup> | 2.133                            | 2.254                            | 1.280 <sup>(2)</sup> | 5.211 <sup>(2)</sup>              | 30             |
| 31        | 2.29         | -2.34           | 4.63     | 0.312                | 1.038                            | 1.039                            | 0.026                | 0.699                             | 31             |
| 32        | 4.59         | 1.95            | 2.64     | 0.277                | 0.577                            | 0.572                            | 0.007                | 0.353                             | 32             |
| 33        | 6.89         | 10.21           | -3.32    | 0.227                | -0.702                           | -0.697                           | 0.008                | -0.377                            | 33             |
| 34        | 14.94        | 25.48           | -10.54   | 0.191                | -2.176                           | -2.307                           | 0.059                | -1.120                            | 34             |
| 35        | 58.62        | 50.93           | 7.69     | 0.263                | 1.663                            | 1.708                            | 0.052                | 1.020                             | 35             |
| 36        | 80.45        | 81.55           | -1.10    | 0.842 <sup>(1)</sup> | -0.516                           | -0.511                           | 0.075                | -1.181                            | 36             |
| 37        | 4.39         | 3.18            | 1.21     | 0.312                | 0.272                            | 0.268                            | 0.002                | 0.180                             | 37             |

| Run Order | Actual Value | Predicted Value | Residual | Leverage             | Internally Studentized Residuals | Externally Studentized Residuals | Cook's Distance      | Influence on Fitted Value DFFIT S | Standard Order |
|-----------|--------------|-----------------|----------|----------------------|----------------------------------|----------------------------------|----------------------|-----------------------------------|----------------|
| 38        | 9.89         | 11.45           | -1.56    | 0.277                | -0.340                           | -0.336                           | 0.002                | -0.208                            | 38             |
| 39        | 17.58        | 27.66           | -10.08   | 0.227                | -2.130                           | -2.250                           | 0.070                | -1.218                            | 39             |
| 40        | 64.83        | 58.82           | 6.01     | 0.191                | 1.241                            | 1.251                            | 0.019                | 0.607                             | 40             |
| 41        | 125.27       | 116.07          | 9.20     | 0.263                | 1.991                            | 2.084                            | 0.074                | 1.244                             | 41             |
| 42        | 205.49       | 210.27          | -4.78    | 0.842 <sup>(1)</sup> | -2.238                           | -2.383                           | 1.408 <sup>(2)</sup> | -5.508 <sup>(2)</sup>             | 42             |
| 43        | 15.21        | 10.93           | 4.28     | 0.312                | 0.958                            | 0.957                            | 0.022                | 0.644                             | 43             |
| 44        | 22.82        | 21.92           | 0.8966   | 0.277                | 0.196                            | 0.193                            | 0.001                | 0.119                             | 44             |
| 45        | 44.56        | 43.59           | 0.9721   | 0.227                | 0.205                            | 0.203                            | 0.001                | 0.110                             | 45             |
| 46        | 82.60        | 85.65           | -3.05    | 0.191                | -0.630                           | -0.624                           | 0.005                | -0.303                            | 46             |
| 47        | 157.60       | 164.70          | -7.10    | 0.263                | -1.536                           | -1.567                           | 0.044                | -0.936                            | 47             |
| 48        | 306.50       | 302.50          | 4.00     | 0.842 <sup>(1)</sup> | 1.871                            | 1.944                            | 0.985 <sup>(2)</sup> | 4.494 <sup>(2)</sup>              | 48             |
| 49        | 6.81         | 10.65           | -3.84    | 0.312                | -0.860                           | -0.857                           | 0.018                | -0.577                            | 49             |
| 50        | 14.77        | 14.12           | 0.6507   | 0.277                | 0.142                            | 0.140                            | 0.000                | 0.087                             | 50             |
| 51        | 27.27        | 20.74           | 6.53     | 0.227                | 1.380                            | 1.398                            | 0.029                | 0.757                             | 51             |
| 52        | 34.09        | 32.71           | 1.38     | 0.191                | 0.285                            | 0.281                            | 0.001                | 0.136                             | 52             |
| 53        | 44.31        | 51.58           | -7.27    | 0.263                | -1.574                           | -1.609                           | 0.047                | -0.961                            | 53             |
| 54        | 71.59        | 69.03           | 2.56     | 0.842 <sup>(1)</sup> | 1.196                            | 1.203                            | 0.402                | 2.782 <sup>(2)</sup>              | 54             |

<sup>(1)</sup> Observation with leverage  $> 2.00 \times (\text{average leverage})$ . <sup>(2)</sup> Exceeds limits.

residuals, leverage, internally and superficially studentized residuals, Cook's distance, influence on fitted value, DFFITS, and standard order for each data point in the dataset as shown in Table 2. This information can be used to assess the accuracy of the regression model, identify influential data points, and evaluate the overall goodness-of-fit of the model.

A perturbation plot is used in regression analysis to visualize the influence of individual data points on the fitted values of the regression model as represented in Figure 1. It is also known as a partial residual plot plus residual plot. It can help to identify influential data points that may have a significant impact on the model's predictions.<sup>36</sup> To fit the regression model, utilize the provided data and calculate the residuals by subtracting the predicted values from the actual values. Plot the percentage inhibition while varying the numeric factor, concentration, across its range while keeping all other factors constant. Design-Expert, by default, establishes the reference point at the midpoint (coded as 0) of all the factors. A factor exhibiting a steep slope or curvature indicates that the response is highly influenced by that specific factor. Conversely, a relatively flat line suggests that the response variable is less sensitive to changes in that particular

factor. The variation in percentage inhibition with concentration is displayed as a perturbation plot.

An interaction plot in RSM is a graphical representation of the interaction effects between two or more factors on a response variable as shown in Figure 2. It is commonly used to visually analyse the combined possessions of multiple factors and their interactions on the response variable of interest.<sup>37</sup> In an interaction plot, the y-axis represents the response variable, while the X-axis displays the levels of the factors. Each factor is typically represented by a separate line or curve on the plot, and different colours or line styles can be used to differentiate between the levels of each factor. The plot may also include error bars or confidence intervals to show the variability of the response variable. The contour plots will exhibit two non-parallel lines, signifying that the influence of one factor is contingent upon the level of the other. Individual effect of each factor as well as the average effect is obtained.

Contour plots were employed to illustrate the effects of the independent variables and the interactions between them on the response variables within the regression model as shown in Figure 3. The categorical contour plot is a visual representation

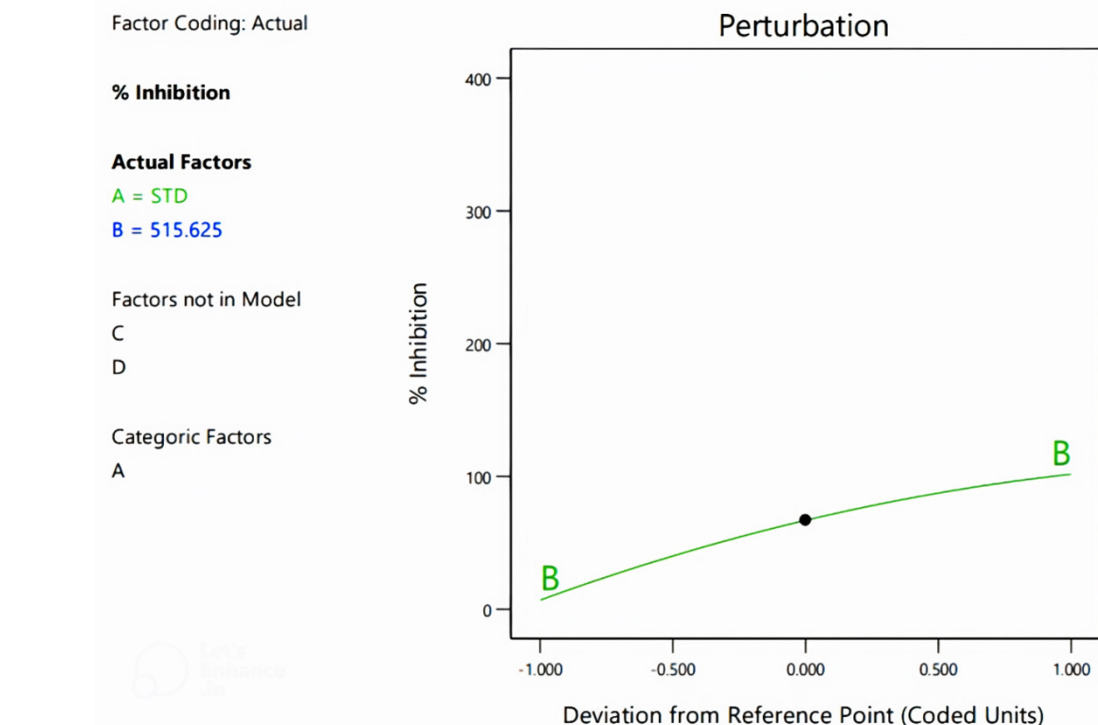


**Figure 1:** Perturbation plot using Response Surface Methodology.

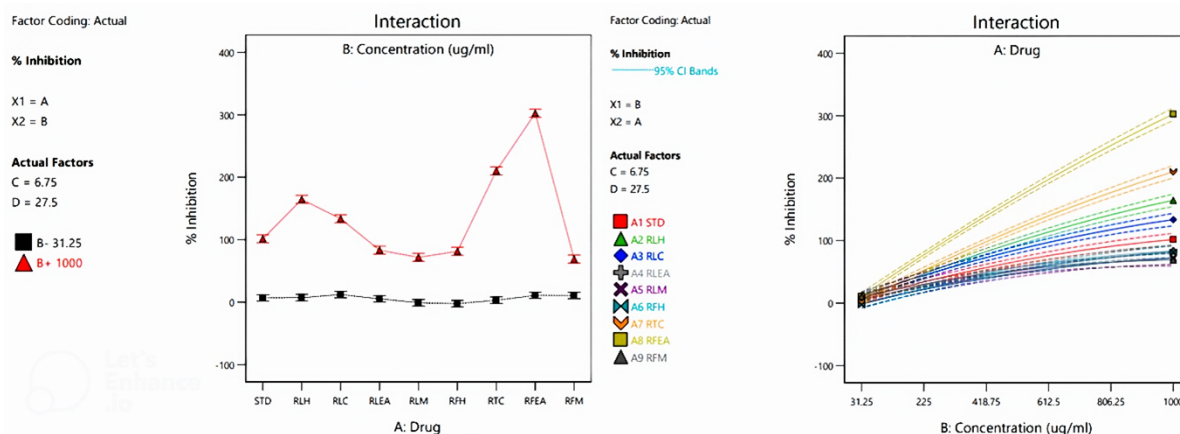


**Figure 2:** Interaction plot of Response Surface Methodology.

in Two Dimensions (2D) that shows the response (% inhibition) plotted against various combinations of categorical factors, such as concentration and pH. In this type of plot, the levels of two factors are depicted on the x-axis and y-axis, while contour lines are used to represent the response variable on the plot. Each contour line represents a constant value of the response variable, and the contour lines are typically drawn at regular intervals to show the shape and direction of the response surface.<sup>38</sup> The contour lines can be plotted in different colours or line styles to indicate the magnitude or direction of the response variable. This plot cannot show numeric factors at the same time.

Figure 4 depicts three-dimensional response surface plots that illustrate the % inhibition for two factors: Concentration

represented by X1 and pH represented by X2. These plots provide an overview of the relationship among the two factors and the response, giving a visual representation of the data that can aid in understanding the trends and patterns in the data. A 3D response surface plot depicts the relationship between two factors, where their levels are plotted on the x-axis and y-axis. The response variable is represented either by the height or colour of the plot.<sup>39</sup> The plot typically shows a three-dimensional surface that represents the response surface, with contours or colour gradients indicating different levels of the response variable. The shape and orientation of the surface can reveal the quadratic nature of the association among the factors and the response. The actual values

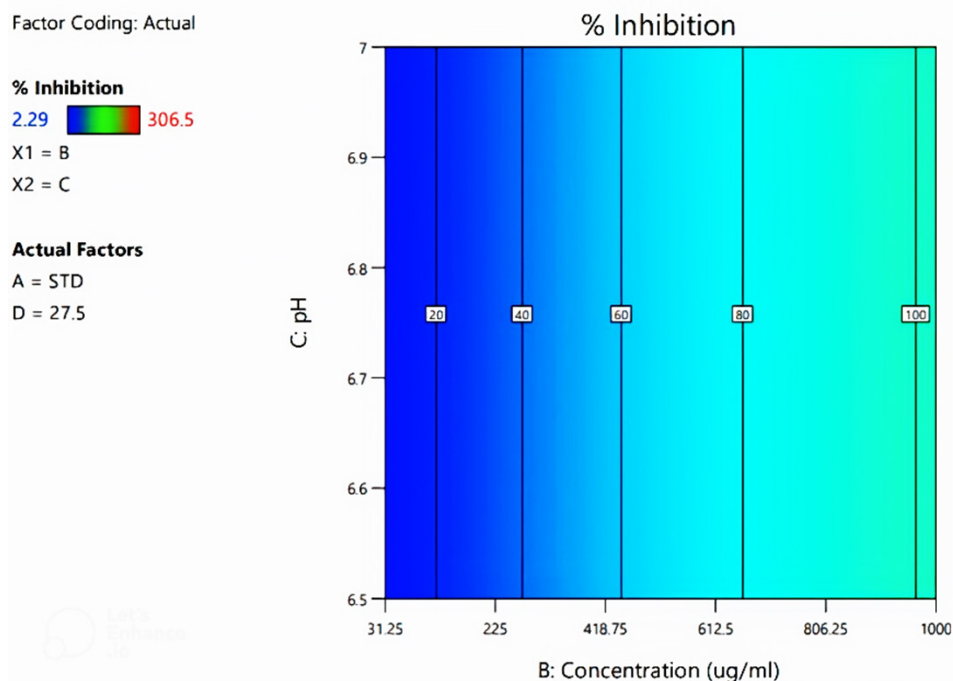


**Figure 3:** Contour plot of inhibition percentage vs concentration and pH.

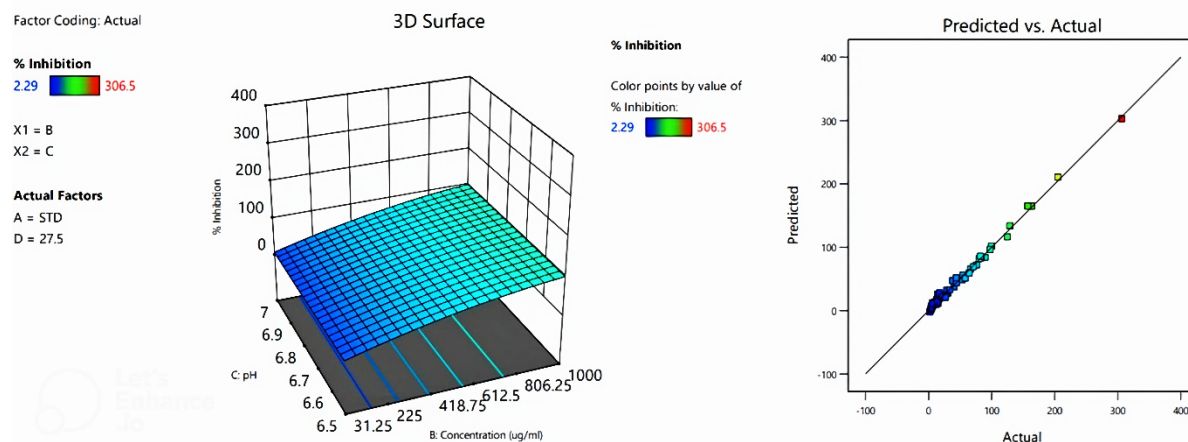


**Figure 4:** Three- Dimensional Response and Predicted vs Actual plot of RSM.

and predicted values are very close to the regression coefficient of  $R^2$  value of 0.9943 and 0.9706 respectively.

The summary of the factors, their goals, lower and upper limits, lower and upper weights, and importance are given for Factor A represents the "Drug" and its goal is to remain within the "STD" to "RFM" range. Factor B represents the "Concentration" and the goal is to maximize it within the range of 1000 to 1500. Factor C represents the "pH" and the goal is to keep it equal to 6.75 while maintaining it within the range of 6.5 to 7. Factor D represents the "Temperature" and the goal is to keep it equal to 27.5 while maintaining it within the range of 25 to 30. Finally, the response variable is "% Inhibition" and the goal is to maximize it within the range of 300 to 400. The factors have equal weights (lower

weight=1, upper weight=1) and the factors are considered to be of high importance (importance=3) in the given context.

The solutions for the 9 combinations of categorical factor levels are presented. Each solution is identified by a number and includes the levels of the factors: Drug, Concentration, pH, and Temperature. Additionally, the corresponding values for percentage inhibition and desirability are provided. The result shows that *R. tetraphylla* ethyl acetate extract show more activity when compared to other extracts. Based on the defined criteria, the optimal solution is obtained with the following factor levels: Drug=RFEA, Concentration=1500.000, pH=6.750, and Temperature=27.500. The resulting percentage inhibition is 413.248, and the Desirability is 1.000, indicating that this solution

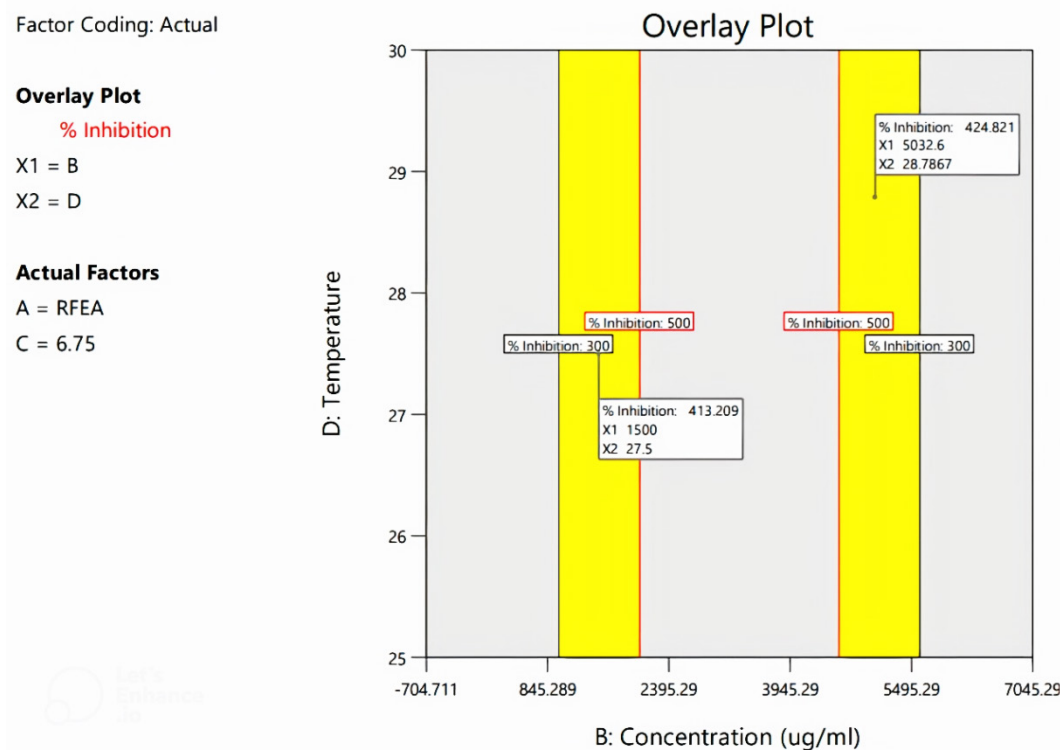


**Figure 5:** Overlay plot of Response Surface Methodology.

has been selected as the best solution based on the predefined goals and limits.

An overlay plot is a graphical representation in which multiple lines are overlaid on a single plot, allowing for visual comparison and analysis as shown in Figure 5. In the context of RSM, an overlay plot can be used to compare the response of a dependent variable (% Inhibition) across different levels of more independent variables (Concentration, Temperature). The plot typically includes multiple lines, each representing a different combination of factor levels, and allows for visual assessment of the effects of each factor on the response variable. Overlay plots are useful for visualizing and interpreting the results of RSM experiments, identifying trends, patterns, and interactions between factors, and guiding decision-making in optimizing the response variable.<sup>40</sup> They can provide valuable insights into the relationships between variables and help in understanding the underlying behaviour of the system.

## CONCLUSION

RSM was employed to optimize the synthesis of anti-inflammatory activity using various crude extracts of *Rauvolfia tetraphylla*. The best observed *in vitro* anti-inflammatory activity was 306.52 µg/mL of ethyl acetate fruit extract among all the variables evaluated. The inhibition percentage was calculated using absorbance values and the anti-inflammatory activity of the various crude extracts

extended from 2.29 to 306.5, indicating the influence of the interaction between the four factors: Drug, Concentration, pH, and Temperature.

The statistical model constructed to evaluate the anti-inflammatory activities of different crude extracts demonstrated a good fit with the actual values. ANOVA confirmed the significance of the model, as evidenced by a high F value (341.92) and a very low *p* value (<0.0001). Additionally, the coefficient of determination ( $R^2=0.9943$ ) specified a high level of accuracy in the model. The verdicts revealed a strong dependence of the inhibition percentage, representing the anti-inflammatory activity, on these factors.

The inhibition activity of different extracts of leaf and fruit of *R. tetraphylla* showed significant anti-inflammatory properties, confirming their traditional use in the action of various inflammatory illnesses. Overall, the results of this study suggest that RSM can be effectively used to optimize the synthesis of anti-inflammatory agents from natural sources, which may provide an alternative to synthetic drugs with potentially harmful side effects.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## ABBREVIATIONS

**RSM:** Response Surface Methodology; **RT:** *Rauvolfia tetraphylla*; **STD:** Standard (Diclofenac sodium); **RLH:** RT Hexane extract of leaves; **RLC:** RT Chloroform extract of leaves; **RLEA:** RT Ethyl acetate extract of leaves; **RLM:** RT methanolic extract of leaves; **RFH:** RT Hexane extract of Fruits; **RFC:** RT Chloroform extract of Fruits; **RFEA:** RT Ethyl acetate extract of Fruits; **RFM:** RT methanolic extract of Fruits; **ANOVA:** Analysis of variance.

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