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Quality Standardization and Statistical Optimization of Polyherbal Blends Targeting Rheumatoid Arthritis

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Abstract
Background: This study investigated the anti-arthritic potential of a polyherbal mixture incorporating turmeric and ginger rhizome, black pepper fruit, and Indian bay leaves, known for their individual antioxidant, analgesic, and anti-inflammatory activity. **Materials and Methods:** Plant parts were standardized according to Ayurvedic Pharmacopoeial guidelines. After Soxhlet extraction, the standard protocols were used to screen phytochemical profiles of each extract. The *in vitro* antiarthritic activities of each extract were evaluated by protein denaturation inhibitory assays, and IC_{50} and IC_{100} values were calculated. Based on the IC_{100}, the DoE13 was utilized to optimize the mixture ratio and check for synergism. **Results:** Standardization complied with pharmacopoeial quality parameters, while phytochemical analysis revealed a remarkable presence of bioactive content and extraction yields. Among the individual extracts, turmeric showed the strongest inhibitory activity, although diclofenac remained superior as a standard reference. Notably, extract combinations outperformed individual components, with MAA ranging from 63.27%-89.23%, indicating synergistic effects. Regression and ANOVA modeling identified the reduced quartic model as the best predictor (R^2=0.96). Optimization recommended mixture "Ratio 1" as the most desirable and effective formulation; however, mixture "Ratio 2" exhibited slightly higher activity (90.28%) despite its lower desirability. **Conclusion:** These findings support the potential of polyherbals comprising turmeric, ginger, black pepper, and Indian bay leaf as effective alternatives for arthritis management, necessitating further pharmacological and clinical investigation.
Keywords: Polyherbal; Anti-arthritic; DoE; Regression; RSM

1. INTRODUCTION

Rheumatoid arthritis (RA) is a systemic autoimmune disorder marked by synovial joint inflammation and destruction, as well as further multi-systemic complications. RA typically manifests higher in women and affects 0.5-1% of adults (30-60 years old) globally. RA-associated complications reduce quality of life and even precipitate mortality.[1] Although the current NSAIDs, DMARDs, and corticosteroids relieve RA symptoms, they often cause ADRs and lose efficacy over time.[2]

Therefore, attention to plant-based therapies has grown, particularly polyherbal formulations, which are rooted in traditional medicine systems such as Ayurveda. These formulations have substantiated enhanced efficacy and reduced toxicity through phytochemical synergism. Scientific validation now supports their multi-targeted action in modulating inflammatory and oxidative pathways.[3]

Consequently, the present study optimized a polyherbal blend of turmeric and ginger rhizome, black pepper fruits, and Indian bay leaves, each known for their anti-inflammatory, antioxidant, and bioavailability-enhancing effects. It was reported that turmeric targets NF-κβ and COX-2, and ginger suppresses prostaglandins. Whereas black pepper can enhance curcumin absorption, and IBL contributes to antioxidant action.[4,5,6,7]

Despite promising evidence, most studies evaluated these botanicals in isolation. This research addressed that gap by developing and statistically optimizing a polyherbal mixture of these plant parts using D-optimal mixture design, followed by *in vitro* validation. The goal was to deliver a scientifically standardized, efficacious, and safe anti-arthritic phytotherapeutic.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

PBS, BSA, trypsin, Tris-HCl, and perchloric acid were procured from Sigma-Aldrich, USA. Diclofenac sodium, HCl, and ethanol were purchased from Merck, Germany. The fresh hen's eggs were procured from local markets. All reagents were of laboratory grade.

2.2 Plant Selection, Collection, and Authentication

Traditionally used anti-inflammatory, analgesic, and antioxidant plants were selected and collected from Golaghat, Assam, during October to November 2023. Specimens were taxonomically authenticated at Gauhati University, and voucher samples (GUBH20533-GUBH20536) were deposited for future reference.

2.3 Physicochemical Properties

Crude plant parts were standardized according to Ayurvedic Pharmacopoeial guidelines to ensure quality and consistency.[8]

2.4 Phytochemical Extraction

The collected plant parts were subjected to Soxhlet extraction (using 95% ethanol) at 60°C for 48-72 hours. The extracts were rotary evaporated and stored at 4°C to preserve phytochemical integrity.[9,10]

2.5 Phytochemical Analysis

Standard methods were used to identify key phytoconstituents, establishing the phytochemical profiles and therapeutic relevance of each extract.[11]

2.6 *In Vitro* Anti-Arthritic Activity

2.6.1 Egg Albumin Denaturation Inhibition Assay (EADIA)

A reaction mixture of 5 ml containing egg albumin (0.2 ml), PBS (2.8 ml, pH 6.4), and test extracts or diclofenac sodium (2 ml) was incubated at 37±2°C for 15 min, and then heated at 70°C for 5 min to induce denaturation. Once cooled, the absorbance of the reaction mixture was measured at 660 nm spectrophotometrically. Distilled water was used as a control.[12]

2.6.2 Bovine Serum Albumin Denaturation Inhibition Assay (BSADIA)

A 0.5 ml mixture of 5% BSA (0.45 ml) and distilled water (0.05 ml), adjusted to pH 6.3, was treated with test extracts or diclofenac sodium and incubated at 37°C for 30 min. Samples were heated at 57°C for 5 min, cooled, and diluted with 2.5 ml PBS. Then, the turbidity was measured at 600 nm spectrophotometrically. The control sample contained BSA and water only.[13]

2.6.3 Anti-Proteinase Assay (APA)

A 2 ml reaction mixture containing trypsin (0.06 mg), Tris-HCl buffer (1 ml, 20 mM, pH 7.4), and test extract or diclofenac sodium (1 ml) was incubated at 37°C for 5 min. Casein (1 ml, 0.8% w/v) was added and further incubated for 20 min. The reaction was stopped with 2 ml of 70% perchloric acid, centrifuged, and the absorbance of the supernatant was measured at 210 nm spectrophotometrically.[14]

Percentage inhibition, mean IC_{50} values were determined in EADIA, BSADIA, and APA for each extract, and IC_{100} values were deduced. For mixture optimization, a combined dose of 1 mg was formulated by incorporating a factor of 25.63% of each extract's IC_{100} value for turmeric, ginger, black pepper, and IBL.

2.7 Optimization of Mixture Ratio

2.7.1 Designing a Model and Evaluation

The design matrix was developed using a coordinate exchange algorithm in DoE13. A D-optimal mixture design utilizing the pseudo-components, 0.10 mg as the lower limit and twice the mean *in-vitro* antiarthritic IC_{100} as the upper limit, was employed to generate the ratio runs. All mixture runs were checked for *in vitro* antiarthritic potential in triplicate as described in EADIA, BSADIA, and APA assays.[15]

Given the limitations of the "Power" metrics in mixture designs, DoE13 suggested comparing FDS to evaluate model predictability across the experimental domain and select a suitable model. Moreover, to ensure model adequacy, sufficient DF for lack-of-fit (≥3) and pure error (≥4) were advised in DoE13.

2.7.2 Model Diagnostics and Confirmation

The selected model further underwent a comprehensive diagnostic evaluation using leverage, ESRs, Cook's distance, and DFFITS to assess potential influence points. Based on diagnostic metrics, the selected model was affirmed for the statistical prediction and mixture optimization.[16]

2.7.3 ANOVA of the Selected Model

ANOVA was performed on the selected model using L-Pseudo coding and Type III sum of squares to evaluate model significance. Model fit was assessed by "fit summary" using adjusted R^2, predicted R^2,

adequate precision, and CV% values.[17] ANOVA identified significant main effects and interactions, and component influence was visualized *via* graphical plots.[18,19]

2.7.4 Numerical Optimization and Validation

Numerical optimization was set to target maximum antiarthritic activity and minimum SE, assigning high priority weights to both. The optimized mixtures were validated by matching the practical and predicted values within 95% PIs.[20]

2.8 Statistical Analysis

All experiments were performed in triplicate, and data were expressed as mean±SEM. The linear regression was used to calculate IC_{50} values. ANOVA was performed through DoE13, and a $p<0.05$ was considered significant.

3. RESULTS

3.1 Physiochemical Properties

All plant materials complied with Ayurvedic Pharmacopoeial standards. Extraneous matter was minimal (0.94-1.95%), total ash ranged from 4.72%-8.46%, and acid-insoluble ash remained low (0.48-1.36%), indicating high purity. IBL showed the highest alcoholic extractive value (13.48%), while turmeric had the highest hydrophilic extractive content (12.38%).

3.2 Extraction of Phytochemicals

Extraction yielded semisolid extracts with distinct organoleptic properties. The yields ranged from 11.18% (ginger)-14.48% (IBL), which confirmed efficient recovery of phytoconstituents.

3.3 Phytochemical Analysis

Phytochemical screening confirmed the presence of flavonoids, alkaloids, glycosides, tannins, carbohydrates, and proteins, whereas saponins were found absent.

3.4 *In Vitro* Anti-Arthritic Activity

3.4.1 Egg Albumin Denaturation Inhibition Assay (EADIA)

In EADIA, diclofenac sodium exhibited MAA ranging from 18.01±2.84%-87.38±1.31%. Among the extracts, turmeric exhibited the highest MAA (12.14±0.73%-76.19±0.17%), followed by ginger (11.25±0.24%-68.51±1.20%), black pepper (8.10±1.82%-57.50±0.59%), and IBL (6.40±0.59%-45.37±3.53%). IC_{50} values were in a sequence of 68.48 µg/ml, 82.35 µg/ml, 90.28 µg/ml, 108.19 µg/ml, 127.91 µg/ml for diclofenac sodium, turmeric, ginger, black pepper, and IBL, respectively (Figure 1).

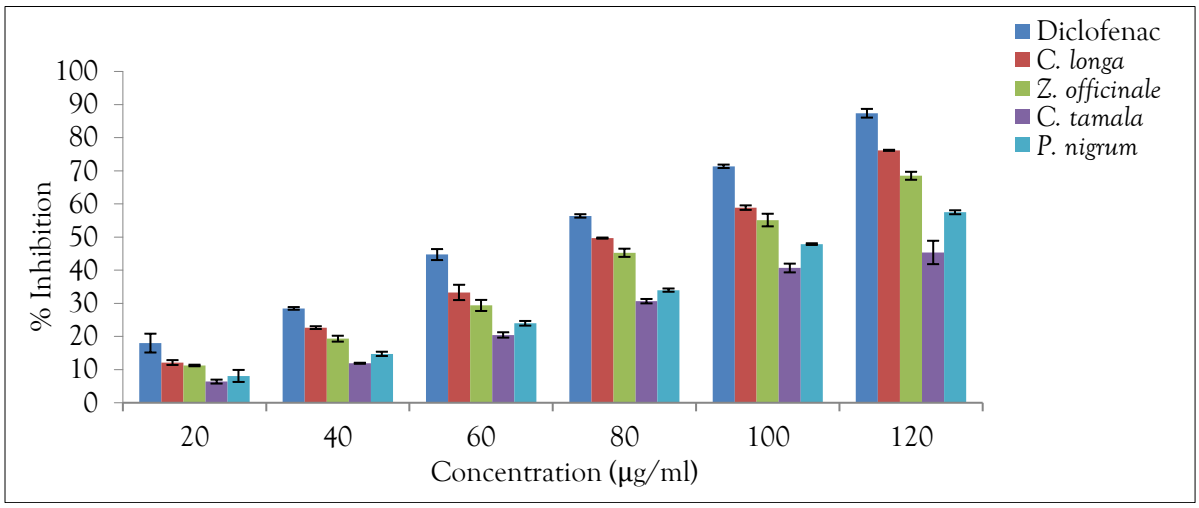

Figure 1. % inhibition in EADIA

3.4.2 Bovine Serum Albumin Denaturation Inhibition Assay (BSADIA)

In BSADIA, diclofenac showed 27.08±0.29%-93.10±0.21% inhibition. Extracts followed a similar potency order for turmeric (18.15±0.29%-77.59±0.27%), ginger (15.30±0.39%-68.69±0.41%), black pepper (9.23±0.29%-59.29±0.57%), and IBL (7.25±0.48%-48.23 ± 0.71%). The respective IC_{50} values for diclofenac sodium, turmeric, ginger, black pepper, and IBL were found to be 51.13 µg/ml, 78.33 µg/ml, 88.71 µg/ml, 105.12 µg/ml, and 125.40 µg/ml (Figure 2).

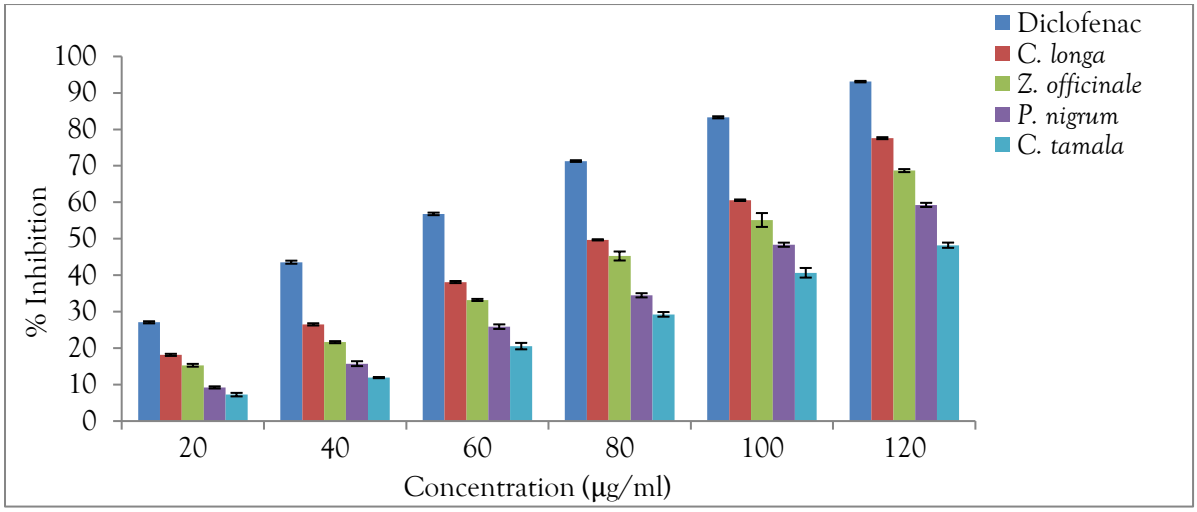

Figure 2. % inhibition in BSADIA

3.4.3 Anti-Proteinase Assay (APA)

In APA, diclofenac inhibited proteinase activity from 25.00±0.23%-88.00±0.16%. Turmeric showed 19.98±0.28%-68.15±0.20%, followed by ginger (12.84±0.44%-65.61±0.41%), black pepper (11.01±0.21%-63.15±0.21%), and IBL (7.16±0.16%-50.89±0.02%). Diclofenac sodium, turmeric, ginger, black pepper, and IBL were found to exhibit IC_{50} values of 57.40 µg/ml, 78.99 µg/ml, 89.24 µg/ml, 96.35 µg/ml, and 122.53 µg/ml, respectively (Figure 3).

The mean IC_{100} values for the extracts were 0.16 mg (turmeric), 0.18 mg (ginger), 0.21 mg (black pepper), and 0.25 mg (IBL). After incorporating a factor of 25.63% of each extract's IC_{100}, the final derived doses were 0.20 mg (turmeric), 0.22 mg (ginger), 0.26 mg (black pepper), and 0.31 mg (IBL) for optimization purposes. This proportional scaling reflected extract potency and ensured sufficient activity within the mixture.

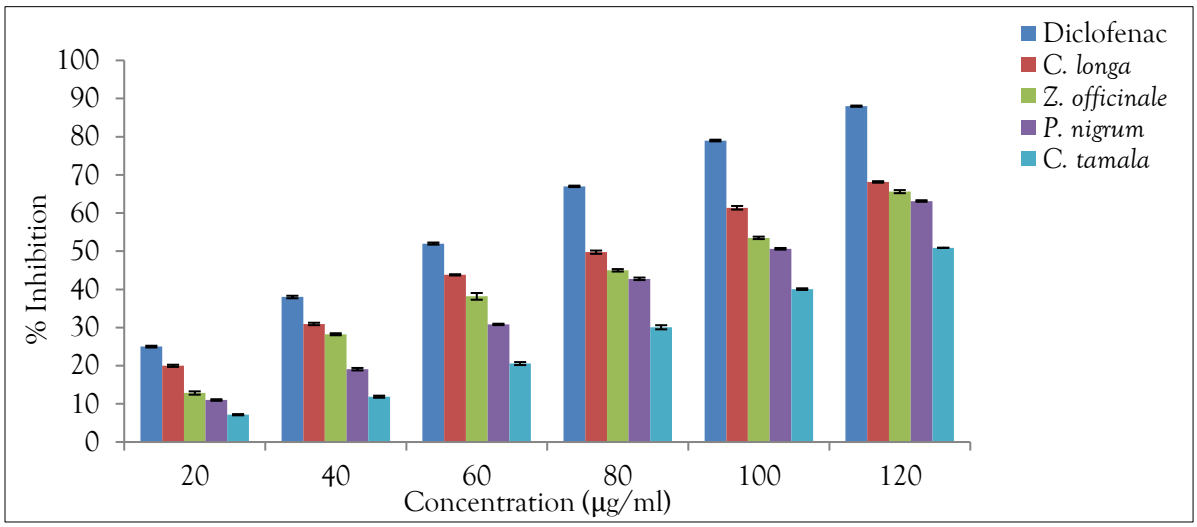

Figure 3. % inhibition in APA

3.5 Optimization of Mixture Ratio

3.5.1 Designing a Model and Evaluation

Twenty-four experimental runs were generated, each with defined ratios of turmeric, ginger, black pepper, and IBL, where mean % inhibition or MAA across the *in vitro* assays served as the response variable. MAA values ranged from 63.27%-89.23%, indicating substantial variation in activity based on composition. Runs with higher proportions of turmeric and black pepper consistently showed MAA>85%, suggesting a synergistic interaction. In contrast, mixtures with lower turmeric and higher IBL content exhibited moderate activity.

Among evaluated models, the reduced quartic model demonstrated optimal fit, with a low average SEM of 0.67 and a stable SEM range (0.446-1.108) in FDS, indicating consistent predictive accuracy across the mixture space (Figure 4). Furthermore, the model exhibited 4 DF for lack of fit and 5 DF for pure error, ensuring the model's adequacy. These findings suggested the reduced quartic model for optimizing our polyherbal mixtures.

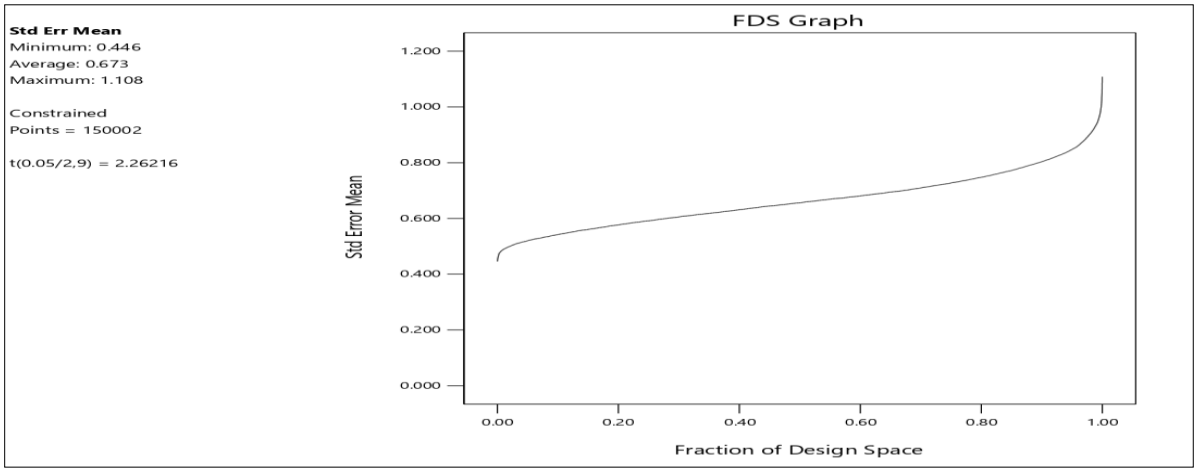

Figure 4. FDS of the reduced quartic model

3.5.2 Model Diagnostics and Confirmation

In diagnostics, the leverage values exceeding the threshold of 0.62 were noted in a few runs, indicating moderate influence; however, these did not correspond with elevated ESRs. ESRs were randomly distributed around zero and within acceptable limits (±4.46), confirming the absence of systematic error. Cook's distance values remained below the critical threshold of 1 for all runs, indicating no data point exerted disproportionate influence on the model. Similarly, all DFFITS values were within the acceptable range (±2.37), supporting the model's stability and predictive reliability. Collectively, these diagnostic metrics affirmed the statistical soundness of the reduced quartic model for the formulation optimization.

3.5.3 ANOVA of the Selected Model

Based on 24 experimental runs, the reduced quartic model exhibited an SS^2 of 47.41 with a corrected total SS of 1093.05. The model yielded an F-value of 14.18 (p=0.0002), confirming statistical significance. The Lack-of-fit was not significant (F=0.18, p=0.9386), indicating good model fit.

"Fit statistics" confirmed the model's acceptability as predicted R^2=0.71, and adjusted R^2=0.89 was with a <0.2 difference, indicating reliable predictive performance. Adequate precision was 14.84, exceeding the threshold of 4, while a CV of 2.96% reflected low experimental variability (Mean of MAA=77.49; SD=2.30).

The linear mixture terms denoted as A (turmeric or *C. longa*), B (ginger or *Z. officinale*), C (black pepper or *P. nigrum*), and D (IBL or *C. tamala*) significantly influenced MAA (SS=620.38, p<0.0001). Among interactions, ABD (p=0.0036) and ABCD (p=0.0013) were statistically significant, indicating key synergistic effects (Eq.1 and Eq.2).

L-Pseudo coded equation (Eq.1):

Y=+130.07A+79.07B+69.26C+68.23D−61.85AB−57.80AC−56.82AD−2.27BC−1.64BD+16.22CD+126.60ABC−393.96ABD−81.50ACD+67.63BCD+3687.16ABCD

Real terms equation (Eq.2):

Y=+163.01A+541.17B+46.19C+19.94D+236.47AB+103.07AC+346.77AD+188.26BC+431.03BD+335.99CD−2258.90ABC−4668.93ABD−3222.33ACD−2531.92BCD+28450.31ABCD

Where, Y= Mean antiarthritic activity

Trace plots revealed that turmeric had the strongest positive effect on anti-arthritic activity, followed by ginger. Black pepper showed a mild negative influence, while IBL significantly reduced activity with increasing concentration (Figure 5). Contour (Figure 6) and 3D response surface plots (Figure 7) displayed that maximum activity occurred at high turmeric and moderate ginger or black pepper, with IBL held constant at 0.1 mg. The Predicted versus Actual plot (Figure 8) showed close alignment, confirming minimal prediction error.

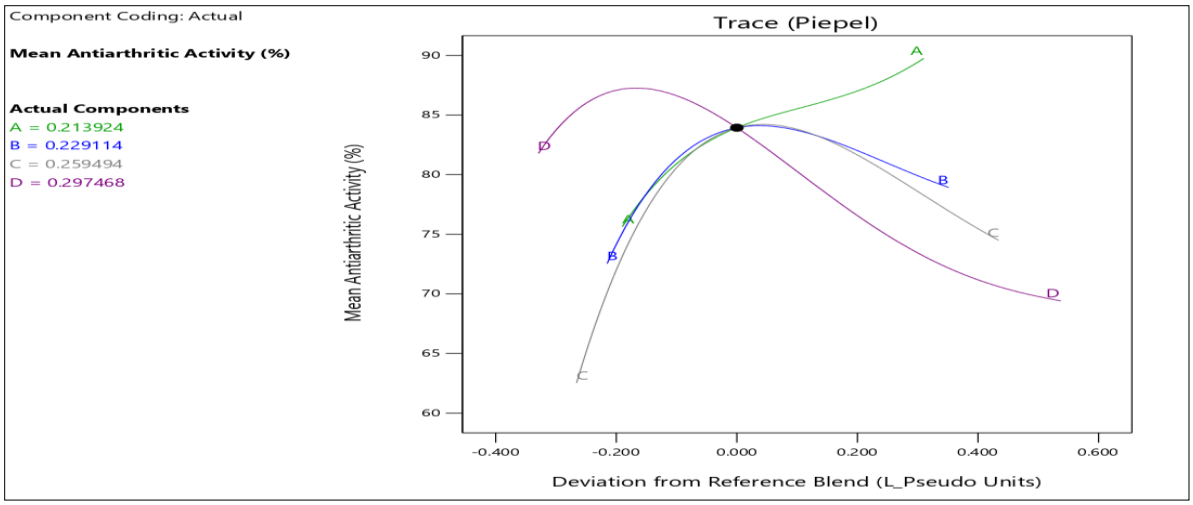

Figure 5. Trace (piepel) plot

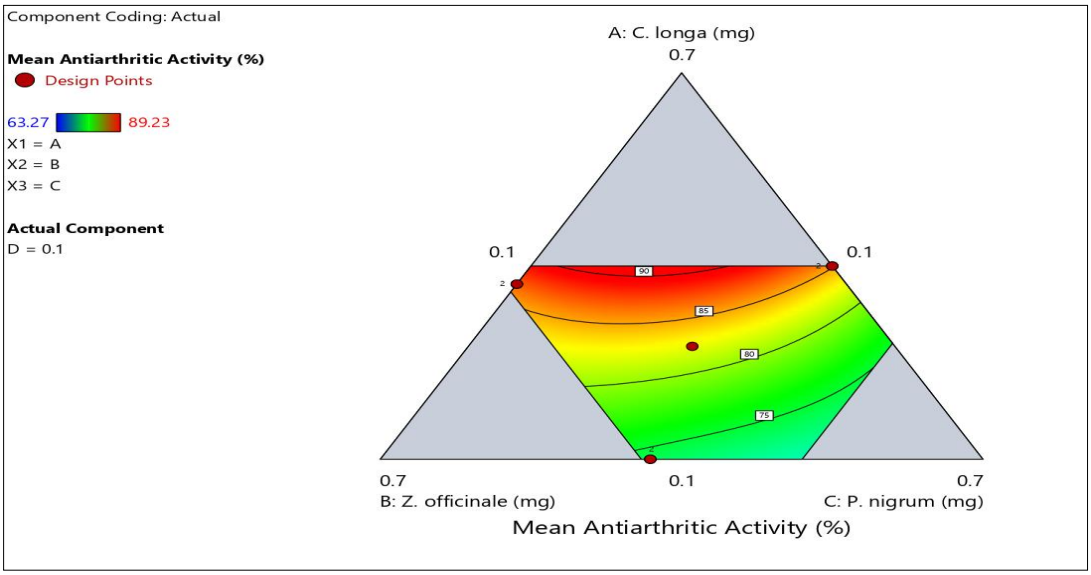

Figure 6. Contour (Ternary) plot

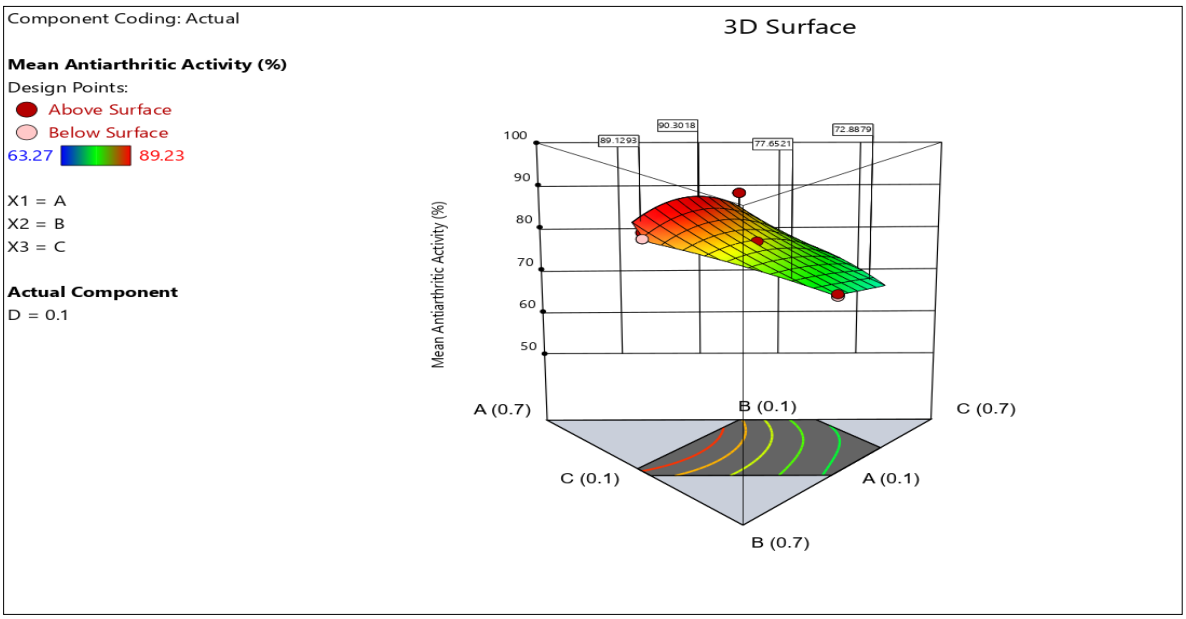

Figure 7. 3D response surface plot

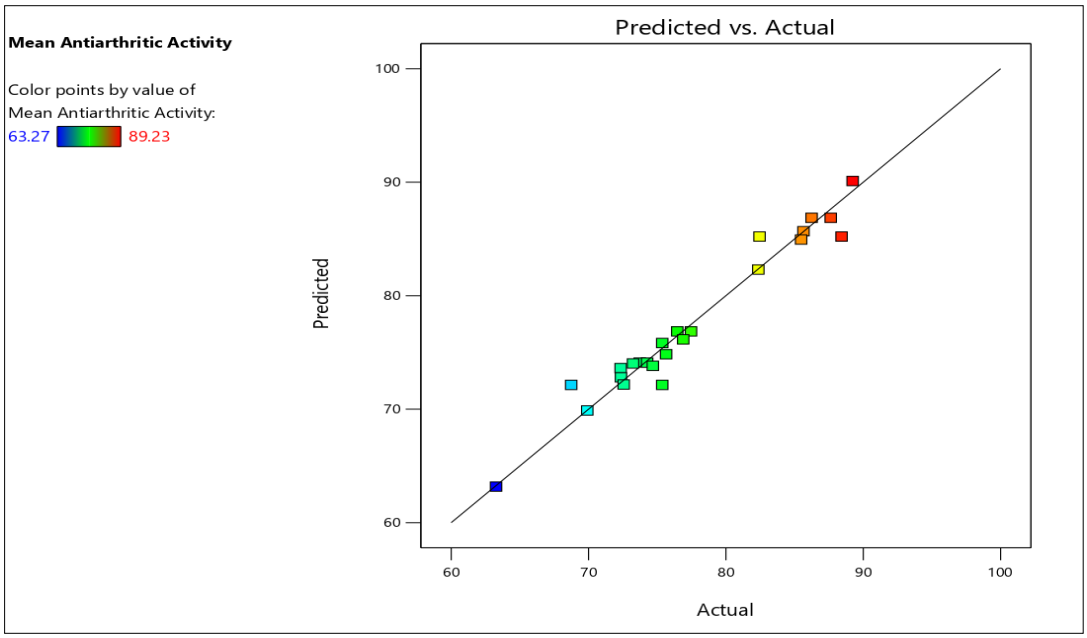

Figure 8. Predicted versus actual plot

3.5.4 Numerical Optimization and Validation

Seven optimized mixtures were generated through numerical optimization, each with defined component ratios. The observed anti-arthritic activities for all formulations fell within the 95% PI, confirming the model's validity. Among them, the mixture "Ratio 2" exhibited the highest MAA (90.28%), outperforming the others.

4. DISCUSSIONS

This study was undertaken to evaluate the anti-arthritic potential of a polyherbal mixture comprising turmeric and ginger rhizomes, black pepper fruits, and IBL selected based on their traditional use and pharmacologically active phytoconstituents[4,5,6,7].

The physicochemical properties of crude plant parts agreed with the Ayurvedic Pharmacopoeial limits, ensuring quality standards[8]. The extractive yields and preliminary phytochemical investigations aligned with previous reports, confirming effective extraction and presence of bioactives[9,10]. *In vitro* assays revealed turmeric as the most effective extract, followed by ginger, black pepper, and IBL. However, diclofenac sodium outperformed all extracts in this regard. These findings guided the selection of initial input constraints in DoE13 for optimization of mixture ratios.

The D-optimal mixture design generated 24 mixture ratios with mean MAA ranging from 63.27%-89.23%. Then, the statistical analysis identified a reduced quartic model as the best fit ($F=14.18$, $p=0.0002$, $R^2=0.96$), reflecting complex interactions among extracts, which was further supported by the model diagnostics. Moreover, the regression equations confirmed the synergism of turmeric, ginger, black pepper, and IBL extracts, revealing turmeric as the principal contributor to MAA. In contrast, the high levels of IBL and black pepper slightly reduced efficacy. These findings were also supported through further graphical representations. Optimization identified seven promising mixture ratios amongst which mixture "Ratio 1" (0.36:0.16:0.33:0.15) was the most balanced, with high efficacy (86.13%) and desirability (0.97). Though mixture "Ratio 2" had lower desirability, it showed a slightly higher activity (90.28%), underscoring the importance of practically validating the optimized mixture ratios.

The results corroborated the traditional rationale of synergistic herbal combinations and demonstrated the utility of modern statistical tools in optimizing polyherbal therapies. It also emphasized that by modulating multiple inflammatory pathways, the polyherbal formulation offers potential scope over monotherapies in terms of safety and efficacy. Noteworthy is that the reproducibility of recent *in silico* tools is deemed questionable, necessitating further validation in preclinical and clinical setups. Nevertheless, this research lays a foundation for *in vivo* validation and mechanistic studies. The approach can be extended to other chronic inflammatory diseases, supporting the integration of traditional medicine with evidence-based drug development.

5. CONCLUSION

This study investigated the anti-arthritic potential of a polyherbal mixture comprising turmeric and ginger rhizomes, black pepper fruits, and Indian bay leaves, selected for their anti-inflammatory and antioxidant properties. Standardization and phytochemical analysis confirmed quality and bioactive content. *In vitro* assays showed turmeric had the highest individual efficacy, though all single extracts were less effective than diclofenac sodium. Polyherbal combinations demonstrated superior activity, confirming synergistic effects. A reduced quartic model optimized the ratios, and subsequent observations identified the mixture "Ratio 1" as the most balanced with high MAA and desirability. However, the mixture "Ratio 2" showed peak MAA but had lower desirability. These findings support further *in vivo* and clinical studies of polyherbal formulations containing turmeric and ginger rhizomes, black pepper fruits, and Indian bay leaves as potential alternatives or adjuncts in RA management.

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Declaration of competing interest: The authors declare that there is no conflict of interest in the paper.

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