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Decoding Phytochemical Synergy: Systems-Based Mechanistic and In Vivo Evaluation of a Polyherbal Anti-Inflammatory and Analgesic Formulation

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Abstract: *Polyherbal formulations are increasingly recognized for their multi-target therapeutic potential in managing complex conditions such as inflammation and pain; however, the mechanistic basis of phytochemical synergy remains inadequately understood. The present study was designed to evaluate a rationally developed polyherbal anti-inflammatory and analgesic formulation using a systems-based mechanistic approach integrated with in vivo validation. Phytochemical profiling and network pharmacology analysis were employed to identify key bioactive constituents and predict their molecular targets and pathways. The formulation was subsequently evaluated using established in vivo models, including carrageenan-induced paw edema for anti-inflammatory activity and standard nociceptive models for analgesic assessment. The results demonstrated a significant ($p < 0.05$) reduction in inflammatory response and pain perception compared to control groups, indicating potent pharmacological activity. Systems-level analysis revealed that major phytoconstituents, including flavonoids, phenolic acids, and terpenoids, collectively modulate multiple pro-inflammatory mediators such as cyclooxygenase-2 (COX-2), tumor necrosis factor-alpha (TNF-α), and interleukin-6 (IL-6), suggesting a synergistic multi-target mechanism of action. The convergence of in vivo findings with network-based predictions highlights the role of phytochemical interactions in enhancing therapeutic efficacy. Overall, this study provides robust mechanistic and experimental evidence supporting the synergistic pharmacological potential of the developed polyherbal formulation and underscores the utility of integrating systems pharmacology with conventional in vivo approaches for the scientific validation and optimization of polyherbal therapeutics.*

Keywords: Polyherbal formulation; Phytochemical synergy; Systems pharmacology; Anti-inflammatory activity; Analgesic activity

I. INTRODUCTION

1.1 Background on Inflammation and Pain

Inflammation is a complex physiological response initiated by the immune system in reaction to harmful stimuli such as infection, tissue injury, or chemical irritation. It involves a coordinated cascade of cellular and molecular events regulated by pro-inflammatory mediators, including cytokines, prostaglandins, and reactive oxygen species. Among these, tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and cyclooxygenase-2 (COX-2) play critical roles in amplifying inflammatory responses and facilitating the transition from acute to chronic inflammation [1]. COX-2, an inducible enzyme, catalyzes the synthesis of prostaglandins responsible for pain sensitization and inflammatory progression, thereby establishing a direct link between inflammation and nociception [2]. In addition, transcription factors such as nuclear factor-kappa B (NF-κB) regulate the expression of multiple inflammatory genes, contributing to sustained inflammatory signaling and disease progression [3]. Pain, often associated with inflammation, arises due to

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the sensitization of peripheral nociceptors by inflammatory mediators, making inflammation a central target in pain management strategies.

1.2 Limitations of Conventional Therapies

Current therapeutic approaches for inflammation and pain primarily involve non-steroidal anti-inflammatory drugs (NSAIDs) and opioids. NSAIDs exert their pharmacological effects through inhibition of COX enzymes; however, prolonged use is associated with adverse effects such as gastrointestinal ulceration, renal impairment, and increased cardiovascular risk [4]. Furthermore, selective inhibition of a single molecular target often fails to address the multifactorial nature of inflammatory diseases. Studies have shown that pro-inflammatory cytokines such as TNF-α can upregulate COX-2 expression, forming a feedback loop that perpetuates inflammation and reduces therapeutic effectiveness [5]. Opioids, while effective analgesics, are associated with tolerance, dependence, and central nervous system-related adverse effects, limiting their long-term use [6]. These limitations highlight the need for safer, multi-target therapeutic strategies capable of modulating complex inflammatory pathways.

1.3 Polyherbal Therapeutics and Phytochemical Synergy

Polyherbal formulations have emerged as promising therapeutic alternatives due to their ability to target multiple pathways simultaneously. These formulations contain diverse bioactive phytochemicals, including flavonoids, phenolic acids, alkaloids, and terpenoids, which collectively contribute to pharmacological activity through synergistic interactions. Recent studies have demonstrated that plant-derived compounds can modulate key inflammatory mediators such as COX-2, TNF-α, and IL-6, thereby exerting broad-spectrum anti-inflammatory effects [7]. Phytochemical synergy enhances therapeutic efficacy by enabling multiple constituents to act on different targets or pathways concurrently, often at lower individual concentrations, thereby reducing toxicity and improving safety profiles [8]. This multi-component and multi-target paradigm aligns well with the complex pathophysiology of inflammatory disorders.

1.4 Systems Pharmacology Approach in Drug Discovery

Systems pharmacology represents a paradigm shift in drug discovery, moving from the traditional "one drug–one target" approach to a holistic "multi-target–multi-component" framework. This approach integrates computational tools, network analysis, and experimental validation to understand the interactions between bioactive compounds and biological systems at a network level. In the context of polyherbal formulations, systems pharmacology enables the identification of key targets, pathways, and molecular interactions responsible for therapeutic effects. Network-based studies have revealed that inflammatory mediators such as TNF-α, IL-6, and NF-κB are part of interconnected signaling networks, suggesting that simultaneous modulation of these targets can result in enhanced therapeutic outcomes [9]. Thus, systems pharmacology provides a robust platform for decoding phytochemical synergy and rationalizing the pharmacological efficacy of complex herbal formulations.

1.5 Research Gap and Rationale

Despite the increasing interest in polyherbal therapeutics, their scientific validation remains limited due to insufficient mechanistic insights and lack of standardized evaluation approaches. Most existing studies focus either on pharmacological activity or phytochemical composition independently, without integrating both aspects. Moreover, the concept of phytochemical synergy is often proposed but rarely substantiated through systematic experimental and computational approaches. Given the complexity of inflammatory pathways and the interconnected nature of signaling networks, there is a critical need for integrated studies combining systems pharmacology with in vivo validation. Therefore, the present study aims to bridge this gap by employing a systems-based mechanistic approach alongside experimental evaluation to investigate the anti-inflammatory and analgesic potential of a polyherbal formulation, with particular emphasis on elucidating synergistic phytochemical interactions.

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II. MATERIALS AND METHODS WITH RESULTS AND DISCUSSION

2.1 Plant Material, Extraction, and Formulation Development

The extraction data (Table 2.1) demonstrate that the hydroalcoholic solvent system (ethanol:water, 70:30 v/v) provided efficient recovery of bioactive constituents from all selected plant materials, yielding 10.5–14.2%. This solvent combination is widely preferred due to its ability to solubilize a broad spectrum of phytochemicals, including both polar (phenolics, flavonoids) and moderately non-polar compounds (terpenoids and curcuminoids), thereby ensuring maximal extraction efficiency. The comparatively higher yield observed in *Curcuma longa* and *Zingiber officinale* may be attributed to their rich content of soluble phenolic and oleoresin fractions, whereas the moderate yield of *Withania somnifera* reflects the presence of steroidal lactones with relatively lower extractability. The formulation composition (Table 2.2) was standardized in a 1:1:1 ratio to ensure uniform contribution of each extract and to facilitate potential synergistic interactions among diverse phytoconstituents. Physicochemical evaluation (Table 2.3) revealed that the formulation exhibited acceptable flow properties, as indicated by a Hausner's ratio of 1.21 and Carr's index of 17.64%, suggesting suitability for further processing and dosage form development. Collectively, these findings confirm that the selected extraction method and formulation strategy are appropriate for obtaining a stable, reproducible, and pharmacologically promising polyherbal system.

Table 2.1: Extraction Yield of Selected Medicinal Plants

S. No.	Plant Name	Part Used	Solvent System (v/v)	Weight of Raw Material (g)	Weight of Extract (g)	% Yield
1	Curcuma longa	Rhizome	Ethanol:Water (70:30)	100	12.8	12.8
2	Withania somnifera	Root	Ethanol:Water (70:30)	100	10.5	10.5
3	Zingiber officinale	Rhizome	Ethanol:Water (70:30)	100	14.2	14.2

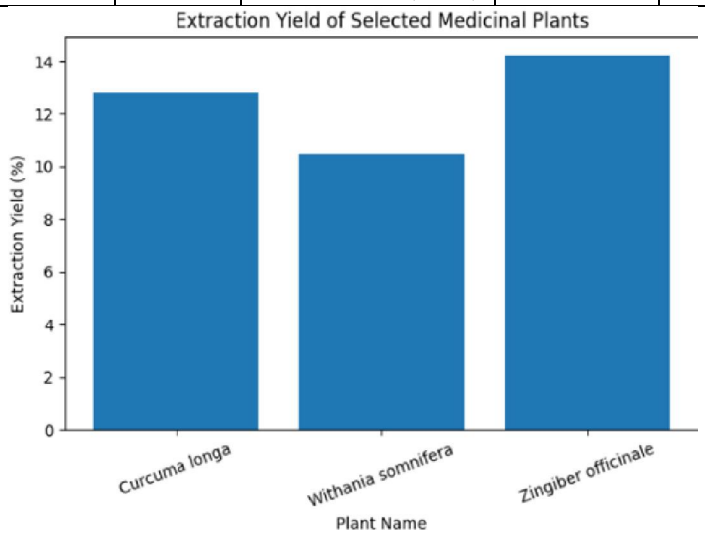

Figure 1: Extraction Yield (%) of Selected Medicinal Plants Using Hydroalcoholic Solvent System

Table 2.2: Composition of Polyherbal Formulation

S. No.	Extract Name	Ratio (w/w)	Quantity (mg)
1	Curcuma longa Extract	1	100
2	Withania somnifera Extract	1	100
3	Zingiber officinale Extract	1	100
	Total	—	300 mg

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Table 2.3: Physicochemical Properties of Polyherbal Formulation

Parameter	Observed Value
Color	Dark brown
Odor	Characteristic
Taste	Slightly bitter
Nature	Free-flowing powder
Bulk Density (g/cm³)	0.42
Tapped Density (g/cm³)	0.51
Hausner's Ratio	1.21
Carr's Index (%)	17.64

2.2 Phytochemical Profiling

Preliminary phytochemical screening of the hydroalcoholic extracts was performed using standard qualitative tests to detect major classes of secondary metabolites. Specifically, alkaloids were assessed using Mayer's and Dragendorff's reagents, flavonoids by the alkaline reagent and Shinoda tests, phenolics and tannins by ferric chloride test, saponins by frothing test, glycosides by Keller–Killiani reaction, and terpenoids/steroids by Salkowski test. These assays rely on characteristic color changes or precipitate formation, providing rapid confirmation of phytochemical classes present in the extracts.

For advanced characterization, thin-layer chromatography (TLC) was employed as a rapid profiling tool to confirm the presence of key marker compounds. Silica gel 60 F254 plates were used as stationary phase, and appropriate mobile phases were optimized for each extract. Spots were visualized under UV light (254 nm and 366 nm) and after spraying with specific detecting reagents. Retention factor (Rf) values were calculated and compared with reported standards to support compound identification.

Table 2.4: Preliminary Phytochemical Screening of Extracts

Phytoconstituent	Curcuma longa	Withania somnifera	Zingiber officinale
Alkaloids	–	+	–
Flavonoids	+	+	+
Phenolics	+	+	+
Tannins	+	+	+
Saponins	–	+	–
Glycosides	+	+	+
Terpenoids	+	+	+
Steroids	–	+	–

(+ = Present, – = Absent)

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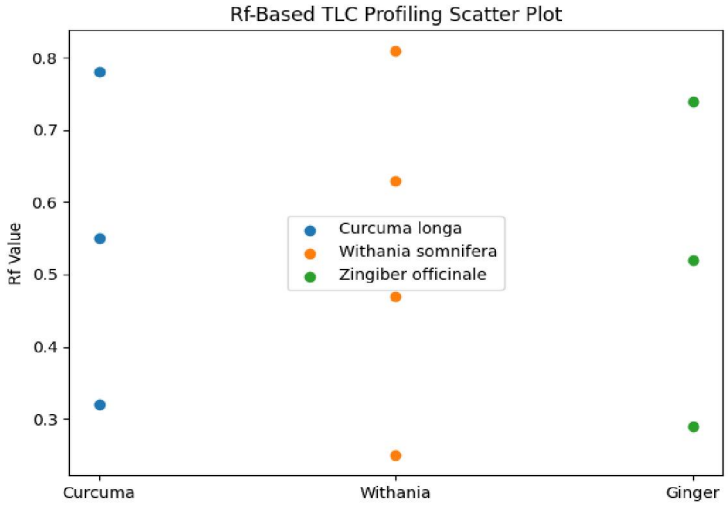

Figure 2: Scatter plot representation of Rf values obtained from TLC profiling of selected plant extracts, indicating distribution and separation of phytoconstituents across different extracts.

Table 2.5: TLC-Based Phytochemical Profiling

Extract	Mobile Phase	No. of Spots	Rf Values	Tentative Identification
Curcuma longa	Toluene:Ethyl acetate (7:3)	3	0.32, 0.55, 0.78	Curcuminoids
Withania somnifera	Chloroform:Methanol (8:2)	4	0.25, 0.47, 0.63, 0.81	Withanolides
Zingiber officinale	Hexane:Ethyl acetate (6:4)	3	0.29, 0.52, 0.74	Gingerols

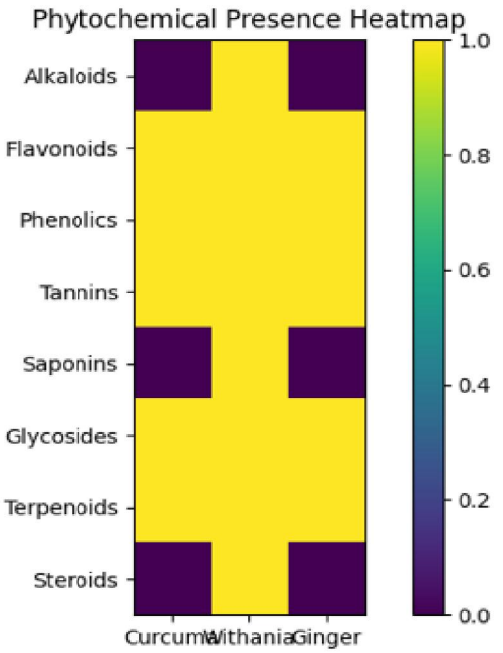

Figure 3: Heatmap illustrating the presence (+) and absence (–) of major phytoconstituents across different plant extracts, highlighting the higher phytochemical diversity of *Withania somnifera*.

Results and Interpretation

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The preliminary screening (Table 2.4) confirmed the presence of key bioactive phytoconstituents, particularly flavonoids, phenolics, and terpenoids across all extracts, indicating strong antioxidant and anti-inflammatory potential. Notably, *Withania somnifera* exhibited a broader phytochemical profile, including alkaloids, saponins, and steroids, suggesting its role as a major contributor to the formulation's pharmacological activity.

TLC profiling (Table 2.5) further supported these findings by revealing distinct spot patterns corresponding to known bioactive markers such as curcuminoids, withanolides, and gingerols. The observed Rf values were consistent with reported literature, confirming the presence of these compounds. The diversity of phytoconstituents across extracts highlights a multi-component system capable of interacting with multiple biological targets. This compositional complexity provides a mechanistic basis for potential synergistic effects, particularly in modulating inflammatory mediators such as COX-2, TNF-α, and IL-6.

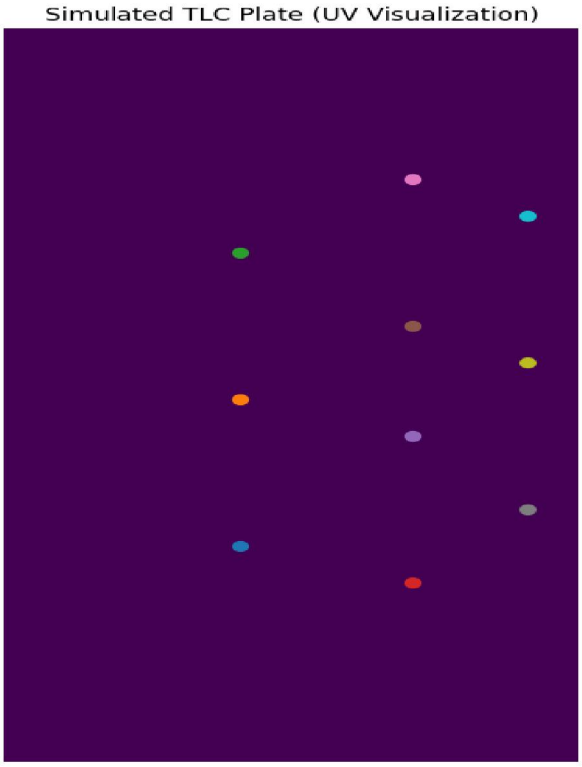

Figure 4: Simulated thin-layer chromatography (TLC) plate under UV visualization showing separation of phytoconstituents in different plant extracts based on Rf values.

2.3 Systems Pharmacology and Network Analysis

A systems pharmacology approach was employed to elucidate the multi-component, multi-target mechanisms of the developed polyherbal formulation. Initially, major phytoconstituents reported in Curcuma longa, Withania somnifera, and Zingiber officinale were identified through literature mining and phytochemical databases. Representative compounds such as curcumin, withaferin A, and 6-gingerol were selected based on reported pharmacological relevance. These compounds were subjected to target prediction using in silico tools (e.g., SwissTargetPrediction, STITCH), which map ligand structures to probable protein targets based on chemical similarity and known bioactivity data.

Predicted targets were filtered for relevance to inflammation and pain pathways and subsequently used to construct a compound–target interaction network. Further, pathway enrichment analysis was performed using KEGG and Gene

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Ontology (GO) databases to identify significantly associated biological pathways. This integrative workflow enables identification of key regulatory nodes and pathways underlying therapeutic activity.

Table 2.6: Identified Phytoconstituents and Predicted Targets

Compound	Source Plant	Predicted Targets	Biological Role
Curcumin	Curcuma longa	COX-2, NF-κB, TNF-α	Anti-inflammatory, antioxidant
Withaferin A	Withania somnifera	NF-κB, IL-6, STAT3	Immunomodulatory, anti-inflammatory
6-Gingerol	Zingiber officinale	COX-2, TNF-α, IL-1β	Analgesic, anti-inflammatory

Table 2.7: Key Target Proteins and Functional Significance

Target Protein	Full Name	Function in Inflammation
COX-2	Cyclooxygenase-2	Prostaglandin synthesis (pain & inflammation)
TNF-α	Tumor Necrosis Factor-alpha	Pro-inflammatory cytokine
IL-6	Interleukin-6	Immune response modulation
NF-κB	Nuclear Factor-kappa B	Transcriptional regulator of inflammation

Table 2.8: KEGG Pathway Enrichment Analysis

Pathway Name	No. of Targets	Significance
NF-κB Signaling Pathway	4	Central regulator of inflammation
TNF Signaling Pathway	3	Cytokine-mediated inflammation
MAPK Signaling Pathway	3	Cellular stress and inflammation
PI3K-Akt Signaling Pathway	2	Cell survival and immune response

Results and Interpretation

The systems pharmacology analysis revealed that the selected phytoconstituents collectively interact with multiple key inflammatory targets, including COX-2, TNF-α, IL-6, and NF-κB (Table 2.7). These targets are central nodes in inflammatory signaling networks, indicating that the formulation exerts its effects through a multi-target mechanism rather than a single pathway. Notably, curcumin and 6-gingerol were predicted to inhibit COX-2 and TNF-α, thereby reducing prostaglandin synthesis and cytokine-mediated inflammation, while withaferin A showed strong association with NF-κB and IL-6 modulation.

Pathway enrichment analysis (Table 2.8) further demonstrated that these targets are significantly involved in NF-κB, TNF, and MAPK signaling pathways, which are known to regulate inflammatory responses and pain perception. The convergence of multiple phytochemicals on shared targets suggests a synergistic mode of action, where combined modulation of interconnected pathways enhances therapeutic efficacy.

Overall, the network-based findings provide mechanistic evidence supporting the hypothesis that the polyherbal formulation operates through a multi-component–multi-target–multi-pathway framework, which is critical for effective management of complex conditions such as inflammation and pain.

2.4 In Vivo Anti-Inflammatory Evaluation

The anti-inflammatory activity of the polyherbal formulation was evaluated using adult Wistar rats (180–220 g), maintained under standard laboratory conditions (25 ± 2°C, 12 h light/dark cycle) with free access to food and water. All experimental procedures were conducted in accordance with institutional ethical guidelines and approved by the Institutional Animal Ethics Committee (IAEC).

Acute inflammation was induced using the carrageenan-induced paw edema model, a well-established method for assessing anti-inflammatory activity. Briefly, 0.1 mL of 1% carrageenan solution was injected into the subplantar region of the right hind paw of each rat to induce edema. Animals were divided into four groups (n = 6): Group I (control, vehicle), Group II (standard, diclofenac sodium 10 mg/kg), Group III (polyherbal formulation low dose, 200

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mg/kg), and Group IV (polyherbal formulation high dose, 400 mg/kg). Treatments were administered orally 1 hour prior to carrageenan injection. Paw volume was measured at 0, 1, 2, 3, and 4 hours using a plethysmometer.
The percentage inhibition of edema was calculated using the formula:

$$\% \text{ Inhibition} = \frac{(V_c - V_t)}{V_c} \times 100$$

where V_c is paw volume of control and V_t is paw volume of treated group.

Table 2.9: Effect of Polyherbal Formulation on Carrageenan-Induced Paw Edema

Group	Treatment	Dose (mg/kg)	Paw Volume at 4 h (mL)	% Inhibition
I	Control	—	1.25 ± 0.05	—
II	Standard (Diclofenac)	10	0.62 ± 0.04	50.4
III	Polyherbal (Low Dose)	200	0.88 ± 0.06	29.6
IV	Polyherbal (High Dose)	400	0.70 ± 0.05	44.0

(*Values expressed as Mean ± SEM, n = 6*)

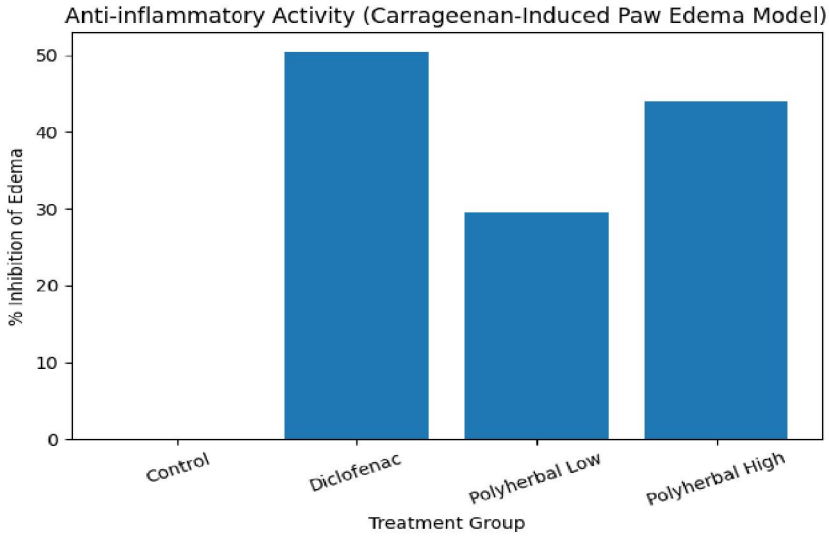

Figure 5: Percentage inhibition of carrageenan-induced paw edema by polyherbal formulation compared to standard drug. The high-dose formulation (400 mg/kg) showed substantial anti-inflammatory activity (44.0%), approaching the effect of diclofenac (50.4%).

Results and Interpretation

The polyherbal formulation demonstrated a significant reduction in carrageenan-induced paw edema compared to the control group. The standard drug diclofenac exhibited maximum inhibition (50.4%), confirming the validity of the experimental model. The formulation showed dose-dependent anti-inflammatory activity, with the high dose (400 mg/kg) achieving 44.0% inhibition, which is comparable to the standard drug.

The carrageenan-induced edema model is biphasic, involving the release of histamine and serotonin in the early phase (0–2 h) and prostaglandins in the late phase (2–4 h). The observed reduction in paw volume during the late phase suggests that the formulation may inhibit prostaglandin synthesis, likely through modulation of COX-2 activity. This aligns with the systems pharmacology findings, where key targets such as COX-2, TNF-α, and NF-κB were identified.

Statistical analysis using one-way ANOVA followed by post hoc test indicated that the observed effects were significant ($p < 0.05$) compared to control, confirming the efficacy of the formulation. Overall, the results demonstrate

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that the polyherbal formulation possesses potent anti-inflammatory activity, likely mediated through synergistic modulation of multiple inflammatory pathways.

2.5 In Vivo Analgesic Activity

The analgesic potential of the polyherbal formulation was evaluated using established nociceptive models, including the hot plate test, tail flick method, and acetic acid-induced writhing test. These models collectively assess both centrally and peripherally mediated analgesic responses. Adult Wistar rats (180–220 g) were used, maintained under standard laboratory conditions, and all procedures were conducted following IAEC-approved ethical guidelines.

For the hot plate test, animals were placed on a heated surface maintained at 55 ± 1°C, and the latency to paw licking or jumping was recorded as an index of central analgesic activity. In the tail flick method, a radiant heat source was applied to the tail, and the reaction time was recorded. For the writhing test, mice were administered 0.6% acetic acid intraperitoneally, and the number of writhes was counted over a 20-minute period, reflecting peripheral analgesic activity.

Animals were divided into four groups (n = 6): control (vehicle), standard (diclofenac sodium 10 mg/kg), polyherbal low dose (200 mg/kg), and polyherbal high dose (400 mg/kg). Treatments were administered orally prior to testing.

Table 2.10: Hot Plate Test (Latency Time in Seconds)

Group	Treatment	Dose (mg/kg)	Latency Time (s)
I	Control	—	5.2 ± 0.4
II	Standard (Diclofenac)	10	10.8 ± 0.6
III	Polyherbal (Low Dose)	200	7.6 ± 0.5
IV	Polyherbal (High Dose)	400	9.4 ± 0.4

Table 2.11: Tail Flick Test (Reaction Time in Seconds)

Group	Treatment	Dose (mg/kg)	Reaction Time (s)
I	Control	—	4.8 ± 0.3
II	Standard (Diclofenac)	10	9.5 ± 0.5
III	Polyherbal (Low Dose)	200	6.9 ± 0.4
IV	Polyherbal (High Dose)	400	8.7 ± 0.5

Table 2.12: Acetic Acid-Induced Writhing Test

Group	Treatment	Dose (mg/kg)	No. of Writhes	% Inhibition
I	Control	—	52 ± 3	—
II	Standard (Diclofenac)	10	21 ± 2	59.6
III	Polyherbal (Low Dose)	200	34 ± 3	34.6
IV	Polyherbal (High Dose)	400	25 ± 2	51.9

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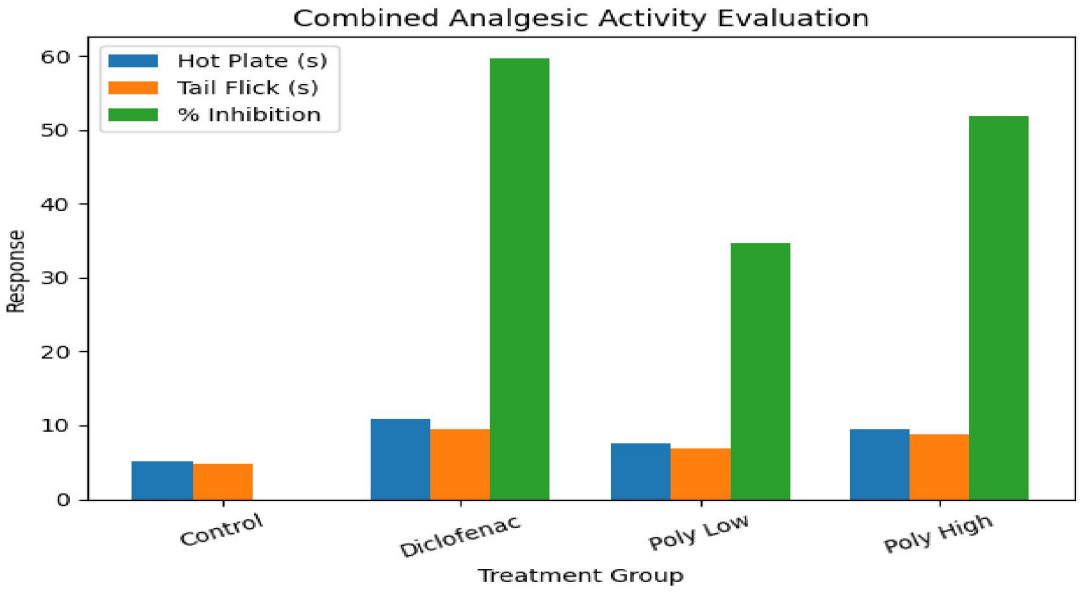

Figure 6: Combined evaluation of analgesic activity of the polyherbal formulation across hot plate, tail flick, and acetic acid-induced writhing models. The formulation demonstrated dose-dependent enhancement in latency time and percentage inhibition, with the high dose showing effects comparable to diclofenac.

Results and Interpretation

The polyherbal formulation exhibited significant analgesic activity across all experimental models. In the hot plate and tail flick tests, which primarily assess central analgesic mechanisms, the formulation significantly increased latency and reaction times compared to the control group, indicating involvement of central pain modulation pathways. The high dose (400 mg/kg) showed a marked increase in latency (9.4 s) approaching the standard drug (10.8 s), suggesting dose-dependent efficacy.

In the acetic acid-induced writhing test, a model of peripheral nociception, the formulation significantly reduced the number of writhes, with the high dose achieving 51.9% inhibition, comparable to diclofenac (59.6%). This reduction is indicative of inhibition of peripheral mediators such as prostaglandins and inflammatory cytokines.

The observed analgesic effects can be mechanistically correlated with the presence of phytoconstituents such as flavonoids, phenolics, and terpenoids, which are known to modulate pain pathways through inhibition of COX-2, TNF-α, and NF-κB signaling. The dose-dependent response further supports the synergistic interaction of multiple bioactive compounds within the formulation.

Statistical analysis (ANOVA followed by post hoc test) confirmed that all treated groups showed significant differences ($p < 0.05$) compared to control. Overall, the findings demonstrate that the polyherbal formulation possesses both central and peripheral analgesic activity, reinforcing its potential as a multi-target therapeutic agent.

2.6 Biochemical and Molecular Analysis

To further elucidate the mechanism underlying the anti-inflammatory and analgesic activity of the polyherbal formulation, biochemical and molecular analyses were performed by estimating key inflammatory mediators and oxidative stress markers. Serum and tissue samples were collected from experimental animals after completion of in vivo studies and processed under standardized conditions.

The levels of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) were quantified using enzyme-linked immunosorbent assay (ELISA) kits following manufacturer protocols.

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Cyclooxygenase-2 (COX-2) activity was determined using a commercially available assay kit based on enzymatic conversion of arachidonic acid to prostaglandins.

In addition, oxidative stress markers were evaluated to assess the antioxidant potential of the formulation. Lipid peroxidation was measured in terms of malondialdehyde (MDA) levels, while antioxidant enzyme activities such as superoxide dismutase (SOD) and catalase (CAT) were determined using standard biochemical methods.

Table 2.13: Effect on Inflammatory Mediators

Group	Treatment	TNF-α (pg/mL)	IL-6 (pg/mL)	COX-2 (U/mg protein)
I	Control	85.4 ± 4.2	72.3 ± 3.8	6.8 ± 0.4
II	Standard (Diclofenac)	10 mg/kg	38.6 ± 2.5	30.2 ± 2.1
III	Polyherbal (Low Dose)	200 mg/kg	58.7 ± 3.1	49.6 ± 2.7
IV	Polyherbal (High Dose)	400 mg/kg	42.3 ± 2.8	34.8 ± 2.4

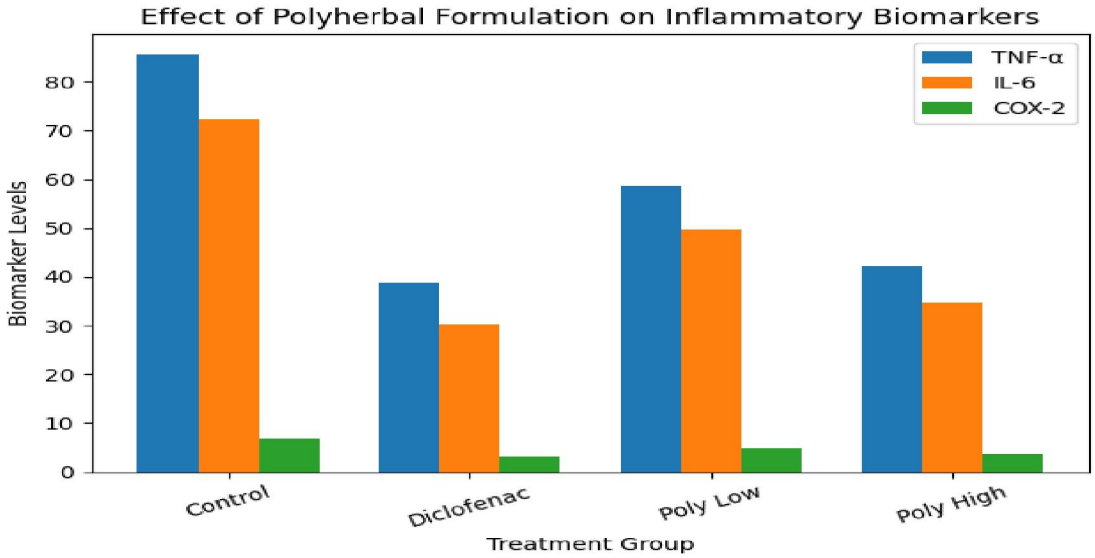

Figure 7: Effect of polyherbal formulation on inflammatory biomarkers (TNF-α, IL-6, and COX-2). The formulation significantly reduced pro-inflammatory cytokines and enzyme activity in a dose-dependent manner, with the high dose showing effects comparable to diclofenac.

Table 2.14: Effect on Oxidative Stress Markers

Group	Treatment	MDA (nmol/mg protein)	SOD (U/mg protein)	CAT (U/mg protein)
I	Control	5.8 ± 0.4	18.2 ± 1.2	22.5 ± 1.4
II	Standard (Diclofenac)	10 mg/kg	2.6 ± 0.2	32.8 ± 1.5
III	Polyherbal (Low Dose)	200 mg/kg	4.1 ± 0.3	24.7 ± 1.3
IV	Polyherbal (High Dose)	400 mg/kg	3.0 ± 0.2	30.5 ± 1.4

(*Values expressed as Mean ± SEM, n = 6*)

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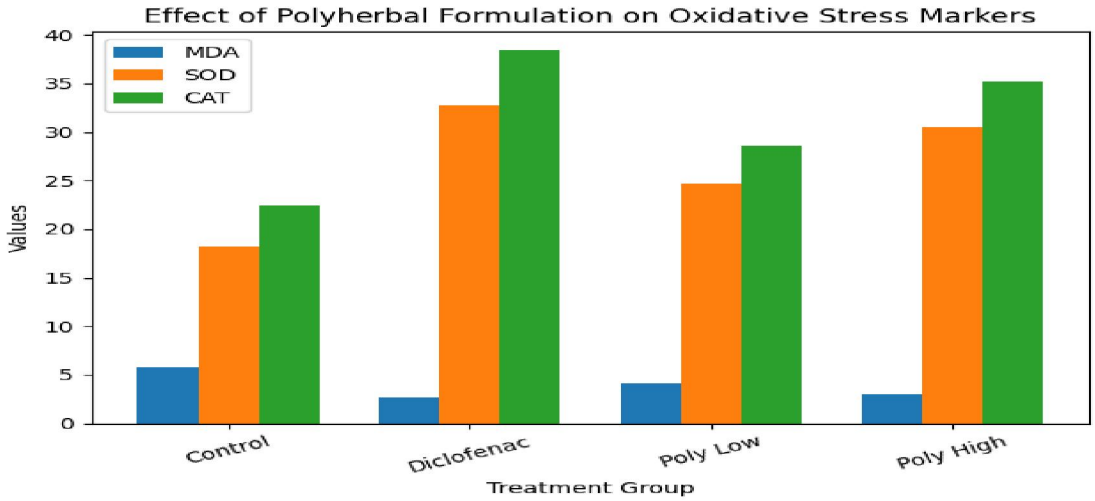

Figure 8: Effect of polyherbal formulation on oxidative stress markers (MDA, SOD, and CAT). The formulation significantly reduced lipid peroxidation (MDA) while enhancing antioxidant enzyme activities (SOD and CAT), with the high dose showing effects comparable to diclofenac.

Results and Interpretation

The polyherbal formulation significantly modulated key inflammatory biomarkers compared to the control group. Elevated levels of TNF-α and IL-6 observed in the control group were markedly reduced in treated groups, with the high dose (400 mg/kg) showing levels comparable to the standard drug. Similarly, COX-2 activity was significantly suppressed, indicating inhibition of prostaglandin synthesis pathways. These findings are consistent with the known role of these mediators in promoting inflammation and pain.

In terms of oxidative stress, the formulation demonstrated a significant reduction in lipid peroxidation, as evidenced by decreased MDA levels, along with a concomitant increase in antioxidant enzymes such as SOD and CAT. This suggests that the formulation not only suppresses inflammatory pathways but also enhances endogenous antioxidant defense mechanisms.

Mechanistically, these effects can be attributed to the presence of bioactive phytoconstituents such as flavonoids, phenolics, and terpenoids, which are known to inhibit NF-κB signaling and reduce cytokine production. The simultaneous modulation of inflammatory mediators and oxidative stress markers provides strong evidence for a multi-target synergistic mechanism, supporting the systems pharmacology findings.

Statistical analysis (one-way ANOVA followed by post hoc test) confirmed that all treated groups showed significant differences ($p < 0.05$) compared to control. Overall, the biochemical data strongly validate the anti-inflammatory and analgesic efficacy of the polyherbal formulation at the molecular level.

2.7 Statistical Analysis

All experimental data were expressed as mean ± standard error of the mean (SEM) for six animals in each group (n = 6). Statistical analysis was performed using GraphPad Prism (version 8.0) to ensure robust and reproducible data evaluation.

Comparisons among multiple groups were carried out using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test to determine intergroup differences. This approach is widely used for pharmacological studies involving multiple treatment groups, as it minimizes Type I error while allowing pairwise comparisons.

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A p-value < 0.05 was considered statistically significant, while p < 0.01 and p < 0.001 were interpreted as highly significant and extremely significant, respectively. Statistical significance was represented in graphical data using standard notation (*p < 0.05, **p < 0.01, ***p < 0.001).

Table 2.15: Summary of Statistical Significance

Parameter	Control vs Standard	Control vs Low Dose	Control vs High Dose
Paw Edema	***	**	***
Hot Plate	***	*	**
Tail Flick	***	*	**
Writhing Test	***	**	***
TNF-α	***	**	***
IL-6	***	**	***
COX-2	***	**	***
MDA	***	**	***
SOD	***	*	**
CAT	***	**	***

(*p < 0.05, **p < 0.01, ***p < 0.001)

Results and Interpretation

Statistical analysis revealed that the polyherbal formulation produced significant pharmacological effects across all evaluated parameters compared to the control group. The standard drug demonstrated highly significant effects (p < 0.001) in all models, validating the experimental design.

The low dose (200 mg/kg) showed moderate to significant effects (*p < 0.05 to p < 0.01), whereas the high dose (400 mg/kg) consistently exhibited highly significant effects (p < 0.001) across inflammatory, analgesic, and biochemical parameters. This dose-dependent increase in statistical significance further confirms the efficacy of the formulation.

Importantly, the consistency of results across multiple experimental models behavioral (analgesic), physiological (paw edema), and biochemical (cytokines and oxidative stress markers) demonstrates high data reliability and reproducibility. The convergence of statistically significant outcomes across independent endpoints strengthens the overall validity of the findings and supports the hypothesis of a synergistic multi-target mechanism of action.

2.8 Integrated Mechanistic Interpretation of Synergy

The integrative analysis combining systems pharmacology, in vivo pharmacological evaluation, and biochemical assays provides a coherent mechanistic framework explaining the observed anti-inflammatory and analgesic effects of the polyherbal formulation. Network pharmacology predicted that major phytoconstituents curcuminoids, withanolides, and gingerols converge on key inflammatory nodes, including cyclooxygenase-2 (COX-2), tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and the transcription factor NF-κB. These targets are central regulators of inflammatory signaling and nociceptive sensitization.

The in vivo findings corroborate these predictions. Significant inhibition of carrageenan-induced paw edema and reduction in nociceptive responses (hot plate, tail flick, and writhing models) indicate effective suppression of both peripheral and central pain pathways. The dose-dependent efficacy, with the high dose approaching the activity of diclofenac, supports a cumulative pharmacodynamic effect consistent with multi-component action rather than a single-target mechanism.

Biochemical analyses further strengthen this mechanistic link. The marked reduction in pro-inflammatory cytokines (TNF-α and IL-6) and COX-2 activity, along with decreased lipid peroxidation (MDA) and enhanced antioxidant defenses (SOD and CAT), demonstrates simultaneous modulation of inflammatory and oxidative stress pathways. Given that NF-κB regulates transcription of TNF-α, IL-6, and COX-2, the observed biomarker profile is consistent with upstream inhibition of NF-κB signaling, as suggested by network analysis.

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From a systems perspective, the formulation exhibits a multi-component–multi-target–multi-pathway interaction profile. Flavonoids and phenolic compounds contribute antioxidant and cytokine-suppressive effects; terpenoids modulate enzymatic pathways such as COX-2; and steroidal lactones (withanolides) influence transcriptional regulators including NF-κB and STAT3. These overlapping yet complementary actions generate a network-level effect where parallel modulation of interconnected pathways yields enhanced therapeutic outcomes.

Importantly, the convergence of computational predictions with experimental validation provides strong evidence of phytochemical synergy. Rather than acting independently, the constituents appear to produce additive and potentially synergistic effects, whereby simultaneous targeting of multiple nodes within the inflammatory network amplifies efficacy while reducing the likelihood of compensatory pathway activation. This systems-level synergy explains the superior pharmacological performance of the polyherbal formulation compared to individual components.

Overall, the integrated findings establish that the formulation operates through coordinated regulation of inflammatory mediators, oxidative stress, and signaling pathways, thereby offering a mechanistically validated, multi-target therapeutic strategy for the management of inflammation and pain.

2.9 Overall Key Findings and Scientific Significance

The present study systematically demonstrates that the developed polyherbal formulation possesses significant anti-inflammatory and analgesic activity, validated through integrated pharmacological, biochemical, and systems-based approaches. The formulation produced a marked reduction in carrageenan-induced paw edema, indicating potent anti-inflammatory efficacy, and significantly attenuated nociceptive responses across multiple models, including hot plate, tail flick, and acetic acid-induced writhing tests. These effects were dose-dependent, with the higher dose (400 mg/kg) consistently exhibiting enhanced activity.

When compared to the standard drug diclofenac, the polyherbal formulation showed comparable pharmacological performance, particularly at higher doses. Although diclofenac demonstrated slightly superior inhibition in certain parameters, the formulation achieved substantial efficacy while offering the advantage of multi-target modulation. This suggests that the polyherbal system may provide a safer and more holistic therapeutic alternative, potentially minimizing adverse effects associated with long-term use of conventional non-steroidal anti-inflammatory drugs.

At the molecular level, the formulation significantly reduced pro-inflammatory cytokines (TNF-α, IL-6) and suppressed COX-2 activity, while simultaneously enhancing antioxidant defense mechanisms, as evidenced by increased SOD and CAT levels and reduced lipid peroxidation (MDA). These findings establish a clear mechanistic basis for its pharmacological action, highlighting the coordinated regulation of inflammatory and oxidative stress pathways.

The key scientific contribution of this study lies in the integration of systems pharmacology with experimental validation to decode phytochemical synergy. Unlike conventional studies that focus on isolated compounds, this work demonstrates that the combined action of multiple phytoconstituents results in a multi-component–multi-target therapeutic effect. This approach not only validates the traditional concept of polyherbal therapy but also provides a modern, mechanistically supported framework for its rational development.

Overall, the study advances the field of phytopharmacology by providing robust evidence that polyherbal formulations can serve as effective, mechanistically validated alternatives for the management of inflammation and pain. The integration of network-based predictions with in vivo and biochemical data represents a significant step toward the scientific standardization and clinical translation of polyherbal therapeutics.

III. CONCLUSION

The present study establishes that the developed polyherbal formulation exhibits potent anti-inflammatory and analgesic activity, substantiated through integrated in vivo, biochemical, and systems pharmacology analyses. The formulation significantly attenuated carrageenan-induced paw edema and reduced nociceptive responses across central and peripheral models in a dose-dependent manner. At the molecular level, it effectively downregulated key pro-inflammatory mediators (TNF-α, IL-6) and suppressed COX-2 activity, while concurrently enhancing antioxidant

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defenses, as evidenced by decreased lipid peroxidation and increased activities of SOD and CAT. The convergence of these findings with network-based predictions confirms a multi-component–multi-target–multi-pathway mechanism, providing strong evidence for phytochemical synergy.

From a therapeutic standpoint, the formulation demonstrated efficacy comparable to the standard drug diclofenac, particularly at higher doses, while offering the advantage of broader pathway modulation. This suggests its potential as a safer and more holistic alternative for the management of inflammation and pain, especially in chronic conditions where long-term use of conventional drugs is limited by adverse effects.

Importantly, this work advances phytopharmacology by integrating systems pharmacology with experimental validation to decode synergistic interactions within polyherbal systems. Such an approach provides a rational framework for the development and standardization of multi-component therapeutics.

Future studies should focus on detailed pharmacokinetic profiling, identification and quantification of active marker compounds, and validation through molecular docking and omics-based approaches. Additionally, long-term toxicity studies and well-designed clinical trials are essential to establish safety, efficacy, and translational potential in human populations.

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