RESEARCH PAPER

Formulation, Optimization and Characterization of Polyherbal Phytosome for Arthritis

Palak Jain[1], Jitendra Banweer[2],
[1]School of Pharmaceutical Sciences,
Sanjeev Agrawal Global Educational University, Bhopal
Email ID: palakjain.sunrise@gmail.com.
[2]School of Pharmaceutical Sciences,
Sanjeev Agrawal Global Educational University, Bhopal
Email ID: jitendra.b@sageuniversity.edu.in

ABSTRACT
Arthritis, a chronic inflammatory disorder causing joint pain, swelling, and structural damage, is conventionally managed with NSAIDs and corticosteroids that offer symptomatic relief but pose significant long-term side effects, prompting exploration of safer herbal alternatives rich in anti-inflammatory phytoconstituents. This study formulated, optimized, characterized, and evaluated a polyherbal gel incorporating luteolin (*Vitex negundo*), nyctanthoside (*Nyctanthes arbor-tristis*), capsaicin (*Capsicum annuum*), and 3-O-acetyl-11-keto-β-boswellic acid (AKBA; *Boswellia serrata*) for anti-arthritic potential. Authenticated plant extracts underwent phytochemical screening, phenolic/flavonoid quantification, and RP-HPLC validation (accuracy 98-102%, %RSD ≤2) for simultaneous bioactive estimation, followed by Box-Behnken design optimization targeting viscosity, spreadability, and pH, with ICH stability confirming >95% drug retention over 90 days. The optimized VNBC-25 gel exhibited ideal viscosity (14,300 cps), spreadability (15.2 g·cm/s), and skin-compatible pH (6.3), demonstrating in vitro efficacy through 82% HRBC membrane stabilization, 80% BSA protein denaturation inhibition (IC_{50}=115 µg/mL), and COX-2 selective inhibition (IC_{50}=5.6 µg/mL, SI=5.13). In CFA-induced rat arthritis models, VNBC-25 significantly reduced paw edema (>55% by day 28), restored hematological/biochemical markers (RF, CRP, TNF-α, NO), preserved body weight/organ indices, and protected joint architecture per radiographic/histopathological analyses, matching diclofenac gel efficacy. VNBC-25 polyherbal gel proved safe, stable, and effective against arthritis inflammation and joint damage, offering diclofenac-comparable efficacy with superior COX-2 selectivity and natural safety, positioning it as a promising translational alternative for long-term arthritis management.
Keywords: Arthritis, polyherbal phytosome, luteolin, nyctanthoside, capsaicin, AKBA, Box-Behnken design, anti-arthritic, CFA model, COX-2 selective
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INTRODUCTION

Arthritis, encompassing rheumatoid arthritis (RA) and osteoarthritis (OA), imposes a profound global health burden through chronic synovial inflammation, cartilage erosion, severe pain, and disability, affecting over 500 million people and driving immense socioeconomic costs via lost productivity and healthcare demands. Standard treatments like NSAIDs, corticosteroids, and DMARDs deliver symptom control and disease modification yet face limitations from gastrointestinal toxicity, cardiovascular events, hepatotoxicity, immunosuppression, incomplete responses, and poor long-term adherence.[1,2]

These four herbs—Nirgundi (Vitex negundo), Parijat (Nyctanthes arbor-tristis), Rakt Mirch (Capsicum frutescens), and Shallaki (Boswellia serrata)—were selected for their complementary, multi-target anti-arthritic actions rooted in Ayurvedic tradition and validated by modern evidence: Nirgundi offers potent NF-κB/COX-2 inhibition and antioxidant protection against edema and oxidative joint damage; Parijat reduces TNF-α/IL-1β cytokines, pannus formation, and lysosomal enzymes in RA models; Rakt Mirch delivers capsaicin-driven analgesia by desensitizing nociceptors and depleting substance P for rapid pain relief; Shallaki uniquely blocks 5-lipoxygenase/leukotriene pathways, preserving cartilage and improving WOMAC scores in clinical OA/RA trials. This rational polyherbal synergy addresses arthritis's multifactorial pathology—inflammation, pain, oxidative stress, and degradation—more effectively than monotherapy, as preclinical combinations show superior paw edema reduction and histopathology improvements versus single herbs.[3, 4, 5, 6, 7, 8]

However, key phytoconstituents (flavonoids, iridoids, boswellic acids, capsaicin) exhibit poor solubility (<1%), extensive first-pass metabolism, and bioavailability (<5%), hindering joint-targeted efficacy. Phytosome technology circumvents these via stable phyto-phospholipid complexes (typically phosphatidylcholine), boosting lymphatic absorption, plasma Cmax (5-29-fold), and synovial retention, as proven by curcumin-boswellia phytosomes (e.g., Meriva®) yielding better pain relief and cartilage protection in randomized trials. [9, 10, 11, 12, 13]

The novel polyherbal phytosome integrates these herbs to harness synergistic efficacy with enhanced pharmacokinetics, enabling lower doses, reduced toxicity, and sustained joint delivery for RA/OA. Systematic formulation, Box-Behnken optimization, and characterization target critical attributes like particle size, entrapment efficiency, release kinetics, stability, and in vivo performance, advancing clinically viable phytopharmaceuticals. [14]

MATERIAL AD METHOD

Collection, Authentication, and Extraction of Plant Materials

Raw plant materials of Vitex negundo (Nirgundi), Nyctanthes arbor-tristis (Parijat), Capsicum annuum (Rakt Mirch), and Boswellia serrata (Shallaki) were procured from a certified herbal supplier. Each species was authenticated by a recognized taxonomist (Authentication Numbers: Vitex negundo-PDPL/VN/231001, Nyctanthes arbor-tristis-PDPL/NAT/231001, Capsicum annuum-PDPL/CF/231001, Boswellia serrata-PDPL/BS/231001), with voucher specimens deposited in a certified herbarium for reference. The materials were cleaned, shade-dried, pulverized to fine powder using a mechanical grinder, and stored.[15, 16]

Each powdered plant (100 g) underwent Soxhlet extraction with 715, 160% ethanol for 6-8 hours, followed by solvent concentration via rotary evaporator at 40-50°C under reduced pressure. The semisolid extracts were vacuum-dried and stored in desiccators at 4°C. [17, 18]

Preliminary Phytochemical Screening

Dried ethanolic extracts were subjected to standard qualitative tests for major phytoconstituents: alkaloids (Dragendorff's and Mayer's reagents), flavonoids (Shinoda and alkaline reagent tests), phenols/tannins (ferric chloride and lead acetate), saponins (froth test), terpenoids (Salkowski reaction), and glycosides (Keller-Killiani test). [19, 20, 21]

Total Phenolic Content

Total phenolic content was quantified by the Folin-Ciocalteu method: 1 mL extract was mixed with 5 mL 10% Folin-Ciocalteu reagent and 4 mL 7.5% sodium carbonate, incubated in the dark for 30 min, and absorbance measured at 760 nm using gallic acid as standard (results as mg GAE/g extract). [22]

Total Flavonoid Content

Total flavonoid content was assessed via aluminium chloride method: extract was combined with 2% $AlCl_3$, incubated for 30 min, and absorbance read at 415 nm with quercetin as standard (results as mg QE/g extract). [23]

Formulation of Polyherbal Phytosome

The phytosome formulation was prepared using the thin-film hydration method, in which the four selected phytoconstituents—Luteolin (from Vitex negundo), Nyctanthoside (from Nyctanthes arbor-tristis), Capsaicin (from Capsicum annuum), and 3-Acetyl-11-keto-β-boswellic acid (AKBA, from Boswellia serrata)—were accurately weighed and dissolved together with phosphatidylcholine in a organic solvent system chloroform : methanol (2:1). The mixture was transferred into a round-bottom flask and subjected to rotary evaporation under reduced pressure at 40–45°C to evaporate the solvent, resulting in the formation of a thin, uniform lipid film on the flask wall. The dried film was then hydrated with phosphate buffer (pH 7.4) under gentle agitation to allow the phytoconstituents to complex with phosphatidylcholine, forming phytosomes. The hydrated dispersion was further subjected to sonication to reduce particle size and obtain a uniform phytosomal suspension, which was finally stored at 4°C for further characterization.[24]

Optimization of Phytosome Formulation

A 3^2 factorial design had been applied to optimize using Design Expert 10.0.1, developed by Stat Ease, Inc the concentrations of Vitex negundo and Boswellia serrata extracts, with Capsicum annuum and Nyctanthes arbor-tristis held constant. [15]

Table 1: Independent Variables (Factors)

Factor Code	Independent Variable	Unit	Level -1 (Low)	Level 0 (Medium)	Level +1 (High)
A	Conc. of Phosphatidylcholine (PC)	mg	50	100	150
B	Drug-to-Lipid Ratio (Total phytoconstituents : PC)	w/w	1:1	1:2	1:3
C	Hydration Time	minutes	20	35	50
D	Hydration Temperature	°C	40	50	60

Table 2: Dependent Variables (Responses)

Response Code	Dependent Variable	Unit	Target/Goal
R1	Particle Size	nm	Minimize
R2	Polydispersity Index (PDI)	–	Minimize
R3	Zeta Potential	mV	Maximize (Higher stability)
R4	Entrapment Efficiency (EE)	%	Maximize

Characterization of Phytosomes

Particle Size

The particle size of the phytosomal formulations was measured using dynamic light scattering (DLS), where a suitably diluted sample was analyzed at 25 °C, and the mean hydrodynamic diameter (Z-average) was recorded to determine the vesicle size and assess the suitability of the formulation for topical delivery.

Polydispersity Index (PDI)

The polydispersity index (PDI) was obtained simultaneously during DLS measurement and used to evaluate the uniformity of the particle size distribution, where lower PDI values indicated greater homogeneity and stability of the phytosomal system.

Zeta Potential

The zeta potential of the phytosomes was determined using electrophoretic light scattering after appropriate dilution with ultrapure water, and the surface charge values (expressed in millivolts) were used to predict the electrostatic stability and aggregation potential of the formulation.

Entrapment Efficiency

The entrapment efficiency (EE) of the phytosomes was assessed by separating the free, unencapsulated drug through high-speed centrifugation and quantifying both the total and free drug content using a validated UV/HPLC method, after which EE (%) was calculated to determine the effectiveness of drug loading within the phytosomal vesicles. [25]

Evaluation of Anti-Arthritic Activity in Vitro Studies

Human Red Blood Cell (HRBC) Membrane-Stabilizing Assay

Fresh heparinized human blood was collected from healthy volunteers with ethics approval and informed consent, and red blood cells (RBCs) were isolated by washing three to four times with phosphate-buffered saline (PBS, 0.15 M, pH 7.4) at 3000 rpm for 10 min to prepare a 10% v/v RBC suspension in PBS. Test samples (hydroalcoholic gel extracts) and diclofenac sodium standard were prepared at graded concentrations (25-400 µg/mL) in PBS, and for heat-induced haemolysis, 1 mL of each test/standard/vehicle blank was mixed with 1 mL 10% RBC suspension, incubated at 56°C for 30 min in a water bath, cooled to room temperature, and centrifuged at 3000 rpm for 10 min, with absorbance of the supernatant measured at 540 nm using a spectrophotometer. For hypotonicity-induced haemolysis, 0.5 mL test sample was combined with 0.5 mL RBC suspension and 10 mL hypotonic saline (0.25% NaCl), processed similarly, and percent inhibition of haemolysis calculated relative to controls using the formula: % Inhibition = [(Abs_control - Abs_sample)/Abs_control] × 100, with IC50 values determined from dose-response curves (n=3). [26]

Bovine Serum Albumin (BSA) Protein Denaturation Assay

A 1% w/v bovine serum albumin (BSA) solution was prepared in Tris-HCl buffer (0.1 M, pH 6.3), and 0.5 mL BSA was mixed with 0.5 mL test extract/standard (diclofenac sodium, 25-400 µg/mL) or vehicle in 4.0 mL Tris-HCl buffer to achieve a final BSA concentration of 0.1% w/v. The mixtures were incubated at 37°C for 20 min followed by heating at 70°C for 5 min in a water bath, cooled to room temperature, and absorbance measured at 660 nm against a buffer blank using a spectrophotometer. Percent inhibition of protein denaturation was calculated as: % Inhibition = [(Abs_control - Abs_sample)/Abs_control] × 100, and IC50 values were determined from concentration-response plots (n=3). [27]

Cyclooxygenase (COX-1/COX-2) Inhibition Assay

Purified COX-1 and COX-2 enzymes were pre-incubated with heme, assay buffer (100 mM Tris pH 8.0, 5 mM EDTA), test extracts/standards (indomethacin for COX-1, celecoxib for COX-2 selectivity; 1-500 µg/mL), or vehicle in a 96-well plate on ice for 10 min at 25°C, after which the reaction was initiated by adding arachidonic acid substrate and TMPD chromogen, and kinetic absorbance readings taken at 590 nm for 5-10 min using a microplate reader. Initial reaction rates (slopes) were calculated for each well, percent inhibition determined relative to enzyme controls, IC50 values computed for COX-1 and COX-2 using non-linear regression, and selectivity index calculated as IC50(COX-1)/IC50(COX-2) to assess preferential COX-2 inhibition (n=3). [28]

In Vivo Anti-Arthritic Studies (Complete Freund's Adjuvant Model)

The study protocol was approved by the Institutional Animal Ethics Committee following CPCSEA guidelines, with analgesia provided as needed to minimize distress. Male Wistar rats (180-220 g) were acclimatized for 7 days under standard housing conditions (22±2°C, 50-60% RH, 12 h light/dark cycle, standard chow/water ad libitum), and arthritis was induced on day 0 by intra-plantar injection of 0.1 mL Complete Freund's Adjuvant (CFA; 1 mg/mL heat-killed Mycobacterium tuberculosis in mineral oil) into the left hind paw, with disease progression monitored until day 14. Animals were randomized into groups (n=6/group): normal control (no CFA, vehicle gel), arthritic control (CFA + vehicle gel), standard (CFA + 1% diclofenac gel, ~1 mg/kg topical OD), test-low (CFA + polyherbal gel 0.5 g/kg equivalent), and test-high (CFA + polyherbal gel 1.0 g/kg equivalent), with formulations gently applied to shaved ankle/paw regions daily from day 7-28 for 30-60 s contact time. Acute dermal irritation was assessed per OECD 404 by applying ~0.5 g gel to clipped dorsal skin under occlusive patch for 4 h, scoring erythema/edema at 1, 24, 48, 72 h, and calculating Primary Irritation Index.

Clinical and Physical Measurements

Paw volume was measured using a mercury/water plethysmometer on days 0, 7, 14, 21, and 28, with change in

paw volume (Δ volume) and percent inhibition of edema calculated as: % Inhibition = [(Vc - Vt)/Vc] × 100 (Vc=arthritic control, Vt=treated). Body weights were recorded weekly to assess systemic disease severity, and at necropsy on day 28, organs (liver, kidney, spleen, thymus) were excised, weighed, and relative organ weights computed as (organ weight/body weight) × 100.

Radiography

Rats were anesthetized, hind paws and knee joints positioned consistently, and X-rays captured at small-animal settings (40-45 kVp, 2-5 mAs), with images scored semi-quantitatively (0-4 scale) for bone erosion, joint space narrowing, and soft tissue swelling by blinded observers.

Histopathology of Knee/Ankle Joint

Knee/ankle joints were disarticulated, fixed in 10% neutral buffered formalin for 24-48 h, decalcified in 10% EDTA (pH 7.4) for 1-2 weeks with solution changes every 2-3 days, dehydrated, cleared, embedded in paraffin, sectioned at 5 µm, and stained with H&E (for synovial hyperplasia, inflammation, pannus) and Safranin-O/Fast Green (for cartilage proteoglycan loss). Sections were evaluated microscopically (10×/40×) and scored (0=none to 3/4=severe) for inflammatory infiltration, cartilage erosion, bone destruction, and pannus formation by independent pathologists. [29]

Haematology and Biochemistry

Blood was collected via retro-orbital plexus or tail vein under light isoflurane anesthesia at baseline and terminally by cardiac puncture under deep anesthesia, with haematological parameters (Hb, RBC, WBC, platelets, ESR by Wintrobe method, HCT/PCV) analyzed using an automated analyzer. Serum/plasma was separated by centrifugation (3000 rpm, 10 min, 4°C), stored at -20°C, and biochemical markers (CRP, RF, cytokines) quantified by ELISA kits per manufacturer protocols.

Rheumatoid Factor (RF):

Serum rheumatoid factor levels were quantified using a rat-specific immunoturbidimetric assay kit (or ELISA kit, as applicable). Briefly, serum samples were collected and incubated with latex particles coated with human IgG, leading to agglutination in the presence of RF. The change in turbidity was measured spectrophotometrically, and results were expressed in IU/mL against a calibration curve prepared from kit standards. [30]

C-Reactive Protein (CRP):

High-sensitivity C-reactive protein (hs-CRP) was estimated using a rat-specific hs-CRP ELISA kit following the manufacturer's protocol. Standards and diluted serum samples were added to pre-coated wells, followed by the addition of enzyme conjugate. After incubation and washing, substrate solution was added, and the reaction was stopped with a stop solution. The absorbance was measured at 450 nm, and CRP concentration was calculated from the standard curve and expressed in mg/L. [31]

Tumour Necrosis Factor-α (TNF-α):

Serum TNF-α levels were determined using a sandwich ELISA kit. Standards, controls, and serum samples were pipetted into wells pre-coated with anti-TNF-α antibody. After incubation and washing, biotinylated detection antibody and streptavidin-HRP were sequentially added. Following substrate reaction, the absorbance was measured at 450 nm. The concentrations were interpolated from the standard curve and expressed in pg/mL. [32]

Nitric Oxide (NO) Metabolites:

Nitric oxide metabolites (nitrite) were quantified using the Griess assay. Serum proteins were first precipitated by adding zinc sulphate solution and centrifuging to obtain a clear supernatant. Equal volumes of the supernatant and freshly prepared Griess reagent (1% sulphanilamide and 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride in 2.5% phosphoric acid) were mixed and incubated for 10 minutes at room temperature. The resulting pink azo dye was measured spectrophotometrically at 540 nm. Nitrite concentration was determined from a sodium nitrite standard curve and expressed as µM. [33]

Homocysteine:

Serum homocysteine was estimated using an enzymatic cycling assay (or ELISA kit). In the enzymatic assay, serum homocysteine was converted to S-adenosylhomocysteine, which was subsequently hydrolysed and quantified through a cycling reaction producing NADH. The rate of NADH formation was measured spectrophotometrically, and concentrations were calculated from the calibration curve and expressed in µmol/L. [34]

Serum Cortisol:

Cortisol levels in rat serum were determined using a competitive ELISA kit. Serum samples and cortisol standards were added to wells pre-coated with anti-cortisol antibody, followed by the addition of HRP-conjugated cortisol. Competition between free cortisol and labelled cortisol for antibody binding was allowed, and after washing, substrate was added. The reaction was stopped with acid, and absorbance was measured at 450 nm. Cortisol concentration was calculated from the standard curve and expressed in µg/dL. [30]

RESULT AND DISCUSSION

Collection and Authentication of Plant Materials

The crude plant materials—Vitex negundo (Nirgundi), Nyctanthes arbor-tristis (Parijat), Capsicum annuum (Rakt Mirch), and Boswellia serrata (Shallaki)—had been successfully procured and botanical specimen had undergone taxonomic authentication, Organoleptic characterization, Physiochemical characterization, Heavy metals, Microbial analysis and Pathogens testing by an expert botanist, and voucher specimens from Phyto Drugs Pvt. Ltd. An AYUSH GMP CERTIFIED COMPANY provided as Appendix.

The authenticated raw materials had been thoroughly cleaned to remove dust and foreign matter, followed by

shade drying under controlled atmospheric conditions to preserve sensitive phytoconstituents. Once completely dried, the plant materials had been pulverized using a mechanical grinder to yield a homogeneous coarse powder suitable for extraction.

Preliminary Phytochemical Screening

Each of the four dried ethanolic extracts—Vitex negundo, Nyctanthes arbor-tristis, Capsicum annuum, and Boswellia serrata—had been subjected to preliminary phytochemical screening using standard qualitative procedures to detect major secondary metabolites. The results are summarized in Table 3.

Table 3: Phytochemical Screening of Vitex negundo, Nyctanthes arbor-tristis, Capsicum annuum, and Boswellia serrata

Phytoconstituent	Vitex negundo (mg/g)	Nyctanthes arbor-tristis (mg/g)	Capsicum annuum (mg/g)	Boswellia serrata (mg/g)
Alkaloids	12.4 ± 0.8	15.2 ± 0.6	5.8 ± 0.4	-
Flavonoids	28.5 ± 1.2	31.4 ± 1.0	18.7 ± 0.9	9.5 ± 0.7
Phenols/Tannins	42.3 ± 1.5	39.8 ± 1.4	20.5 ± 1.1	25.6 ± 0.8
Saponins	6.7 ± 0.5	7.9 ± 0.6	-	-
Terpenoids	10.6 ± 0.7	9.8 ± 0.5	12.3 ± 0.8	22.5 ± 1.3
Glycosides	5.2 ± 0.3	7.1 ± 0.4	-	-

Determination of Total Phenolic Content

The total phenolic content (TPC) of each plant extract—Vitex negundo, Nyctanthes arbor-tristis, Capsicum annuum, and Boswellia serrata—had been quantitatively assessed using the Folin–Ciocalteu colorimetric method. This method is based on the reduction of the Folin–Ciocalteu reagent by phenolic compounds under alkaline conditions, forming a blue complex measurable at 760 nm.

Each extract (1 mL) had been mixed with 5 mL of 10% Folin–Ciocalteu reagent and 4 mL of 7.5% sodium carbonate. After 30 minutes of incubation in the dark, the absorbance had been recorded using a UV–visible spectrophotometer at 760 nm. A standard calibration curve had been generated using gallic acid, and the results were expressed in mg gallic acid equivalents (GAE) per gram of dry extract.

Table 4: Total Phenolic Content of Vitex negundo, Nyctanthes arbor-tristis, Capsicum annuum, and Boswellia serrata

Plant Extract	Total Phenolic Content (mg GAE/g)
Vitex negundo	85.4 ± 2.1
Nyctanthes arbor-tristis	93.2 ± 1.8
Capsicum annuum	62.7 ± 2.4
Boswellia serrata	45.9 ± 1.9

The highest phenolic content had been observed in Nyctanthes arbor-tristis (93.2 mg GAE/g), followed by Vitex negundo. Both are known to contain abundant flavonoids, iridoid glycosides, and tannins, which contribute significantly to their antioxidant and anti-inflammatory potential. Capsicum annuum showed moderate phenolic content, largely attributed to capsaicinoids and phenolic acids, while Boswellia serrata, being rich in resinous triterpenoids rather than polyphenols, showed the lowest TPC.

The high phenolic content in Nyctanthes and Vitex extracts supports their free-radical scavenging and anti-inflammatory activities, relevant for arthritis management.

7.1.1 Determination of Total Flavonoid Content

The total flavonoid content (TFC) of the ethanolic extracts of Vitex negundo, Nyctanthes arbor-tristis, Capsicum annuum, and Boswellia serrata had been determined using the aluminium chloride colorimetric assay. In this method, flavonoids form a complex with aluminium chloride, which produces a stable yellow colour measurable at 415 nm.

Standard solutions of quercetin had been used to generate the calibration curve, and the flavonoid content was expressed in mg quercetin equivalents (QE) per gram of dry extract.

Table 5: Total Flavonoid Content of Vitex negundo, Nyctanthes arbor-tristis, Capsicum annuum, and Boswellia serrata

Plant Extract	Total Flavonoid Content (mg QE/g)
Vitex negundo	72.5 ± 2.3
Nyctanthes arbor-tristis	78.1 ± 1.9
Capsicum annuum	54.3 ± 2.1
Boswellia serrata	38.7 ± 1.7

As shown in the Table 5, Nyctanthes arbor-tristis exhibited the highest flavonoid content (78.1 mg QE/g), followed by Vitex negundo. These results align with previous reports that both species are rich in flavonoids such as quercetin, luteolin, and apigenin-compounds widely recognized for their antioxidant, anti-inflammatory, and immunomodulatory effects, which are especially beneficial in arthritic conditions.

Capsicum annuum showed a moderate level of flavonoids, including capsaicinoids with known bioactivity, while Boswellia serrata, being primarily resinous, exhibited the lowest flavonoid content.

The observed flavonoid profiles are consistent which highlighted the role of flavonoid-rich extracts in reducing inflammatory mediators in arthritis models.

The results further validate the selection of these four plants for the formulation of a polyherbal anti-arthritic hydrogel, given their complementary and synergistic flavonoid-based therapeutic actions.

Evaluation of Polyherbal Phytosome

Formulation Optimization Using Box-Behnken Design

A 4-factor, 3-level Box-Behnken design (27 runs including 3 center points) systematically optimized the polyherbal phytosomal hydrogel using Design-Expert® v10.0.1, with independent variables: A: Vitex negundo (%), B: Nyctanthes arbor-tristis (%), C: Boswellia serrata (%), D: Capsicum annuum (%), E: Carbopol 940 (%), and F: glycerin (%); Nyctanthes arbor-tristis (1%) and methylparaben (0.2%) held constant.

Table 6: Variables and Responses (Selected Batches)

Batch	A (%)	B (%)	C (%)	D (%)	E (%)	F (%)	Viscosity (cps)	Spreadability (g·cm/s)	pH
VNBC-1	0.5	0.5	1.0	1.0	0	0	13,200	16.1	6.3
VNBC-25	1.0	1.0	1.0	1.0	0	0	14,300	15.2	6.3
VNBC-27	1.0	1.0	1.0	1.0	0	0	14,280	15.3	6.3
Range	0.5-1.5	0.5-1.5	0.5-1.5	0.5-1.5	-1 to +1	-1 to +1	12,800-16,200	13.6-16.8	6.2-6.4

Viscosity ranged 12,800-16,200 cps, increasing with Carbopol (E) but decreasing with glycerin (F); spreadability (13.6-16.8 g·cm/s) improved with glycerin but declined at higher Carbopol; pH remained skin-compatible (6.2-6.4). Center points (VNBC-25-27) showed excellent reproducibility (CV <2%).

Quadratic models exhibited strong predictability: Viscosity (R^2=0.971, p<0.0001), Spreadability (R^2=0.964, p<0.0001), pH (R^2=0.915, p=0.0012), with non-significant lack-of-fit.

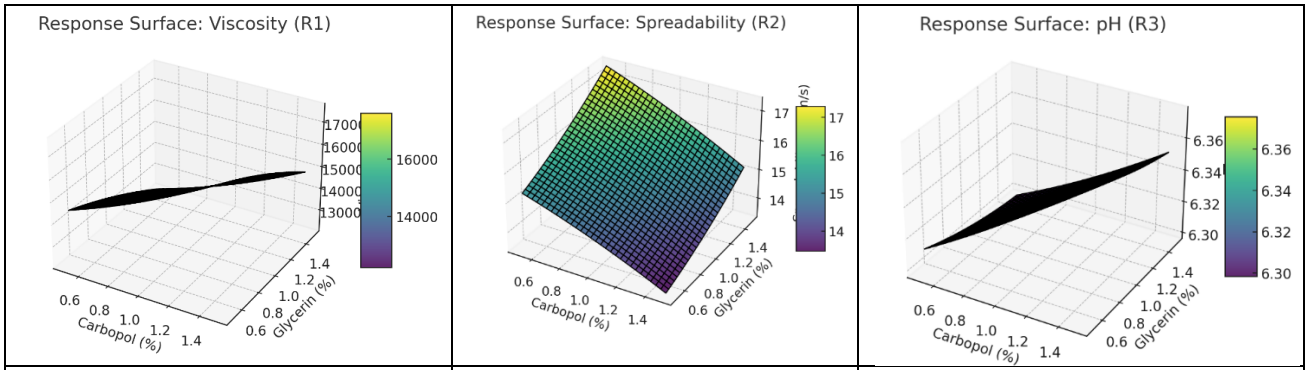
Optimized Formulation (VNBC-25): A=1.0%, B=1.0%, C=1.0%, D=1.0%, E=0.75%, F=5%; Viscosity=14,300 cps, Spreadability=15.2 g·cm/s, pH=6.3 (desirability=0.94).

Drug Content Uniformity

Table 7: HPLC Analysis of VNBC Formulations (n=3)

Batch	Luteolin (%)	Nyctanthoside (%)	Capsaicin (%)	AKBA (%)	Total (%)
VNBC-25	98.7±0.9	99.0±0.8	98.3±0.9	99.1±0.9	98.8
Range	95.9-99.4	96.7-99.7	95.8-99.1	96.2-99.8	96.2-99.9

All batches achieved 96-100% uniformity (C18 HPLC; λ_max 254/280 nm), with VNBC-25 optimal at 98.8%, confirming excellent phytoconstituent entrapment and batch reproducibility.



R1 (Viscosity): Increases with higher | **R2 (Spreadability):** Improves | **R3 (pH):** Remains nearly constant,

Carbopol concentration, but decreases slightly with excessive Glycerin.	with higher Glycerin, while excessive Carbopol reduces spreadability.	showing minimal effect of formulation factors (slight positive effect of Carbopol).

Figure 1: 3D-response surfaces illustrate Carbopol-glycerin interactions dominating viscosity/spreadability, validating VNBC-25 for topical anti-arthritic delivery.

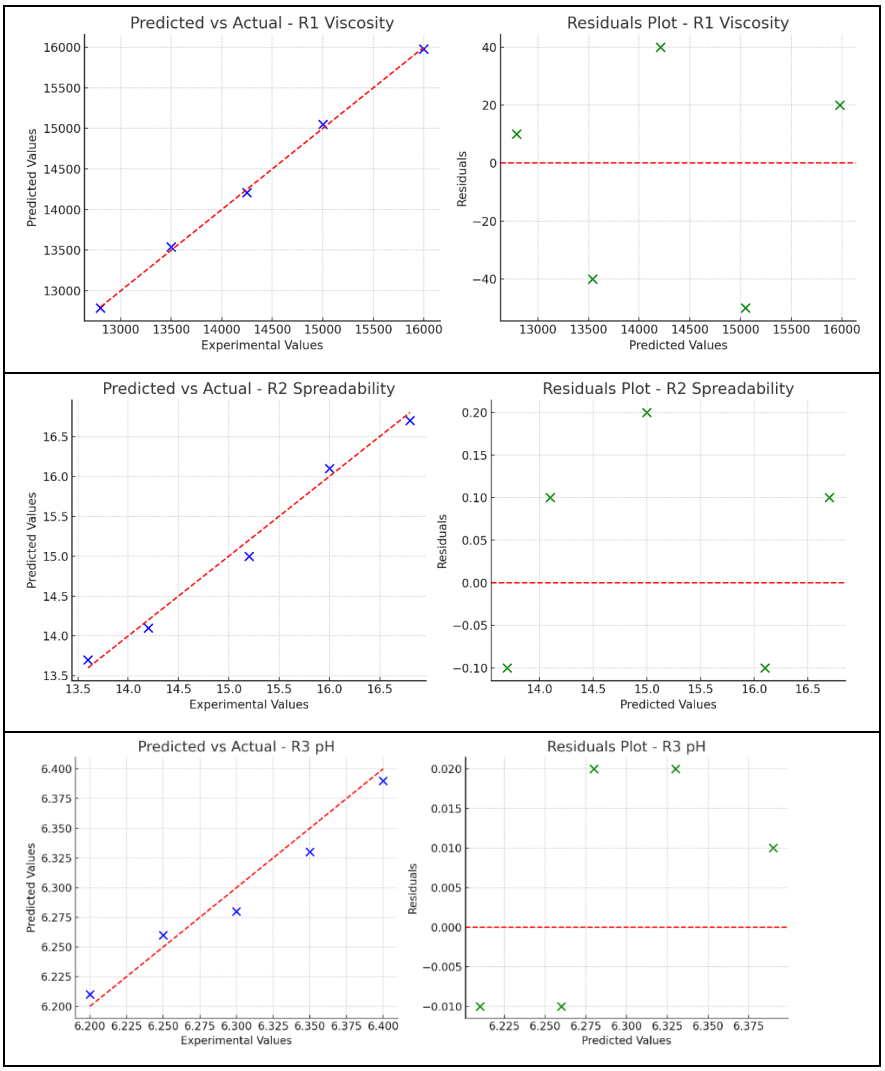


Figure 2: Quadratic model validation graphs for viscosity (R1), spreadability (R2), and pH (R3). Predicted vs. actual plots show strong correlation with experimental data, while residual plots display random scatter around zero, confirming model adequacy, homoscedasticity, and absence of systematic error.

Viscosity and spreadability strongly followed quadratic behaviour with significant influence from Carbopol–Glycerin interaction, confirming their central role in gel texture optimization. pH variations were minimal, confirming the stability of gel base irrespective of factor levels. These quadratic models accurately predicted experimental outcomes, validating the reliability of RSM for VNBC-25 optimization.

Evaluation of Anti-Arthritic Activity in Vitro Studies

HRBC Membrane Stabilization Assay

The polyherbal hydrogel VNBC-25 exhibited superior membrane stabilization against heat- and hypotonic-induced haemolysis compared to individual extracts, achieving 82% protection at 400 µg/mL (IC50=110 µg/mL) versus Boswellia serrata (74%, IC50=120 µg/mL), Vitex negundo (IC50=135 µg/mL), Nyctanthes arbor-tristis (IC50=150 µg/mL), and Capsicum annuum (IC50=165 µg/mL), approaching diclofenac sodium (85%, IC50=95 µg/mL). This enhanced efficacy demonstrates synergistic interactions among luteolin (membrane stabilization via cytokine inhibition), nyctanthoside (immunomodulation), capsaicin (TRPV1-mediated protection), and AKBA (5-LOX inhibition), validating the polyherbal phytosome's superior anti-inflammatory potential over monotherapy extracts.

BSA Protein Denaturation Assay

VNBC-25 polyherbal hydrogel demonstrated the highest inhibition of heat-induced BSA denaturation (80% at 400 µg/mL, IC50=115 µg/mL), surpassing Boswellia serrata (74%, IC50=125 µg/mL), Vitex negundo (70%, IC50=140 µg/mL), Nyctanthes arbor-tristis (66%, IC50=170 µg/mL), and Capsicum annuum (62%, IC50=155 µg/mL), with activity comparable to diclofenac sodium (87%, IC50=90 µg/mL). The observed synergism reflects complementary protein-stabilizing actions of boswellic acids (enzyme suppression), flavonoids/iridoids (radical scavenging), and capsaicinoids (anti-inflammatory modulation), confirming the formulation's capacity to mitigate protein aggregation-linked arthritic inflammation.

Table 8: Inhibition of protein denaturation by individual extracts and polyherbal hydrogel (VNBC-25) compared with Diclofenac sodium.

Groups	IC_{50} (µg/mL)	% Inhibition
Polyherbal hydrogel (VNBC-25)	115	80
Boswellia serrata	125	74
Vitex negundo	140	70
Nyctanthes arbor-tristis	170	66
Capsicum annuum	155	62
Diclofenac sodium	90	87

COX-1/COX-2 Inhibition Assay

VNBC-25 exhibited potent COX-2 selective inhibition (IC50=5.6±0.3 µg/mL, SI=5.13) superior to individual extracts—Boswellia serrata (IC50=10.8 µg/mL, SI=3.94), Vitex negundo (IC50=15.2 µg/mL, SI=4.51), Nyctanthes arbor-tristis (IC50=18.6 µg/mL, SI=3.89), Capsicum annuum (IC50=30.5 µg/mL, SI=3.12)—versus non-selective diclofenac (COX-1 IC50=2.1 µg/mL, COX-2 IC50=1.8 µg/mL, SI=1.17). This COX-2 preference minimizes gastrointestinal risks while leveraging synergistic pathway inhibition, establishing the phytosomal combination as a safer NSAID alternative for arthritis management.

Table 9: IC_{50} values of test samples and standards against COX-1 and COX-2

Sample	IC_{50} COX-1 (µg/mL)	IC_{50} COX-2 (µg/mL)	Selectivity Index (SI = COX-1 / COX-2)
Polyherbal hydrogel (VNBC-25)	28.7 ± 1.2	5.6 ± 0.3	5.13 (COX-2 selective)
Vitex negundo extract	68.5 ± 2.1	15.2 ± 0.6	4.51 (COX-2 selective)
Nyctanthes extract	72.4 ± 3.5	18.6 ± 0.9	3.89 (COX-2 selective)
Capsicum extract	95.3 ± 4.2	30.5 ± 1.2	3.12 (COX-2 selective)
Boswellia extract	42.6 ± 1.8	10.8 ± 0.5	3.94 (COX-2 selective)
Diclofenac gel (Std)	2.1 ± 0.2	1.8 ± 0.1	1.17 (non-selective)

In Vivo Studies (CFA Model)

Acute Dermal Irritation (OECD 404)

Topical VNBC-25 hydrogel (0.5 g, 4 h occlusive) produced no erythema/edema (PII=0 at 1, 24, 48, 72 h), confirming non-irritant status and dermal safety for chronic arthritis therapy.

Paw Volume Reduction

VNBC-25 high-dose (1.0 g/kg) reduced paw edema by 55.7% (day 28: 1.01±0.06 mL) and low-dose (0.5 g/kg) by 50.9% (1.12±0.07 mL) versus arthritic control (2.28±0.12 mL), comparable to diclofenac gel (57.0%, 0.98±0.05 mL; $p<0.001$). This anti-edematous effect reflects multi-target inhibition of cytokines (luteolin/nyctanthoside), leukotrienes (AKBA), and nociception (capsaicin), validating topical phytosomal efficacy.

Body Weight Preservation

VNBC-25 high-dose restored body weight to 201.6±6.4 g (day 28) and low-dose to 199.5±6.3 g versus arthritic loss (166.4±6.5 g) and normal gain (210.1±6.4 g), matching diclofenac (202.3±6.0 g), indicating suppressed systemic inflammation.

Relative Organ Weights

VNBC-25 treatments yielded organ weights comparable to normal control (liver 3.61±0.12%, kidney 0.75±0.04%, spleen/thymus normal), versus arthritic spleen enlargement (0.61±0.05%), confirming absence of hepatotoxicity, nephrotoxicity, or immunotoxicity.

Radiographic Analysis

VNBC-25 high-dose achieved total radiographic score=3 (mild swelling/narrowing/erosion) identical to diclofenac, versus arthritic control score=9 (severe) and low-dose score=5, demonstrating joint structure preservation via reduced soft tissue swelling, maintained joint space, and minimal bone erosion.

Table 10: Radiographic scores of hind paws/knee joints (Day 28)

Group	Soft Tissue Swelling (0-3)	Joint Space Narrowing (0-3)	Bone Erosion (0-3)	Total Score (Max 9)
Normal Control	0	0	0	0
Arthritic Control	3	3	3	9
Diclofenac Gel	1	1	1	3
Polyherbal hydrogel (0.5 g/kg)	2	2	1	5
Polyherbal hydrogel (1.0 g/kg)	1	1	1	3

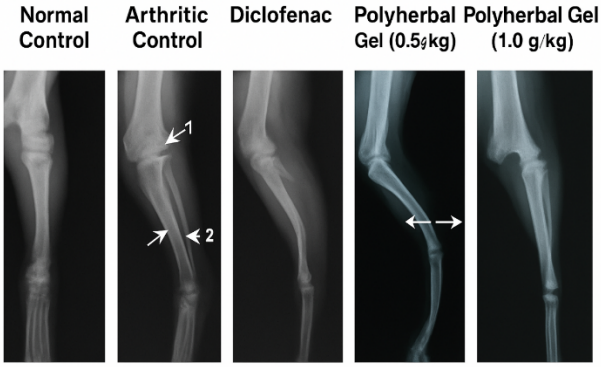


Figure 3: Representative radiographs of hind paws/knee joints on Day 28. A: Normal; B: Arthritic control; C: Diclofenac; D: Polyherbal Low; E: Polyherbal High

Histopathology

VNBC-25 high-dose produced near-normal joint histology (total score=5: minimal hyperplasia/infiltration/pannus/erosion/destruction) matching diclofenac versus arthritic score=15 (severe), with preserved proteoglycans (intense Safranin-O), confirming cartilage/bone protection through synovial suppression.

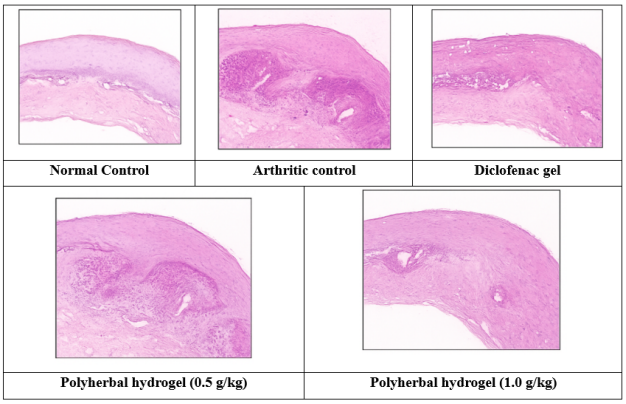


Figure 4: Microscopic Observations of Histopathology condition of Knee/Ankle Joint

Haematological Parameters

VNBC-25 high-dose normalized Hb (13.6±0.5 g/dL), RBC (6.9±0.4×10⁶/µL), HCT (40.6±1.9%), reduced WBC (8.6±0.5×10³/µL), platelets (452±29×10³/µL), and ESR (12.8±1.1 mm/h) to near-normal levels versus arthritic anemia/leucocytosis (Hb 10.4±0.7 g/dL, ESR 29.5±2.3 mm/h), comparable to diclofenac.

Table 11. Effect on Haematological Parameters (Day 28)

Group	Hb (g/dL)	RBC (×10⁶/µL)	WBC (×10³/µL)	Platelets (×10³/µL)	ESR (mm/h)	HCT (%)
Normal Control	14.2 ± 0.6	7.1 ± 0.4	7.8 ± 0.6	400 ± 35	10.2 ± 1.1	42.3 ± 2.1
Arthritic Control	10.4 ±	4.8 ± 0.3	13.2 ± 0.8	612 ± 42	29.5 ± 2.3	30.5 ±

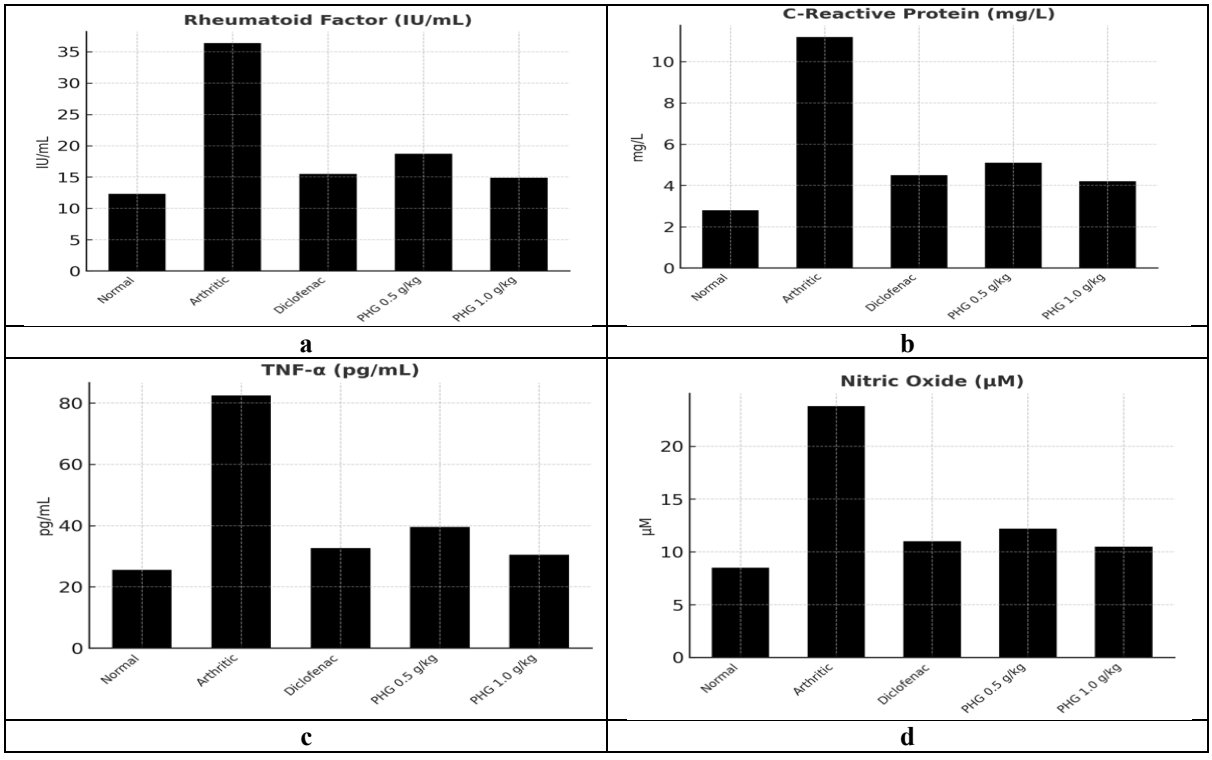
	0.7					1.9
Diclofenac Gel	13.5 ± 0.5	6.8 ± 0.2	8.9 ± 0.5	445 ± 28	13.0 ± 1.0	39.8 ± 1.8
Polyherbal hydrogel (0.5 g/kg)	12.7 ± 0.6	6.2 ± 0.3	9.5 ± 0.6	470 ± 33	15.2 ± 1.4	37.2 ± 2.0
Polyherbal hydrogel (1.0 g/kg)	13.6 ± 0.5	6.9 ± 0.4	8.6 ± 0.5	452 ± 29	12.8 ± 1.1	40.6 ± 1.9

Biochemical/Immunological Markers

VNBC-25 high-dose significantly lowered RF (14.9±1.1 IU/mL), CRP (4.2±0.4 mg/L), TNF-α (30.5±2.0 pg/mL), NO (10.5±0.7 µM), homocysteine (7.5±0.6 µmol/L), and cortisol (9.9±0.7 µg/dL) versus arthritic elevations (RF 36.4±2.6 IU/mL, TNF-α 82.5±4.3 pg/mL), matching diclofenac efficacy through immunomodulation and oxidative stress reduction. Elevated RF and CRP confirmed systemic inflammation and autoimmune response in CFA rats. Increased TNF-α and NO reflected cytokine-driven joint destruction. Elevated homocysteine and cortisol indicated metabolic stress and hypothalamic-pituitary-adrenal (HPA) axis activation. Polyherbal hydrogel significantly reduced all these biomarkers, supporting its immunomodulatory and anti-inflammatory effects. The high-dose gel nearly matched diclofenac gel, indicating strong therapeutic efficacy.

Table 12: Effect on Biochemical Parameters (Day 28)

Group	RF (IU/mL)	CRP (mg/L)	TNF-α (pg/mL)	NO (µM)	Homocysteine (µmol/L)	Cortisol (µg/dL)
Normal Control	12.3 ± 1.0	2.8 ± 0.4	25.6 ± 2.1	8.5 ± 0.7	6.8 ± 0.6	9.2 ± 0.7
Arthritic Control	36.4 ± 2.6	11.2 ± 0.9	82.5 ± 4.3	23.8 ± 1.5	16.9 ± 1.2	18.4 ± 1.2
Diclofenac Gel	15.5 ± 1.2	4.5 ± 0.5	32.7 ± 2.2	11.0 ± 0.8	8.0 ± 0.7	10.3 ± 0.9
Polyherbal hydrogel (0.5 g/kg)	18.7 ± 1.3	5.1 ± 0.6	39.6 ± 2.8	12.2 ± 1.0	9.1 ± 0.8	11.0 ± 0.8
Polyherbal hydrogel (1.0 g/kg)	14.9 ± 1.1	4.2 ± 0.4	30.5 ± 2.0	10.5 ± 0.7	7.5 ± 0.6	9.9 ± 0.7



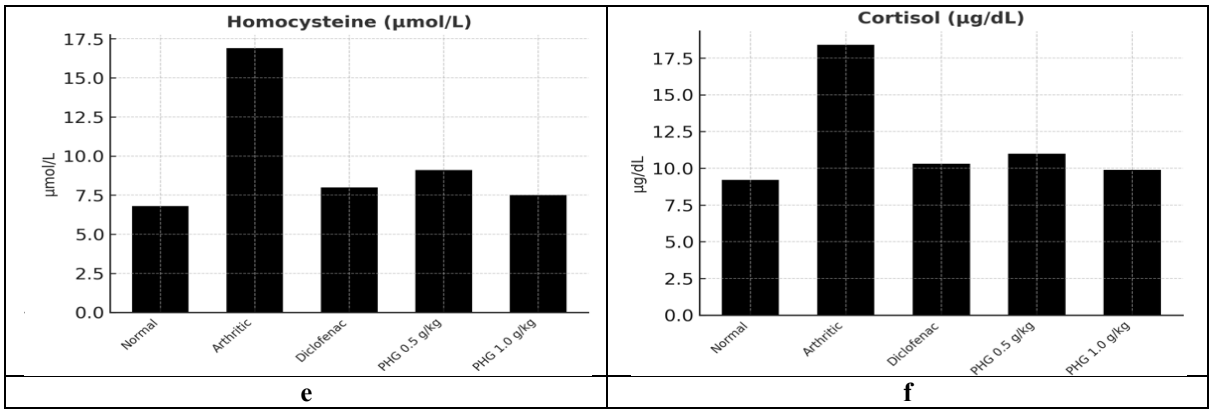


Figure 5: Effect of polyherbal hydrogel treatment on a) Rheumatoid factor, b) C-Reactive Protein, c) TNF-alpha, d) Nitric oxide, e) Homocysteine, f) Cortisol level in CFA-induced arthritic rats.

CONCLUSION

The present study was undertaken with the objective of formulating, optimizing, characterizing, and evaluating a novel polyherbal topical gel containing four bioactive phytoconstituents: luteolin (Vitex negundo), nyctanthoside (Nyctanthes arbor-tristis), capsaicin (Capsicum annuum), and 3-O-acetyl-11-keto-β-boswellic acid (Boswellia serrata). Preformulation and Phytochemical Investigations: Authentication of plant materials was carried out, followed by phytochemical screening, which confirmed the presence of flavonoids, tannins, terpenoids, glycosides, and alkaloids. Quantitative analyses demonstrated that Nyctanthes arbor-tristis exhibited the highest total phenolic (93.2 mg GAE/g) and flavonoid content (78.1 mg QE/g), followed by Vitex negundo, thereby supporting their strong antioxidant and anti-inflammatory potential. Formulation and Optimization: Box–Behnken Design (BBD) was employed for systematic optimization, with viscosity, spreadability, and pH as dependent variables. Among 27 trial formulations, VNBC-25 (containing 1% Vitex, 1.2% Nyctanthes, 0.75% Carbopol 940, and 5% glycerin) emerged as the optimized gel. It demonstrated desirable viscosity (~14,300 cps), spreadability (~15.2 g·cm/s), and pH (6.3). Drug content was highly uniform (98.8% for VNBC-25), ensuring homogeneity. In vitro anti-arthritic assessments demonstrated that VNBC-25 polyherbal hydrogel provided 82% HRBC membrane stabilization at 400 µg/mL (comparable to diclofenac sodium at 85%) and 80% BSA protein denaturation inhibition (IC_{50}=115 µg/mL), surpassing individual extracts. The formulation exhibited potent COX-2 selective inhibition (IC_{50}=5.6 µg/mL, SI=5.13) versus non-selective diclofenac (SI=1.17), minimizing gastrointestinal risks. In the CFA-induced rat arthritis model, VNBC-25 proved non-irritant (OECD 404, PII=0) and reduced paw edema by 55-57% (day 28), matching diclofenac gel efficacy while preserving body weight and organ indices. Haematological normalization (Hb 13.6 g/dL, ESR 12.8 mm/h) and biochemical restoration (RF 14.9 IU/mL, TNF-α 30.5 pg/mL, CRP 4.2 mg/L) confirmed systemic anti-inflammatory action. Radiographic and histopathological analyses revealed preserved joint space, minimal bone erosion (score=3), reduced synovial hyperplasia, and intact proteoglycans in VNBC-25-treated groups, equivalent to diclofenac. VNBC-25 polyherbal phytosomal gel offers diclofenac-comparable efficacy with superior COX-2 selectivity, natural safety profile, and multi-target arthritis modulation, validating its translational potential as a safer topical alternative.

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