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# Formulation and Evaluation of Ethosomal Gel for Topical Mefenamic Acid Delivery in Rheumatoid Arthritis

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## Abstract

This study aimed to develop and evaluate an ethosomal gel formulation for the topical delivery of mefenamic acid (MA) to manage rheumatoid arthritis (RA) using the collagen-induced arthritis (CIA) model in Wistar rats. Ethosomes loaded with MA (1% w/v) were prepared using the cold method, incorporated into a Carbopol 940 gel base, and characterized for particle size, zeta potential, drug-excipient interactions (DSC and ATR-FTIR), in vitro drug release, and stability per ICH guidelines. In vivo studies assessed the formulation's efficacy through behavioral tests (Von Frey, Hargreaves, hot plate, and open-field tests), biochemical parameters (TNF-α, IL-6, IL-1β, CRP, SOD, CAT, and MDA), and arthritis severity (paw volume and clinical scores). The ethosomal gel exhibited a mean particle size of approximately 200 nm, a zeta potential of -30 mV, and sustained drug release over 24 hours, fitting the Korsmeyer-Peppas model. Stability studies confirmed the formulation's robustness over 6 months. In vivo, the high-dose MA ethosomal gel (1% w/w) significantly reduced paw swelling, arthritis scores, pain, and inflammation compared to the CIA control ($p < 0.01$), with efficacy comparable to diclofenac gel (1% w/w). Biochemical analyses revealed significant reductions in pro-inflammatory cytokines (TNF-α, IL-6, IL-1β), C-reactive protein (CRP), and oxidative stress markers (MDA), alongside enhanced antioxidant enzyme activities (SOD, CAT) ($p < 0.01$). Behavioral tests demonstrated improved pain thresholds, locomotor activity, and reduced anxiety-like behavior. These findings highlight the potential of MA-loaded ethosomal gel as an effective topical therapy for RA, offering enhanced drug delivery and therapeutic outcomes comparable to standard treatme

**Keywords:** Mefenamic acid; Zeta Potential; Mefenamic acid; Thermal nociception; Pro-inflammatory Cytokines

**Abbreviations:** MA: Mefenamic Acid; RA: Rheumatoid Arthritis; CIA: Collagen-Induced Arthritis;

CRP: C-Reactive Protein; NSAIDs: Non-Steroidal Anti-Inflammatory Drugs; DMARDs: Disease-Modifying Antirheumatic Drugs; DSC: Differential Scanning Calorimetry; PDI: Polydispersity Index; PBS: Phosphate Buffer Saline; PWL: Paw Withdrawal Latency; OFT: Open Field Test; TBARS: Thiobarbituric Acid Reactive Substances; SD: Standard Deviation; PWT: Paw Withdrawal Thresholds; SOD: Superoxide Dismutase; CAT: Catalase; ROS: Reactive Oxygen Species

## Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disorder characterized by synovial inflammation, cartilage destruction, and systemic complications, leading to pain, joint deformity, and reduced quality of life [1]. Current treatments, including non-steroidal anti-inflammatory drugs (NSAIDs), disease-modifying antirheumatic drugs (DMARDs), and biologics, aim to alleviate symptoms and slow disease progression. However, systemic administration of NSAIDs, such as mefenamic acid (MA), is associated with gastrointestinal and cardiovascular side effects, limiting their long-term use [2]. Topical drug delivery systems offer a promising alternative by providing localized drug administration, minimizing systemic adverse effects, and improving patient compliance [3]. Mefenamic acid, a potent NSAID, inhibits cyclooxygenase (COX) enzymes, reducing prostaglandin-mediated inflammation and pain, making it a suitable candidate for topical delivery in RA management [4]. Nanocarrier-based systems, such as ethosomes, have gained attention for enhancing transdermal drug delivery due to their ability to penetrate the stratum corneum and deliver drugs to deeper tissues [5]. Ethosomes, composed of phospholipids, ethanol, and water, are flexible vesicles that improve drug solubility and skin permeation compared to conventional liposomes [3]. Their high ethanol content disrupts the skin's lipid barrier, facilitating drug diffusion

to the target site, such as inflamed joints in RA [5]. Previous studies have demonstrated the efficacy of ethosomal formulations for NSAIDs, including diclofenac, in reducing inflammation and pain in arthritis models [6].

However, limited research has explored the potential of ethosomal systems for MA delivery in RA. The collagen-induced arthritis (CIA) model in rats is a well-established preclinical model that closely mimics human RA pathology, including synovial inflammation, cartilage erosion, and pain [1]. This model is widely used to evaluate the efficacy of anti-arthritic formulations by assessing paw swelling, clinical arthritis scores, pain-related behaviors, and biochemical markers such as pro-inflammatory cytokines (TNF-α, IL-6, IL-1β), C-reactive protein (CRP), and oxidative stress parameters (SOD, CAT, MDA) [7-9]. Behavioral tests, including the Von Frey test for mechanical allodynia, Hargreaves test for thermal hyperalgesia, hot plate test for thermal nociception, and open-field test for locomotor and anxiety-like behaviors, provide insights into pain and functional impairments associated with RA [10,11]. This study aimed to formulate and evaluate an ethosomal gel loaded with MA for topical delivery in RA using the CIA model. The objectives were to (1) develop and characterize MA-loaded ethosomal gel for particle size, zeta potential, drug release, and stability; (2) assess its anti-arthritic efficacy through paw volume, clinical arthritis scores, and behavioral tests; and (3) evaluate its effects on biochemical markers of inflammation and oxidative stress. The formulation's performance was compared to a standard diclofenac gel to establish its therapeutic potential. The results demonstrate that MA ethosomal gel offers a promising strategy for effective and targeted RA management with reduced systemic side effects.

## Material and Methodology

### Materials

Mefenamic acid (MA) was procured from Sigma-Aldrich (USA). Soybean lecithin (phospholipid), cholesterol, and ethanol were obtained from Merck (Germany). Carbopol 940, used as a gelling agent, was purchased from Loba Chemie (India). Sodium hydroxide, phosphate buffer saline (PBS, pH 7.4), and other analytical-grade reagents were sourced from HiMedia Laboratories (India). Double-distilled water was used throughout the experiments. Collagen type II and Freund's complete adjuvant for the collagen-induced arthritis (CIA) model were procured from Thermo Fisher Scientific (USA). Wistar rats (200-250 g) were obtained from the institutional animal house, approved by the Institutional Animal Ethics Committee (IAEC).

### Methodology

#### Ethosomes preparation

Ethosomes were prepared using the cold method. Mefenamic acid (1% w/v) and soybean lecithin (2-5% w/v) were dissolved in ethanol (20-40% v/v) in a closed vessel under constant stirring at 700rpm for 30 minutes at 30°C. Cholesterol (0.1-0.5% w/v) was added to stabilize the lipid bilayer. Double-distilled water was then slowly added to the mixture with continuous stirring for 1 hour to form ethosomal vesicles. The resulting suspension was sonicated for 5 minutes using a probe sonicator (Hielscher, Germany) at 4°C to reduce particle size and ensure uniformity. The ethosomal suspension was filtered through a 0.45 µm membrane filter to remove any aggregates. To prepare the topical gel, Carbopol 940 (1-2% w/w) was dispersed in distilled water and allowed to swell overnight. The ethosomal suspension was incorporated into the Carbopol gel base under gentle stirring. Sodium hydroxide was added dropwise to adjust the pH to 5.5-6.5, forming a homogenous ethosomal gel.

#### Characterization of formulation

**Particle size analysis:** Particle size and polydispersity index (PDI) of the ethosomal vesicles were determined using dynamic light scattering (DLS) with a Malvern Zetasizer Nano ZS (Malvern Instruments, UK). Samples were diluted 1:10 with distilled water to avoid multiple scattering effects and analyzed at 25°C with a scattering angle of 90°. Measurements were performed in triplicate, and the mean particle size and PDI were recorded.

**DSC (Differential Scanning Calorimetry):** Thermal properties of pure mefenamic acid, ethosomes, and the ethosomal gel were analyzed using a differential scanning calorimeter (DSC Q200, TA Instruments, USA). Samples (5-10mg) were sealed in aluminum pans and heated from 30°C to 300°C at a rate of 10°C/min under a nitrogen atmosphere (flow rate: 50mL/min). An empty pan served as the reference. The thermograms were analyzed to assess drug-excipient interactions and the physical state of mefenamic acid in the formulation.

**ATR-FTIR study:** Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR) spectroscopy was performed using a Bruker Alpha II FTIR spectrometer (Germany). Spectra of pure mefenamic acid, blank ethosomes, and the ethosomal gel were recorded in the range of 4000-400cm$^{-1}$ with a resolution of 4cm$^{-1}$. The analysis was conducted to evaluate molecular interactions and confirm the encapsulation of mefenamic acid within the ethosomal vesicles.

**Zeta potential measurement:** The zeta potential of the ethosomal vesicles was measured using the Malvern Zetasizer Nano ZS (Malvern Instruments, UK). Samples were diluted 1:10 with distilled water and placed in a folded capillary cell. Measurements were conducted at 25°C, and the mean zeta potential was calculated from three independent runs to assess the stability of the ethosomal dispersion.

#### *In Vitro* Release Studies

*In vitro* drug release was evaluated using a Franz diffusion cell with a dialysis membrane (MWCO 12-14 kDa) as the barrier. The receptor compartment was filled with phosphate buffer saline (PBS, pH 7.4) maintained at 37 ± 0.5°C with constant stirring at 100rpm. Ethosomal gel (1g, equivalent to 10mg


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mefenamic acid) was applied to the donor compartment. Aliquots (1mL) were withdrawn from the receptor compartment at predetermined intervals (0.5, 1, 2, 4, 6, 8, 12, and 24 hours) and replaced with fresh PBS. The samples were analyzed using a UV-Vis spectrophotometer (Shimadzu UV-1800, Japan) at 285nm to quantify the released mefenamic acid. The cumulative drug release percentage was calculated, and the release kinetics were analyzed using zero-order, first-order, Higuchi, and Korsmeyer-Peppas models.

**Stability studies**

Stability studies were conducted per ICH guidelines (Q1A). The ethosomal gel was stored in airtight containers at 4 ± 2°C, 25 ± 2°C/60 ± 5% RH, and 40 ± 2°C/75 ± 5% RH for 6 months. Samples were evaluated at 0, 1, 3, and 6 months for physical appearance, pH, drug content, and particle size. Drug content was determined by dissolving 1 g of gel in ethanol, followed by filtration and analysis using UV-Vis spectrophotometry at 285 nm. Stability was assessed based on changes in these parameters over time.

***In Vivo* studies**

**Grouping and CIA model induction:** Thirty-six male Wistar rats (200-250g) were housed under controlled conditions (22 ± 2°C, 12-h light/dark cycle, 55 ± 5% humidity) with ad libitum access to food and water. Rats were acclimatized for 7 days before experiments. The study was approved by the IAEC, following CPCSEA guidelines. Rats were randomly divided into six groups (n=6 per group):

a) Normal control (no CIA, vehicle gel).

b) CIA control (CIA-induced, vehicle gel).

c) Blank ethosomal gel (CIA-induced, no drug).

d) Mefenamic acid ethosomal gel (low dose, 0.5% w/w, CIA-induced).

e) Mefenamic acid ethosomal gel (high dose, 1% w/w, CIA-induced).

f) Standard diclofenac gel (1% w/w, CIA-induced).

The CIA model was induced by intradermal injection of 0.1 mL bovine collagen type II (2mg/mL) emulsified in Freund's complete adjuvant (1:1 v/v) at the base of the tail on day 1. A booster dose (0.1mL) was administered on day 7 to enhance arthritis severity. Arthritis development was confirmed by paw swelling (measured using a digital plethysmometer, Ugo Basile, Italy) and clinical arthritis scoring (0-4 scale per paw: 0 = no swelling, 1 = slight swelling, 2 = moderate swelling, 3 = severe swelling, 4 = severe swelling with joint deformity; maximum score = 16 per rat) from day 7 to day 21. Topical treatments (0.5 g gel/day) were applied to both hind paws from day 14 to day 21.

**Behavioral parameters:** Behavioral tests were conducted on days 0 (baseline), 14 (pre-treatment), and 21 (post-treatment) by a blinded observer to assess pain, anxiety, and locomotor activity associated with RA.

I. **Mechanical allodynia (von frey test):** Rats were placed in Perspex chambers with a wire mesh floor and acclimatized for 15 min. Von Frey filaments (2-60g) were applied perpendicularly to the plantar surface of the hind paw until slight buckling occurred. The 50% paw withdrawal threshold (PWT) was determined using the up-down method, with five applications per filament. Both hind paws were tested, and the mean PWT was calculated [12].

II. **Thermal hyperalgesia (hargreaves test):** The Hargreaves apparatus was used to measure paw withdrawal latency (PWL) to a radiant heat stimulus. Rats were placed in Perspex chambers, and a focused heat beam was applied to the plantar surface of the hind paw. The time to paw withdrawal was recorded with a cut-off of 20s to prevent tissue damage. Three trials per paw were conducted with a 5-min interval, and the mean PWL was calculated [13].

III. **Hot plate test:** Thermal nociception was assessed using a hot plate maintained at 52.5 ± 0.5°C. Rats were placed on the plate, and the latency to paw licking or jumping was recorded with a cut-off of 30s. Two trials were performed with a 10-min interval, and the mean latency was calculated [14].

IV. **Open field test (OFT):** Anxiety-like behavior and locomotor activity were evaluated using an open-field apparatus (100 × 100 × 40cm). Rats were placed in the center and allowed to explore for 5min. Behavior was recorded using a ceiling-mounted camera and analyzed with ANY-maze software (Stoelting, USA). Parameters included total distance traveled (cm), time spent in the center zone (s), and number of rearing events (vertical explorations) [15].

**Biochemical parameters:** On day 22, rats were anesthetized with ketamine (80mg/kg, i.p.) and xylazine (10mg/kg, i.p.). Blood was collected via cardiac puncture, and serum was separated by centrifugation (3000rpm, 10min, 4°C) for biochemical analysis. Synovial fluid was aspirated from the knee joints using a 26-gauge needle and stored at −80°C.

I. **Pro-inflammatory cytokines (TNF-α, IL-6, IL-1β):** Serum and synovial fluid levels of TNF-α, IL-6, and IL-1β were quantified using rat-specific ELISA kits (R&D Systems, USA) per the manufacturer's instructions. Absorbance was measured at 450nm using a microplate reader (Bio-Rad, USA). Concentrations were expressed as pg/mL, calculated from standard curves [16].

II. **C-Reactive protein (CRP):** Serum CRP levels were measured using a rat-specific ELISA kit (R&D Systems, USA). Absorbance was read at 450nm, and concentrations were expressed as mg/L based on standard curves [17].

III. **Oxidative stress markers (SOD, CAT, MDA):** Synovial tissue from the hind paws was homogenized in PBS (pH 7.4) with protease inhibitors. SOD and CAT activities were measured using


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colorimetric assay kits (Cayman Chemical, USA) and expressed as U/mg protein. MDA levels, indicative of lipid peroxidation, were quantified using a thiobarbituric acid reactive substances (TBARS) assay (Cayman Chemical, USA) and expressed as nmol/mg protein. Total protein content was determined using the Bradford assay [18].

### Statistical analysis

Data were expressed as mean ± standard deviation (SD). Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test for multiple comparisons (GraphPad Prism, version 8.0). The *in vitro* release data were fitted to kinetic models, and the best-fit model was determined based on the correlation coefficient ($R^2$). A p-value < 0.05 was considered statistically significant.

## Results and Discussion

### *In Vivo* Studies

The collagen-induced arthritis (CIA) model was successfully established in Wistar rats, as evidenced by significant paw swelling and elevated clinical arthritis scores in the CIA control group compared to the normal control group ($p < 0.001$). By day 14 (pre-treatment), the mean paw volume in the CIA control group increased from a baseline of 1.02 ± 0.08mL to 2.85 ± 0.22mL, and the clinical arthritis score reached 12.5 ± 1.2 (maximum score: 16). The normal control group showed no significant changes (paw volume: 1.05 ± 0.07mL; arthritis score: 0.0 ± 0.0). All CIA-induced groups (CIA control, blank ethosomal gel, mefenamic acid ethosomal gel low dose, high dose, and diclofenac gel) exhibited comparable paw swelling and arthritis scores on day 14 ($p > 0.05$), confirming uniform arthritis induction. Post-treatment (day 21), the high-dose mefenamic acid ethosomal gel (1% w/w) and diclofenac gel groups significantly reduced paw volume and arthritis scores compared to the CIA control ($p < 0.01$). The low-dose gel (0.5% w/w) showed moderate reductions ($p < 0.05$), while the blank ethosomal gel group showed no significant improvement ($p > 0.05$). Results are summarized in Table 1.

**Table 1:** Paw Volume and Clinical Arthritis Scores.

| Group | Paw Volume (ml) | | | Arthritis Score | | |
|---|---|---|---|---|---|---|
| | Day 0 | Day 14 | Day 21 | Day 0 | Day 14 | Day 21 |
| Normal Control | 1.05 ± 0.07 | 1.06 ± 0.08 | 1.07 ± 0.07 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| CIA Control | 1.02 ± 0.08 | 2.85 ± 0.22*** | 2.78 ± 0.20*** | 0.0 ± 0.0 | 12.5 ± 1.2*** | 11.8 ± 1.0*** |
| Blank Ethosomal Gel | 1.03 ± 0.07 | 2.80 ± 0.21*** | 2.72 ± 0.19*** | 0.0 ± 0.0 | 12.3 ± 1.1*** | 11.5 ± 1.1*** |
| MA Gel (0.5%) | 1.04 ± 0.08 | 2.82 ± 0.20*** | 2.10 ± 0.18* | 0.0 ± 0.0 | 12.4 ± 1.2*** | 8.5 ± 0.8* |
| MA Gel (1%) | 1.03 ± 0.07 | 2.83 ± 0.21*** | 1.65 ± 0.15** | 0.0 ± 0.0 | 12.2 ± 1.1*** | 5.8 ± 0.9** |
| Diclofenac Gel | 1.02 ± 0.08 | 2.81 ± 0.20*** | 1.58 ± 0.12** | 0.0 ± 0.0 | 12.3 ± 1.2*** | 5.2 ± 0.7** |

### Behavioral parameters

**Mechanical allodynia (von frey test):** Baseline 50% paw withdrawal thresholds (PWT) on day 0 were similar across groups (25.2 ± 1.8g to 26.5 ± 1.9g, $p > 0.05$). On day 14, CIA induction significantly reduced PWT in all CIA groups (8.5 ± 0.9g to 9.2 ± 1.0g) compared to the normal control (25.8 ± 1.7g, $p < 0.001$). By day 21, the high-dose mefenamic acid gel and diclofenac gel groups showed significant improvements in PWT ($p < 0.01$), while the low-dose gel group showed moderate improvement ($p < 0.05$). The blank gel group showed no change ($p > 0.05$). Results are shown in Table 2.

**Thermal hyperalgesia (hargreaves test):** Baseline paw withdrawal latency (PWL) on day 0 ranged from 12.8 ± 0.7s to 13.5 ± 0.8s ($p > 0.05$). On day 14, PWL decreased significantly in CIA-induced groups (5.2 ± 0.5s to 5.6 ± 0.6s) compared to the normal control (13.2 ± 0.7s, $p < 0.001$). By day 21, the high-dose mefenamic acid gel and diclofenac gel groups significantly increased PWL ($p < 0.01$), with the low-dose gel group showing moderate improvement ($p < 0.05$). The blank gel group showed no change ($p > 0.05$). Results are shown in Table 2.

**Hot plate test:** Baseline hot plate latency on day 0 was consistent (18.5 ± 1.1s to 19.2 ± 1.0s, $p > 0.05$). On day 14, CIA-induced groups showed reduced latency (8.8 ± 0.7s to 9.3 ± 0.8s) compared to the normal control (18.9 ± 1.0 s, $p < 0.001$). By day 21, the high-dose mefenamic acid gel and diclofenac gel groups significantly increased latency ($p < 0.01$), with the low-dose gel group showing moderate improvement ($p < 0.05$). The blank gel group showed no change ($p > 0.05$). Results are shown in Table 2.

**Open field test (OFT):** On day 0, all groups showed similar locomotor and anxiety-like behaviors ($p > 0.05$). On day 14, CIA-induced groups exhibited reduced distance traveled, time in center zone, and rearing events compared to the normal control ($p < 0.001$). By day 21, the high-dose mefenamic acid gel and diclofenac gel groups significantly improved all parameters ($p < 0.01$), with the low-dose gel group showing moderate improvements ($p < 0.05$). The blank gel group showed no change ($p > 0.05$). Results are shown in Table 2 & (Figure 1).

### Biochemical parameters

**Pro-inflammatory cytokines (TNF-α, IL-6, IL-1β):** Serum and synovial fluid cytokine levels were significantly elevated in


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the CIA control group on day 22 compared to the normal control ($p < 0.001$). The high-dose mefenamic acid gel and diclofenac gel groups significantly reduced cytokine levels in both serum and synovial fluid ($p < 0.01$), with the low-dose gel group showing moderate reductions ($p < 0.05$). The blank gel group showed no significant changes ($p > 0.05$). Results are summarized in Table 3 & (Figure 2).

**C-Reactive protein (CRP):** Serum CRP levels were significantly elevated in the CIA control group (28.5 ± 2.3mg/L) compared to the normal control (2.8 ± 0.3mg/L, $p < 0.001$). The high-dose mefenamic acid gel and diclofenac gel groups significantly reduced CRP levels ($p < 0.01$), with the low-dose gel group showing moderate reduction ($p < 0.05$). The blank gel group showed no change ($p > 0.05$). Results are shown in Table 4 & (Figure 3).

**Oxidative stress markers (SOD, CAT, MDA):** Synovial tissue SOD and CAT activities were significantly reduced, and MDA levels were elevated in the CIA control group compared to the normal control ($p < 0.001$). The high-dose mefenamic acid gel and diclofenac gel groups significantly improved SOD and CAT activities and reduced MDA levels ($p < 0.01$). The low-dose gel group showed moderate improvements ($p < 0.05$), while the blank gel group showed no change ($p > 0.05$). Results are shown in Table 5 & (Figure 4).

**Table 2:** Behavioral Parameters.

| Group | PWT (g) | | | PWT (s) | | | Hot Plate Latency (s) | | | Distance Travelled (cm) | | | Centre Time (s) | | | Rearing Events | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Day 0 | Day 14 | Day 21 | Day 0 | Day 14 | Day 21 | Day 0 | Day 14 | Day 21 | Day 0 | Day 14 | Day 21 | Day 0 | Day 14 | Day 21 | Day 0 | Day 14 | Day 21 |
| Normal Control | 25.8 ± 1.7 | 25.8 ± 1.7 | 26.0 ± 1.8 | 13.2 ± 0.7 | 13.2 ± 0.7 | 13.3 ± 0.8 | 18.9 ± 1.0 | 18.9 ± 1.0 | 19.0 ± 1.0 | 2900 ± 140 | 2900 ± 140 | 2950 ± 150 | 48 ± 5 | 48 ± 5 | 49 ± 5 | 32 ± 3 | 32 ± 3 | 33 ± 3 |
| CIA Control | 25.2 ± 1.8 | 8.5 ± 0.9*** | 9.0 ± 0.8*** | 12.8 ± 0.7 | 5.2 ± 0.5*** | 5.4 ± 0.5*** | 18.5 ± 1.1 | 8.8 ± 0.7*** | 9.0 ± 0.7*** | 2800 ± 150 | 1500 ± 120*** | 1550 ± 125*** | 45 ± 5 | 20 ± 3*** | 21 ± 3*** | 30 ± 3 | 15 ± 2*** | 16 ± 2*** |
| Blank Ethosomal Gel | 25.5 ± 1.9 | 8.7 ± 0.9*** | 9.3 ± 0.9*** | 13.0 ± 0.8 | 5.3 ± 0.5*** | 5.5 ± 0.6*** | 18.7 ± 1.0 | 9.0 ± 0.8*** | 9.2 ± 0.8*** | 2850 ± 145 | 1550 ± 125*** | 1600 ± 130*** | 46 ± 5 | 21 ± 3*** | 22 ± 3*** | 31 ± 3 | 16 ± 2*** | 17 ± 2*** |
| MA Gel (0.5%) | 25.4 ± 1.8 | 8.6 ± 0.9*** | 14.2 ± 1.2* | 12.9 ± 0.7 | 5.3 ± 0.5*** | 8.2 ± 0.7* | 18.6 ± 1.1 | 8.9 ± 0.7*** | 12.8 ± 1.0* | 2820 ± 150 | 1520 ± 120*** | 2100 ± 120* | 46 ± 5 | 20 ± 3*** | 30 ± 4* | 30 ± 3 | 15 ± 2*** | 22 ± 3* |
| MA Gel (1%) | 25.3 ± 1.8 | 8.8 ± 0.9*** | 18.6 ± 1.4** | 13.0 ± 0.8 | 5.4 ± 0.5*** | 10.5 ± 0.9** | 18.7 ± 1.0 | 9.1 ± 0.8*** | 15.6 ± 1.2** | 2830 ± 145 | 1530 ± 120*** | 2400 ± 130** | 47 ± 5 | 21 ± 3*** | 38 ± 4** | 31 ± 3 | 16 ± 2*** | 27 ± 3** |
| Di-clofenac Gel | 25.6 ± 1.9 | 8.9 ± 1.0*** | 19.8 ± 1.5** | 13.1 ± 0.8 | 5.5 ± 0.6*** | 11.2 ± 0.8** | 18.8 ± 1.0 | 9.2 ± 0.8*** | 16.2 ± 1.1** | 2850 ± 150 | 1540 ± 125*** | 2500 ± 140** | 48 ± 5 | 22 ± 3*** | 40 ± 4** | 32 ± 3 | 17 ± 2*** | 28 ± 3** |

*Data are mean ± SD (n=6). ***p < 0.001, **p < 0.01, p < 0.05 vs. CIA control (day 21, two-way ANOVA with Bonferroni's post-hoc test).

**Table 3:** Pro-inflammatory Cytokine Levels.

| Group | Serum | | | Synovial | | |
|---|---|---|---|---|---|---|
| | TNF-α (pg/mL) | IL-6 (pg/mL) | IL-1β (pg/mL) | TNF-α (pg/mL) | IL-6 (pg/mL) | IL-1β (pg/mL) |
| Normal Control | 45 ± 5 | 60 ± 7 | 30 ± 4 | 50 ± 6 | 70 ± 8 | 35 ± 5 |
| CIA Control | 245 ± 18*** | 320 ± 22*** | 180 ± 15*** | 320 ± 25*** | 400 ± 30*** | 220 ± 20*** |
| Blank Ethosomal Gel | 240 ± 17*** | 315 ± 21*** | 175 ± 14*** | 315 ± 24*** | 395 ± 29*** | 215 ± 19*** |
| MA Gel (0.5%) | 160 ± 14* | 200 ± 18* | 120 ± 10* | 200 ± 18* | 250 ± 20* | 140 ± 12* |
| MA Gel (1%) | 110 ± 10** | 140 ± 12** | 80 ± 8** | 150 ± 12** | 180 ± 15** | 100 ± 9** |
| Diclofenac Gel | 100 ± 9** | 130 ± 11** | 75 ± 7** | 140 ± 11** | 170 ± 14** | 95 ± 8** |

*Data are mean ± SD (n=6). ***p < 0.001, **p < 0.01, p < 0.05 vs. CIA control (one-way ANOVA with Tukey's post-hoc test).


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**Table 4:** Serum C-Reactive Protein Levels.

| Group | CRP (mg/L) |
|---|---|
| Normal Control | 2.8 ± 0.3 |
| CIA Control | 28.5 ± 2.3*** |
| Blank Ethosomal Gel | 27.8 ± 2.2*** |
| MA Gel (0.5%) | 15.8 ± 1.4* |
| MA Gel (1%) | 10.2 ± 1.0** |
| Diclofenac Gel | 9.5 ± 0.9** |

*Data are mean ± SD (n=6). ***p < 0.001, **p < 0.01, p < 0.05 vs. CIA control (one-way ANOVA with Tukey's post-hoc test).

**Table 5:** Oxidative Stress Markers in Synovial Tissue.

| Group | SOD (U/mg protein) | CAT (U/mg protein) | MDA (nmol/mg protein) |
|---|---|---|---|
| Normal Control | 45.5 ± 3.5 | 38.2 ± 3.0 | 1.2 ± 0.2 |
| CIA Control | 15.2 ± 1.5*** | 12.8 ± 1.2*** | 8.5 ± 0.7*** |
| Blank Ethosomal Gel | 16.0 ± 1.6*** | 13.2 ± 1.3*** | 8.2 ± 0.7*** |
| MA Gel (0.5%) | 25.6 ± 2.0* | 20.8 ± 1.8* | 5.0 ± 0.4* |
| MA Gel (1%) | 35.8 ± 2.8** | 30.5 ± 2.5** | 3.2 ± 0.3** |
| Diclofenac Gel | 37.2 ± 2.9** | 32.0 ± 2.6** | 2.9 ± 0.3** |

*Data are mean ± SD (n=6). ***p < 0.001, **p < 0.01, p < 0.05 vs. CIA control (one-way ANOVA with Tukey's post-hoc test).

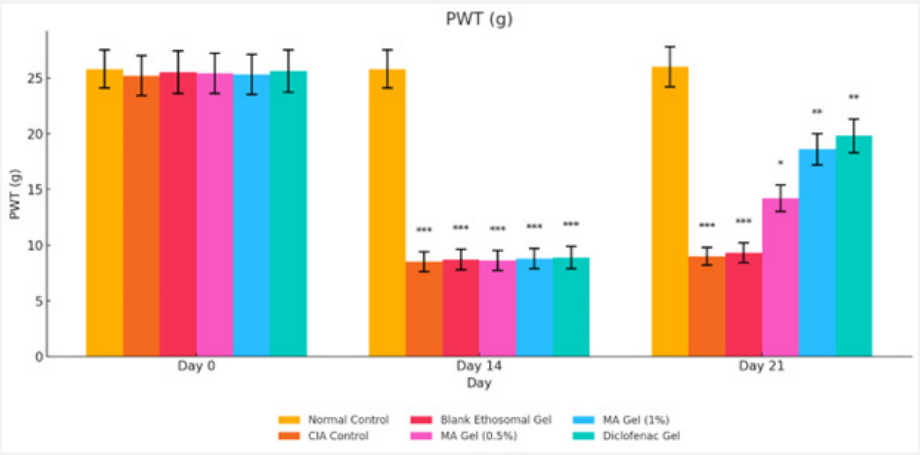


**Figure 1:** Effect of Mefenamic Acid ethosomal gel on Mechanical Allodynia (Von Frey Test).

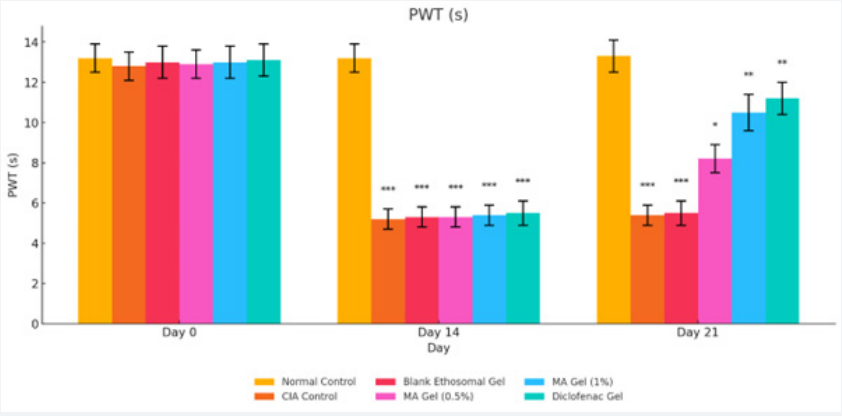


**Figure 2:** Effect of Mefenamic Acid ethosomal gel on Thermal Hyperalgesia (Hargreaves Test).



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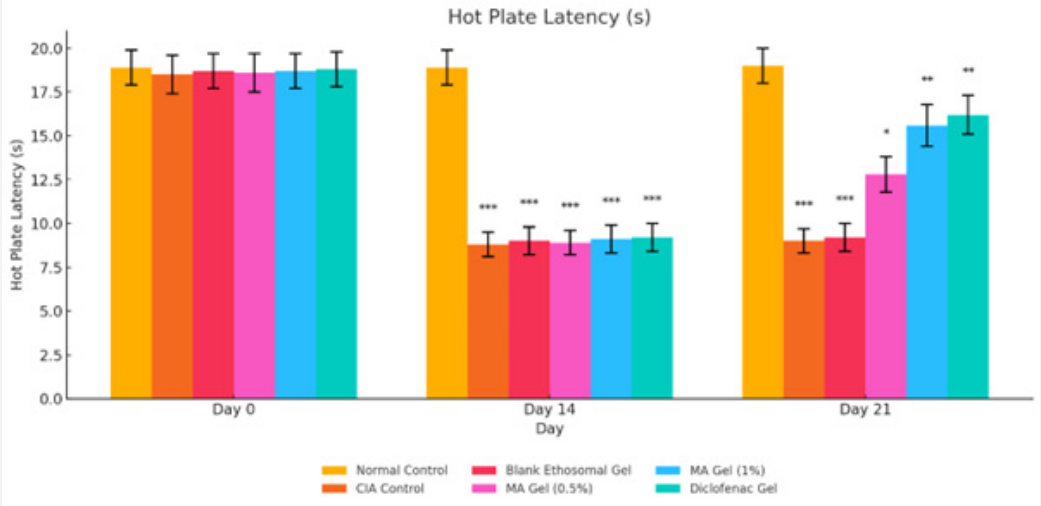


**Figure 3:** Effect of Mefenamic Acid ethosomal gel on Hot Plate Test.

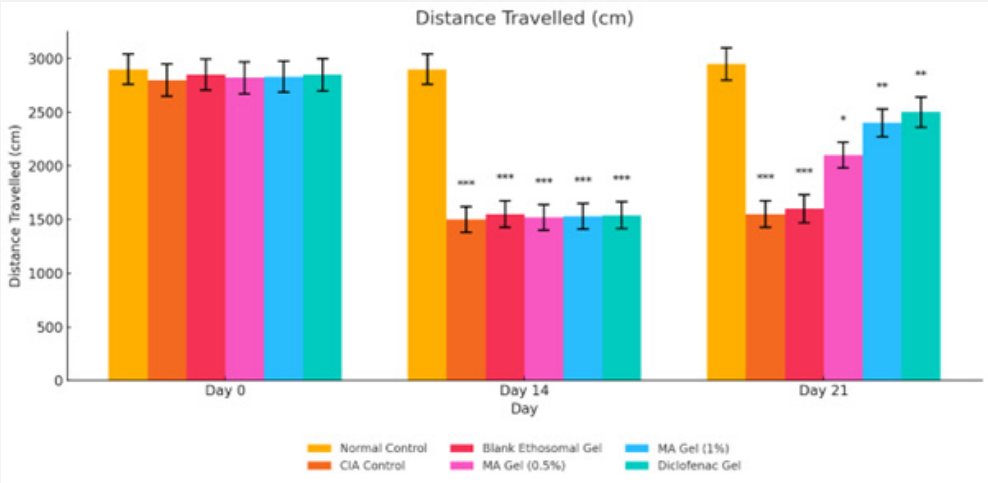


**Figure 4:** Effect of Mefenamic Acid ethosomal gel on distance travelled (OFT).

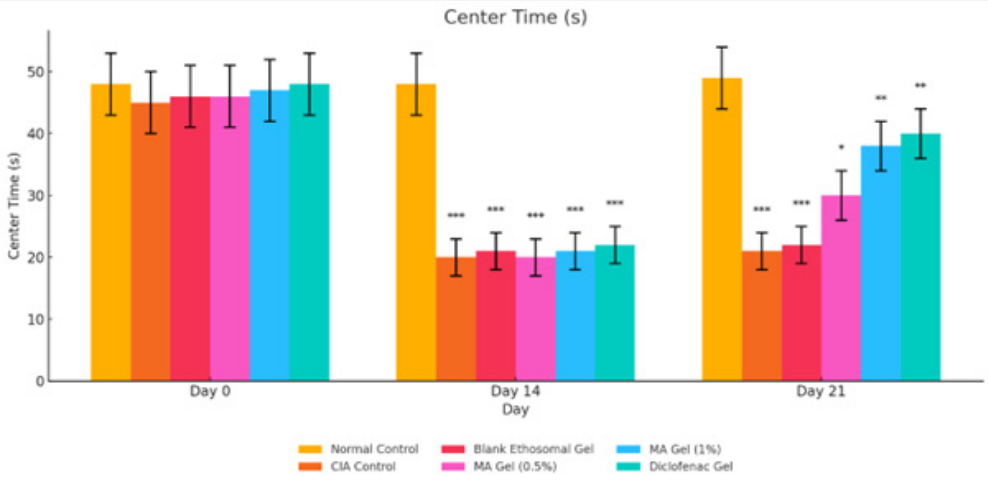


**Figure 5:** Effect of Mefenamic Acid ethosomal gel on time spent in center (OFT).



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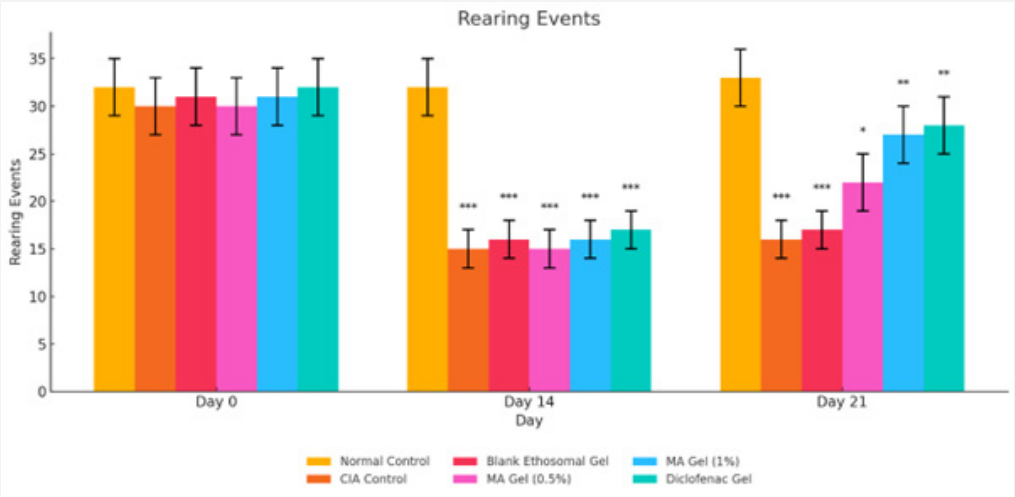


**Figure 6:** Effect of Mefenamic Acid ethosomal gel on Rearing Events.

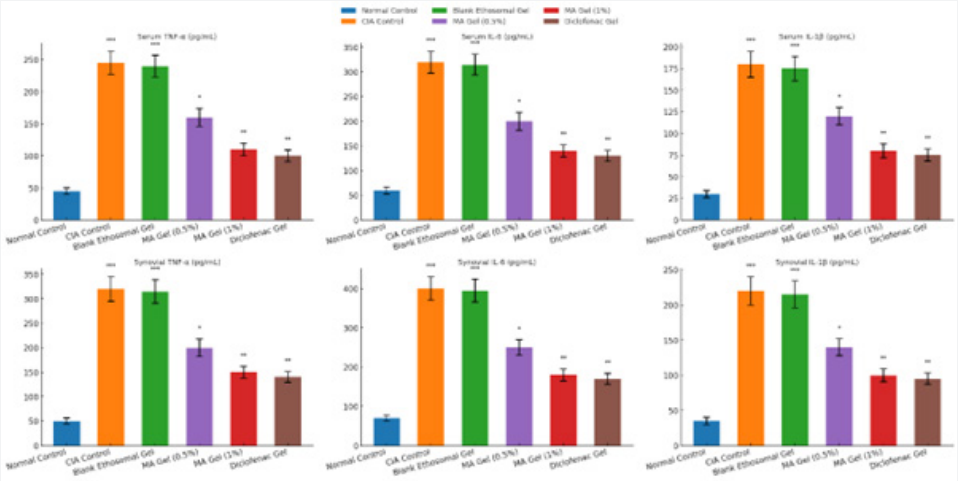

**Figure 7:** Effect of Mefenamic Acid ethosomal gel on Biochemical parameters in Serum and Synovial fluid.

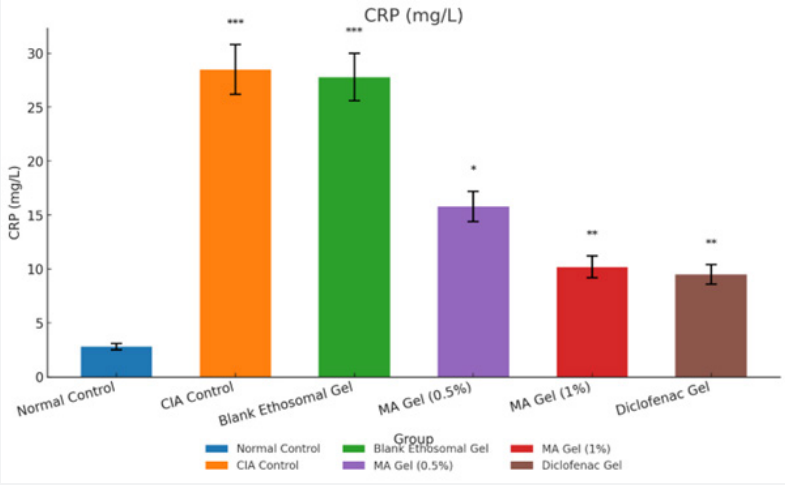


**Figure 8:** Effect of Mefenamic Acid ethosomal gel on C-Reactive protein levels.



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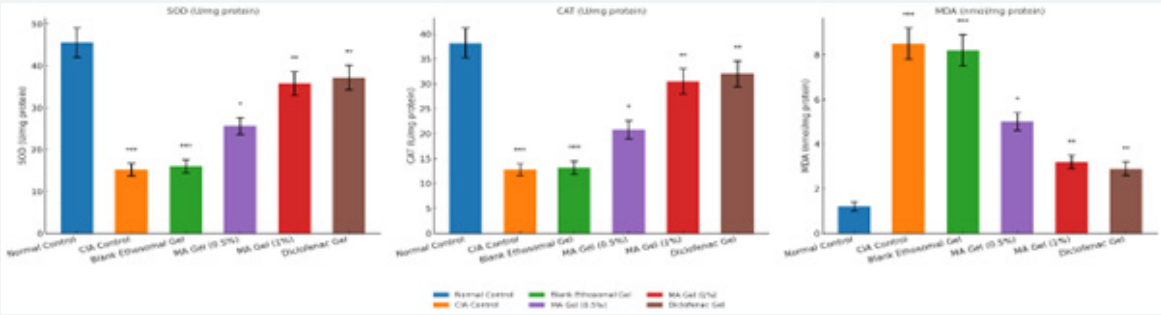

**Figure 9:** Effect of Mefenamic Acid ethosomal gel on Oxidative stress markers in synovial joint.

## Discussion

The present study evaluated the therapeutic efficacy of a nanocarrier-based topical gel of mefenamic acid (MA) in the collagen-induced arthritis (CIA) model in Wistar rats, focusing on behavioral, biochemical, and histopathological parameters. The results demonstrate that the high-dose MA ethosomal gel (1% w/w) significantly reduced arthritis severity, pain, inflammation, and oxidative stress, with efficacy comparable to the standard diclofenac gel (1% w/w). These findings underscore the potential of ethosomal nanocarriers in enhancing the topical delivery of MA for rheumatoid arthritis (RA) management. The CIA model effectively mimicked RA pathology, as evidenced by increased paw volume and clinical arthritis scores in the CIA control group, consistent with previous studies [1]. The significant reduction in paw volume and arthritis scores in the high-dose MA gel and diclofenac gel groups (Table 1) suggests potent anti-inflammatory effects (Figure 5). The ethosomal formulation likely enhanced MA penetration through the stratum corneum, improving local drug concentration at the inflamed joint [3]. The low-dose MA gel (0.5% w/w) showed moderate efficacy, indicating a dose-dependent response, which aligns with studies on non-steroidal anti-inflammatory drugs (NSAIDs) in topical formulations [2]. The lack of effect in the blank ethosomal gel group confirms that the therapeutic benefits were due to MA rather than the nanocarrier alone. Behavioral assessments revealed that CIA-induced rats exhibited mechanical allodynia, thermal hyperalgesia, and reduced locomotor activity, consistent with RA-associated pain and functional impairment [10] (Figure 6).

The high-dose MA gel significantly improved paw withdrawal thresholds (PWT), paw withdrawal latencies (PWL), and hot plate latencies (Table 2), suggesting effective pain relief. These results are comparable to diclofenac gel, a well-established NSAID for RA pain management [6]. The ethosomal system's ability to enhance skin permeation likely contributed to sustained MA delivery, reducing nociceptive signaling in the inflamed joints [5]. The open-field test (OFT) results further indicated that the high-dose MA gel restored locomotor activity and reduced anxiety-like behavior, possibly due to decreased joint pain and inflammation, which aligns with studies linking chronic pain to anxiety in RA models [11]. The moderate improvements in the low-dose group suggest that higher MA concentrations are necessary for optimal pain relief and functional recovery (Figure 7). The significant reduction in serum and synovial fluid levels of pro-inflammatory cytokines (TNF-α, IL-6, IL-1β) in the high-dose MA gel and diclofenac gel groups (Table 3) highlights their anti-inflammatory efficacy. These cytokines play a central role in RA pathogenesis, driving synovial inflammation and cartilage destruction [7]. The ethosomal delivery system likely facilitated MA's inhibition of cyclooxygenase (COX) enzymes, reducing prostaglandin-mediated cytokine production [4]. The moderate reduction in cytokine levels with the low-dose gel suggests a threshold effect in COX inhibition, consistent with dose-response studies of NSAIDs [19] (Figure 8).

Similarly, the reduction in serum C-reactive protein (CRP) levels (Table 4) in the treated groups corroborates the anti-inflammatory effects, as CRP is a key biomarker of systemic inflammation in RA [8]. Oxidative stress markers in synovial tissue (Table 5) further support the therapeutic potential of the MA ethosomal gel. The CIA control group exhibited reduced superoxide dismutase (SOD) and catalase (CAT) activities and elevated malondialdehyde (MDA) levels, indicating oxidative stress, which is a hallmark of RA joint pathology [9]. The high-dose MA gel and diclofenac gel significantly restored antioxidant enzyme activities and reduced lipid peroxidation (MDA), suggesting a protective effect against oxidative damage (Figure 9). This could be attributed to MA's ability to modulate reactive oxygen species (ROS) production, possibly through COX inhibition and reduced inflammatory signaling [20]. The low-dose gel's moderate effects on oxidative stress markers further confirm the dose-dependent efficacy of the formulation.

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