Original article

Exploring the therapeutic potential of *Trigonella foenum-graecum* extract-loaded transferosomal gel in complete Freund's adjuvant-induced rat models

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Abstract

Background: Rheumatoid arthritis (RA) is a debilitating autoimmune disorder that is characterized by inflammation and joint damage.
Objective: This study evaluated the antiarthritic activity of *Trigonella foenum-graecum* (TFG) transferosomal gel in a Freund's adjuvant-induced arthritic rat model and compared it with diclofenac gel, a standard anti-inflammatory drug.
Methods: Arthritis was induced in rats by injecting 0.1 mL (1 mg/mL) of complete Freund's adjuvant suspension into the subplantar region of the left hind foot, and they were allowed to develop arthritis for 21 days.
Results: Arthritis induction led to a considerable increase in paw volume, which is indicative of inflammation. Treatment with both TFG-transferosomal gel and diclofenac gel substantially reduced the paw volume, thus demonstrating their anti-inflammatory effects. In addition, the TFG-transferosomal gel reduced the arthritic scores, indicating its potential to ameliorate arthritic symptoms. Both treatments improved locomotor activity, which suggests relief from pain-induced hypoactivity. Furthermore, biochemical analysis revealed that the TFG-transferosomal gel effectively reduced inflammatory enzyme activity and mediator levels, comparable to that of diclofenac gel.
Conclusion: These results demonstrate the anti-inflammatory efficacy of the TFG-transferosomal gel, thus highlighting its potential as a therapeutic option for managing arthritis-associated inflammation and symptoms. Further research is warranted to elucidate its mechanisms of action and assess its clinical utility.

Keywords: Complete Freund's adjuvant-induced rat models, rheumatoid arthritis, topical drug delivery system, transferosomal gel, *Trigonella foenum-graecum*.

Rheumatoid arthritis (RA) is a complex systemic autoimmune disorder that is characterized by pathological changes such as synovial membrane hyperplasia, inflammatory cell infiltration, neovascularization, cartilage erosion, and joint degeneration. Approximately 1.0% of the world's population suffers from RA, with a notable male-to-female ratio of 3 : 1. Cytokines, such as tumor necrosis factor-alpha (TNF-α), interleukin (IL), IL-1β, and IL-6, play crucial roles in the pathogenesis of RA by promoting inflammation, joint injury, and tissue damage. [1 - 3]

Despite the availability of disease-modifying anti-rheumatoid medications (DMARDs) and non-steroidal anti-inflammatory drugs for RA treatment, their use is often associated with adverse effects, including cardiovascular complications, ulcers, and gastrointestinal issues. Moreover, the high cost, potential adverse effects, and limited efficacy of current RA medications highlight the need for

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alternative, safer, and more effective therapies. [4, 5]

Interest in herbal antiarthritic therapy is growing, with a focus on exploring the potential of plant-derived phyto- or herbal-medications. Fenugreek (*Trigonella foenum-graecum*; Fabaceae) is a widely used plant in traditional medicine for its leaves and seeds, which are known for their potent antioxidant properties attributed to flavonoids and polyphenols. Furthermore, fenugreek's anti-inflammatory properties are linked to the presence of alkaloids and flavonoids, which act as antioxidants and may inhibit inflammatory enzymes such as cyclooxygenase, lipoxygenase, and nitric oxide synthase. [6 - 8]

Transdermal drug delivery is a popular approach for treatment, beginning with the scopolamine patch, which was introduced in 1979. This route has various advantages over oral and needle-based routes, including self-administration, non-invasiveness, substantially decreased drug clearance via the liver, and increased patient compliance. Transferosomes are a promising drug delivery carrier for transdermal treatment because they can transport larger amounts of active substances to deeper layers of the skin. Transferosomes enter the subcutaneous layer via the intra- or trans-cellular pathway, forming an "osmotic gradient." These carriers may hold both hydrophobic and hydrophilic compounds, which allows for the simultaneous administration of various medicines and phytochemical components into the systemic circulation. [9] Transferosomes have several advantages over liposomes and other lipid-based delivery systems, including skin penetration, drug bioavailability, and stability. Their unique deformability, safety profile, and versatility make them a popular choice for various medicinal and cosmetic applications. [10]

Despite fenugreek's historical use in inflammation management, there is limited research on the development of transferosomal gel formulations using fenugreek extract. Therefore, this study aimed to formulate and investigate efficient transferosomal gel formulation with anti-inflammatory properties using a fenugreek ethanolic extract. Transferosomal gel offers advantages, such as ease of administration, localized effects, minimal pain or irritation during application, avoidance of first-pass metabolism, and protection against gastrointestinal degradation, which makes it an attractive delivery system for targeted application to affected areas in RA management. [11, 12]

Materials and methods

Ethical policies

This study received approval from the institutional animal ethical committee of the Lloyd Institute of Management and Technology, Greater Noida (IAEC approval no: 1206/PO/Re/S/08/CPCSEA/08/2023/10/15). The animals were housed and cared for according to the CCSEA guidelines.

Materials

Analytical reagent grade ethanol and Tween-80 were purchased from Loba Chemie Pvt. Ltd., Mumbai, India. Soy phospholipid (PHOSPHOLIPON® 90 G), Carbopol 934 polymer, propylene glycol, complete Freund's adjuvant (1 mg/mL), and triethanolamine were procured from Merck Life Sciences, India. *Trigonella foenum-graecum* (TFG) seeds were purchased from a local market in Baghpat, Uttar Pradesh, India, and authenticated by the Council of Scientific and Industrial Research, National Institute of Science Communication and Policy Research (CSIR-NIScPR), New Delhi (authentication no. NISCPR/RHMD/consult/2021/3956-57). Extraction was performed using the Soxhlet method. TFG-loaded transferosomes were developed using the extract via the thin-layer hydration technique. The resulting mixture was then incorporated into Carbopol to prepare the transferosome-loaded gel.

Experimental animals

Male Wistar rats (200 - 250 g) were obtained from Rodent Research India Pvt. Ltd., Jind, Haryana. All animal experiments adhered to the ARRIVE guidelines and were conducted in accordance with the National Research Council's guide for the care and use of laboratory animals (8th edition). The study received approval from the Institutional Animal Ethics Committee of Lloyd Institute of Management and Technology, Greater Noda (IAEC approval no. 1206/PO/Re/S/08/CPCSEA/08/2023/10/15) on October 15, 2023. The animals were housed and cared for in accordance with the CCSEA guidelines. The rats were acclimatized to the environment for two weeks before being used in the experiments.

Preparation and characterization of the TFG-loaded transferosomal gel

An accurately weighed quantity of Carbopol 934 (1 g) was placed into three separate 200 mL beakers, and each beaker was filled with 70 mL of deionized water. The mixtures were then set aside for 4 h to allow for soaking. Thereafter, polyethylene glycol-400 (10 g) and TFG-extract-loaded transferosomes (equivalent to 2.5 g of extract) were introduced into the gel mixture and stirred continuously for 4 h to achieve a uniform gel formulation. The pH of the gel (pH 6) was subsequently adjusted using triethylamine. [10, 12, 13]

Evaluation of the antiarthritic activity of the TFG-transferosomal gel in vivo in complete Freund's adjuvant-induced rat models

The efficacy of the TFG-transferosomal gel was assessed using a rat model of complete Freund's adjuvant (CFA)-induced arthritis through topical application. The rats were divided into four groups (n = 6 each). Group 1 received a topical application of a gel base, serving as the normal vehicle control. Arthritis was induced in groups 2 - 4 by injecting 0.1 mL (1 mg/mL) of CFA suspension into the subplantar region of the rat's left hind foot. Group 2 served as the arthritic control. Arthritis was allowed to develop in groups 2 - 4 for 21 days. [14 - 16]

Hind paw volume assessment

During the experimental period, the rat paw volume in the control and treatment groups was measured on the 21st day using a plethysmometer to confirm the establishment of arthritis in the animals. Following the confirmation of arthritis development, diclofenac diethylamine gel IP 2.3 w/w (procured from a local community pharmacy) and the TFG transferosomes loaded gel formulation were applied topically from day 22 to 42 to the left knee joint region of Group 3 (serving as the reference standard) and Group 4, respectively. Throughout the treatment duration, the rat paw volume of all animals was measured on days 22, 27, 32, 37, and 42 using a plethysmometer, thereby ensuring the comprehensive assessment of treatment efficacy and arthritis progression over time. [17 - 19]

The increase in paw volume (ΔV) or edema at time (t) was calculated using the following formula:

ΔV = paw volume ($t0$) - paw volume (t)
where $t0$ = paw volume on day 0 before immunization and t = paw volume after immunization.

Arthritis score

During the experimental period, the severity of arthritis in the rats was assessed by visual inspection to evaluate their ability to walk on days 22, 27, 32, 37, and 42. Using a modified version of an established scale, each animal was systematically scored on a scale ranging from 0 to 5. A score of 0 indicated normal walking and running, while a score of 1 denoted difficulty in locomotion. Scores of 2 and 3 represented varying degrees of limping, with or without retraction of the hind paw, respectively. A score of 4 indicated crawling or exclusive lying down, while a score of 5 signified severe inflammation accompanied by crawling or lying down. This standardized scoring system ensured the consistent evaluation of arthritis severity across all experimental rats, thereby providing an effective comparison between the control and treatment groups. [16, 19, 20]

Spontaneous locomotor activity

On days 22, 27, 32, 37, and 42, all rats underwent a 5-min session of the open field test to evaluate spontaneous locomotor activity and anxiety-like behavior. This assessment was performed using automated locomotor activity boxes procured from Aarson Scientific Work (Haryana, India). Ambulatory counts recorded in both the central and peripheral zones of the open field were analyzed to discern the relative anxiety levels among the rats. The methodologies and parameters, including ambulatory and vertical counts, used in these tests were consistent with previously documented protocols. [16, 19, 20]

Biochemical analysis

The hind legs of the experimental rats were photographed to assess the morphological changes associated with arthritis. At the end of the experiment, all animals were euthanized by the administration of a high-dose anesthetic cocktail comprising ketamine (200 mg/kg) and xylazine (20 mg/kg). Blood samples were collected via direct cardiac puncture into plain tubes for serum separation. These serum samples were used for the analysis of various markers, including C-reactive protein (CRP), using an immunoturbidimetric kit (Cat: E-EL-R0506), and COX activity in the paw tissue, using an enzyme-linked immunosorbent assay (ELISA) kit (Cat: E-EL-R0792). Furthermore, the levels of prostaglandin E_2 (PGE_2, Cat: E-EL-0034), TNF-α (Cat: E-EL-R2856), and interleukins (IL-6 (Cat: E-EL-R0015) and IL-10

(Cat: E-EL-R0016)) were quantified using ELISA kits from Elabscience. Lastly, the protein concentration of the samples was determined using the bicinchoninic acid (BCA) protein assay kit (Cat: MBS355529, MyBioSource). [21, 22]

Statistical analysis

All data were expressed as the mean ± standard deviation and analyzed using GraphPad Prism v. 8.0 by one-way analysis of variance followed by Tukey's multiple comparison test. $P < 0.05$ was considered statistically significant.

Results

Preparation of TFG-transferosomal gel

The prepared transferosomes were meticulously analyzed for various parameters, including particle size, zeta potential, entrapment efficiency, and deformability index. The results showed that the particle size of the transferosomes was 269.2 ± 1.2 nm. Moreover, the zeta potential, which indicates the overall charge of the transferosomes, was measured at -28.0 ± 2.2 mV. The entrapment efficiency, which is a critical factor indicating the proportion of the drug successfully encapsulated within the transferosomes, was 63.7% ± 3.2%. In addition, the vesicle elasticity, assessed via the deformability index, was 23.7 ± 0.9. The transferosomes were successfully incorporated into the 1.0% Carbopol gel at pH 6 and maintained at room temperature for further evaluation.

Effect of TFG-transferosomal gel on the paw volume of CFA-induced arthritic rats

Compared with the normal control group (0.4 ± 0.0 mL), the CFA control group exhibited a notable increase in paw volume (0.5 ± 0.0 mL) on day 22, indicating the successful induction of arthritis. Treatment with diclofenac sodium gel and TFG-transferosomal gel resulted in a significant reduction ($P < 0.0001$) in paw volume (0.4 ± 0.0 and 0.4 ± 0.0 mL, respectively) compared with the arthritic control group on day 42 **(Figure 1A).**

Effect of TFG-transferosomal gel on the arthritic score

The arthritic index score was significantly higher in the CFA-induced arthritis rats (3.9 ± 0.6) than in the control group on day 22, and on day 42, this increased to 4.7 ± 0.4. The arthritic score for the TFG-transferosomal gel-treated group on day 42 was 1.8 ± 0.5. However, the lowest arthritic score was observed in the diclofenac gel-treated group, which was 1.6 ± 0.4 on day 42. The arthritis index in the diclofenac gel- and TFG-transferosomal gel-treated rats was found to be significantly lower ($P < 0.0001$) than in the control group **(Figures 1B and 1C).**

Effect of TFG-transferosomal gel on the spontaneous locomotor activity

The CFA control group took a longer time to travel a 2-m distance (226.7 ± 12.0 s) compared to the normal control group (34.3 ± 3.0 s) on day 22, which increased to 268.6 ± 22.2 s on day 42. Rats in the TFG-transferosomal gel and standard diclofenac gel treatment groups took 82.9 ± 10.4 s and 74.7 ± 14.1 s, respectively, to complete the 2-m distance on day 42. Treatment with both TFG-transferosomal gel and diclofenac gel resulted in a significant reduction in spontaneous locomotor activity compared with the untreated CFA control group ($P < 0.0001$) **(Figure 1D).**

Effect of TFG-transferosomal gel on the inflammatory enzymes and mediators

In the CFA control group, the COX activity was elevated to 0.1 ± 0.0 µg/mg of protein, while the PGE_2 levels were substantially increased to 534.4 ± 2.8 pg/mL. Treatment with diclofenac gel reduced the COX activity to 0.1 ± 0.0 µg/mg of protein and the PGE_2 levels to 251.3 ± 17.4 pg/mL. Similarly, treatment with TFG-transferosomal gel resulted in a significant reduction in the COX activity to 0.1 ± 0.0 µg/mg of protein ($P < 0.001$) and the PGE_2 levels to 321.3 ± 1.0 pg/mL ($P < 0.0001$) **(Figures 2A and 2B).**

In addition, to assessing inflammatory enzymes and mediators, the effects of TFG-transferosomal gel and diclofenac gel on inflammatory cytokine levels were evaluated to provide further insight into their anti-inflammatory mechanisms.

IL-6 levels were significantly elevated in the CFA control group (44.0 ± 3.0 pg/mL) compared with the normal control group (24.2 ± 1.2 pg/mL), thus indicating enhanced inflammation due to CFA induction. Treatment with both TFG-transferosomal gel ($P < 0.05$) and diclofenac gel ($P < 0.001$) significantly reduced the level of IL-6 compared with the CFA control group with values of 35.6 ± 4.0 and 25.6 ± 3.2 pg/mL, respectively **(Figure 2C).**

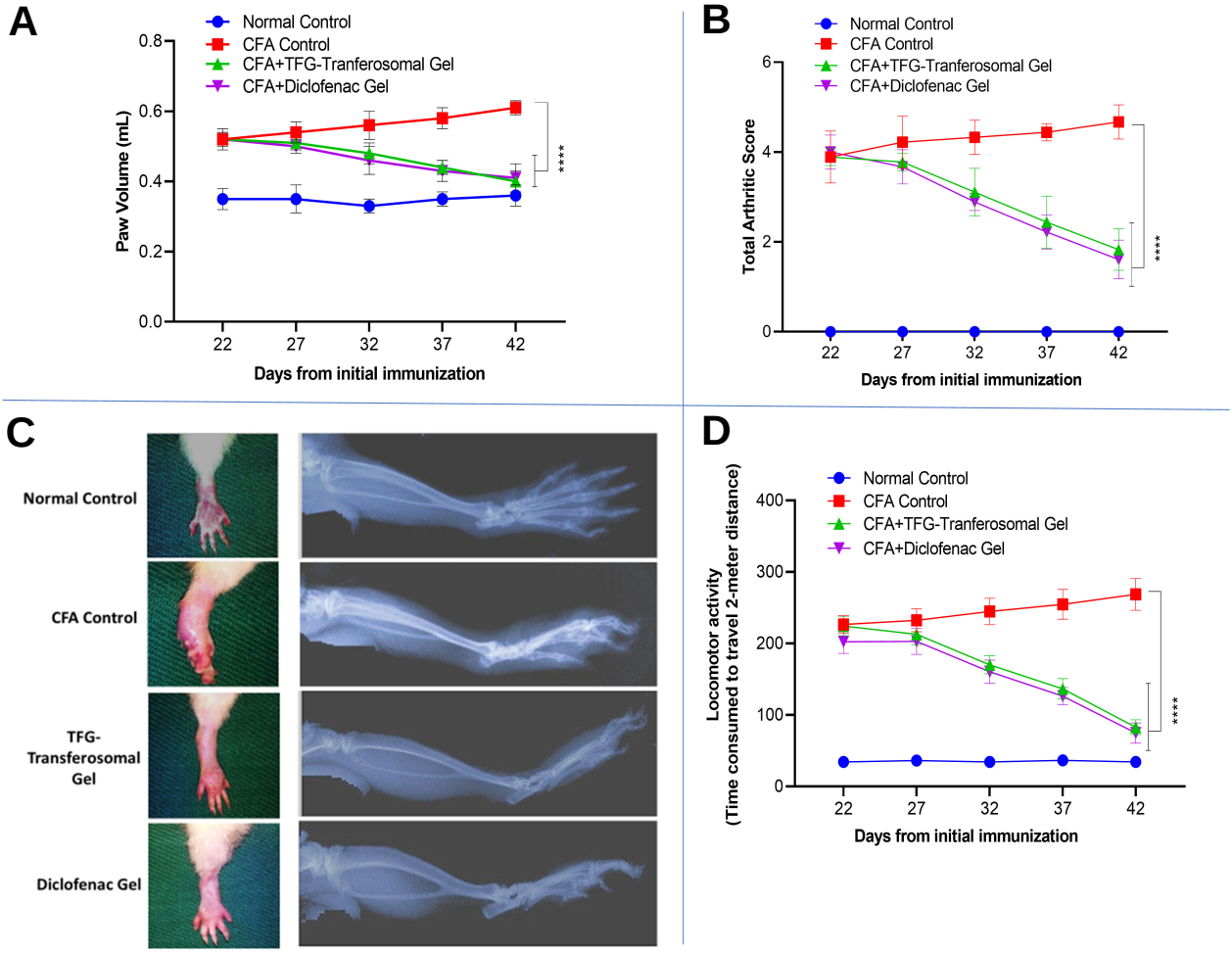

Figure 1. (A) effects of TFG-transferosomal gel and diclofenac gel on paw volume; **(B)** effect of TFG-loaded transferosomal gel on CFA-induced arthritic score; **(C)** photographs and radiographs of rat paws; **(D)** effects of TFG-transferosomal gel and diclofenac gel on locomotor activity.
* indicates a comparison between CFA control and treatment groups; **** indicates $P < 0.0001$.

The IL-10 levels were significantly reduced in the CFA control group (17.5 ± 0.8 pg/mL) compared with the normal control group (25.4 ± 0.9 pg/mL), indicating a decreased anti-inflammatory response in the presence of inflammation induced by CFA. Treatment with both TFG-transferosomal gel (24.1 ± 1.4 pg/mL) and diclofenac gel (23.5 ± 0.8 pg/mL) led to a significant increase in IL-10 levels compared with the CFA control group ($P < 0.001$) **(Figure 2D).**

Furthermore, the TNF-α levels were significantly elevated in the CFA control group (26.9 ± 2.2 pg/mL) compared with the normal control group (9.5 ± 0.3 pg/mL), indicating increased inflammation due to CFA induction. Treatment with both TFG-transferosomal gel (17.4 ± 0.9 pg/mL) and diclofenac gel (12.1 ± 0.3 pg/mL) resulted in a significant reduction in TNF-α levels compared with the CFA control group ($P < 0.0001$) **(Figure 2E).**

Effect of TFG-transferosomal gel on the inflammatory markers CRP and total protein

In the CFA control group, CRP levels were significantly elevated compared with the normal control group (6.7 ± 0.6 mg/L). However, treatment with both TFG-transferosomal gel (4.3 ± 0.5 mg/L) and diclofenac gel (3.7 ± 0.6 mg/L) resulted in a significant reduction in CRP levels compared with the CFA control group ($P < 0.0001$) **(Figure 2F)**. The total protein concentration was notably decreased in the CFA control group (5.5 ± 0.3 g/dL). Treatment with both TFG-transferosomal gel (7.6 ± 0.6 g/dL) and diclofenac gel (7.4 ± 0.3 g/dL) resulted in a significant increase in the total protein concentration compared with the CFA control group ($P < 0.0001$) **(Figure 2G).**

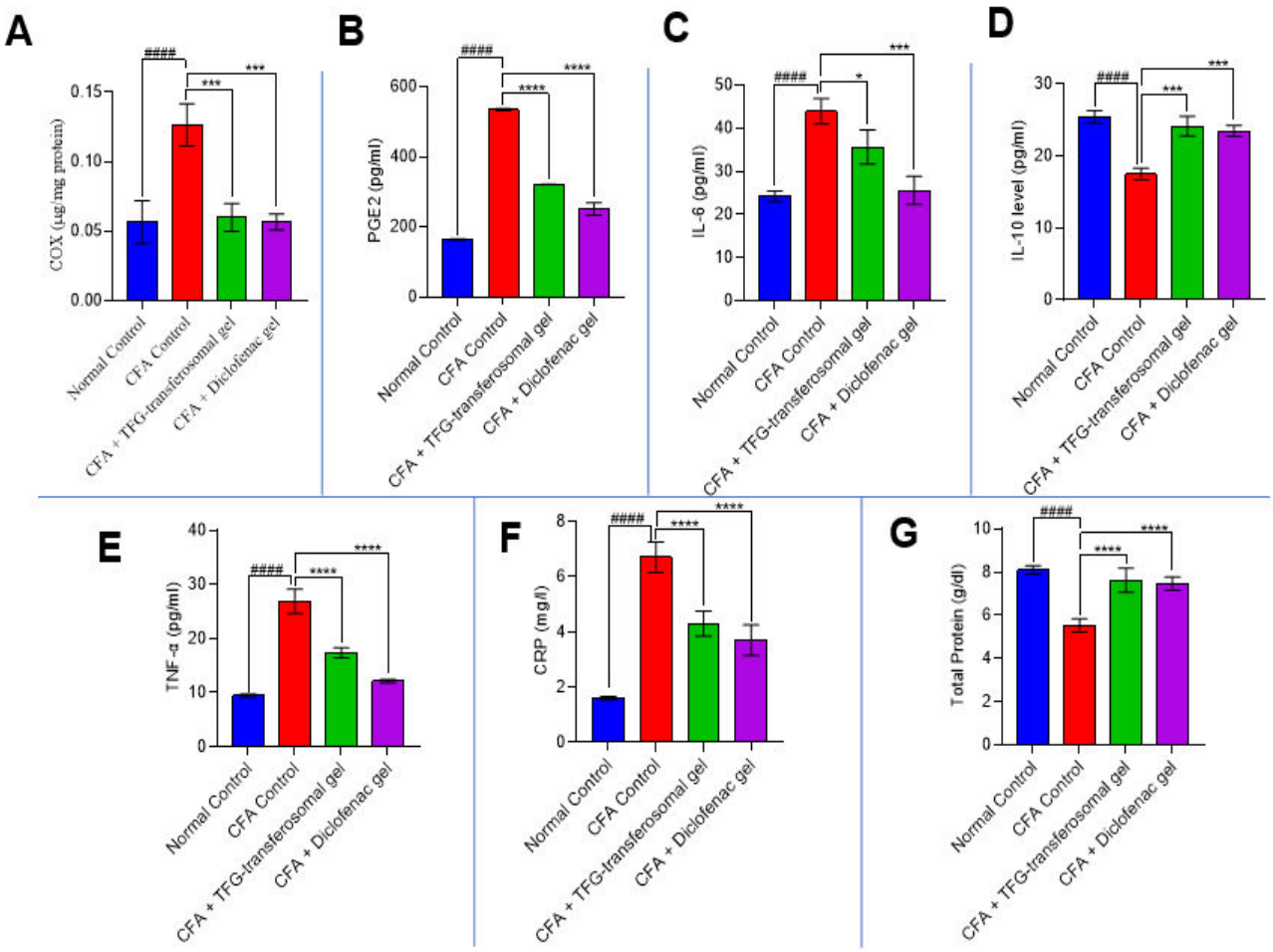

Figure 2. (A) Effects of TFG-transferosomal gel and diclofenac gel on COX; **(B)** Effects of TFG-transferosomal gel and diclofenac gel on PGE2; **(C)** Effects of TFG-transferosomal gel and diclofenac gel on IL-6; **(D)** Effects of TFG-transferosomal gel and diclofenac gel on IL-10; **(E)** Effects of TFG-transferosomal gel and diclofenac gel on TNF-α; **(F)** Effects of TFG-transferosomal gel and diclofenac gel on C-reactive protein (CRP); **(G)** Effects of TFG-transferosomal gel and diclofenac gel on total protein.
# indicates the comparison between normal control and CFA control
* indicates the comparison between CFA control and treatment groups, * indicates $P < 0.05$, *** indicates $P < 0.001$, and **** or #### indicates $P < 0.0001$.

Discussion

The successful preparation and characterization of TFG-loaded transferosomal gel is a substantial advancement in drug delivery systems for the treatment of arthritis. The particle size of 269.2 ± 1.2 nm suggests that the transferosomes are within the optimal range for cellular uptake and subsequent distribution to the inflamed tissues. In addition, the negative zeta potential indicates good stability and dispersion of the transferosomal suspension, which is essential for prolonged circulation and enhanced therapeutic efficacy. Furthermore, the high entrapment efficiency highlights the efficient encapsulation of TFG within the transferosomes, thereby ensuring maximum drug loading while minimizing potential systemic toxicity. Moreover, the deformability index reflects the flexibility of the vesicles, enabling them to traverse biological barriers and effectively reach the target site. These findings collectively demonstrate the suitability of the TFG-transferosomal gel as a promising drug delivery system for arthritis treatment, offering improved pharmacokinetics and therapeutic outcomes compared with conventional formulations.

The in vivo evaluation of the TFG-transferosomal gel in a Freund's adjuvant-induced arthritis rat model revealed its remarkable efficacy in alleviating arthritic symptoms. The significant reduction in paw volume and arthritic scores following treatment with TFG-transferosomal gel emphasizes its potent anti-inflammatory effects, comparable to those of diclofenac gel, a standard antiarthritic medication. Moreover, the observed improvement in locomotor activity in the TFG-transferosomal gel-treated rats further supports its therapeutic potential in enhancing mobility and reducing pain-associated hypoactivity. These findings underscore the promising antiarthritic activity of the TFG-transferosomal gel and indicate its potential as a viable alternative to conventional therapies for arthritis management.

The biochemical analysis of inflammatory enzymes and mediators provides mechanistic insights into the anti-inflammatory effects of the TFG-transferosomal gel. The significant reduction in COX activity and PGE_2 levels following treatment with TFG-transferosomal gel corroborates its ability to suppress the inflammatory cascade, akin to diclofenac gel. Furthermore, the modulation of inflammatory cytokines, including IL-6, IL-10, and TNF-α, highlights the broader immunomodulatory effects of TFG-transferosomal gel, contributing to its antiarthritic properties. In addition, the observed reduction in systemic inflammation, as evidenced by decreased CRP levels and restoration of total protein concentration, further underscores the therapeutic efficacy of TFG-transferosomal gel in mitigating arthritis-associated inflammation and systemic effects. These findings elucidate the multifaceted anti-inflammatory mechanisms of the TFG-transferosomal gel, positioning it as a promising therapeutic option for arthritis treatment with potential benefits beyond symptom alleviation.

The comprehensive evaluation of the TFG-transferosomal gel reiterates its potential as a novel therapeutic approach for arthritis management. The successful preparation and characterization of the TFG-loaded transferosomal gel, coupled with its demonstrated efficacy in preclinical models, highlight its potential as an alternative to conventional arthritis treatment. Further research is required to elucidate the underlying mechanisms of action, optimize dosage regimens, and evaluate the long-term efficacy and safety profiles in clinical settings. In addition, investigations into the pharmacokinetic profile and tissue distribution of the TFG-transferosomal gel will provide valuable insights into its therapeutic potential and pharmacological properties. Overall, this TFG-transferosomal gel represents a promising avenue for the development of innovative and effective therapies for arthritis and other inflammatory conditions, offering benefits in terms of enhanced efficacy, reduced side effects, and improved patient outcomes.

Conclusion

This study on TFG-transferosomal gel highlights its potential as an anti-inflammatory and antiarthritic treatment. The gel effectively reduced the paw volume or edema in arthritic rats, demonstrating a significant anti-inflammatory effect compared with the CFA control group. Its efficacy is comparable to that of diclofenac gel, which is a standard anti-inflammatory treatment, suggesting that TFG-transferosomal gel could be a viable alternative. The gel also reduced the arthritic scores, although with a slightly lower efficacy than diclofenac in some cases, indicating its potential to alleviate arthritis symptoms. In addition, it reduces pain-induced hypoactivity in arthritic rats, thus confirming its potential as an antiarthritic treatment with positive effects on locomotor activity. Biochemical assessments revealed the gel's ability to decrease inflammatory enzymes and mediators, such as COX and PGE_2, along with a significant reduction in IL-6, TNF-α, and CRP levels while increasing IL-10 levels. These findings collectively indicate that the TFG-transferosomal gel has notable anti-inflammatory properties, making it a promising candidate for further research and clinical trials to fully explore its therapeutic potential and establish its efficacy in clinical settings.

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Conflicts of interest statement

The authors have no commercial, proprietary, or financial interest in the products or companies described in this article.

Data sharing statement

All data generated or analyzed during the present study are included in this published article. Further details are available for noncommercial purposes from the corresponding author on reasonable request.

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