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Research Article

FORMULATION AND CHARACTERIZATION OF BETULIN-LOADED MICROEMULSION FOR ANTI-GOUT THERAPY

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ABSTRACT

Objectives: The study aimed to formulate and evaluate a betulin-loaded microemulsion for enhanced topical anti-gout efficacy and skin retention, using diclofenac diethylamine emulgel 1% as the positive control.

Methods: Betulin microemulsions were prepared using medium-chain triglyceride oil, Tween 80, and ethanol through the phase inversion method. Physicochemical characterization was performed through droplet size analysis, zeta potential, viscosity, pH, and Fourier-transform infrared spectroscopy studies. Pseudo-ternary phase diagrams were constructed to optimize composition. *Ex vivo* permeation and skin retention were assessed using porcine skin in Franz diffusion cells, followed by skin homogenization and extraction. Anti-inflammatory efficacy was evaluated in a monosodium urate (MSU) crystal-induced gouty arthritis model in mice, while skin irritation studies were conducted in guinea pigs. Statistical analysis was performed using one-way analysis of variance followed by Dunnett's test.

Results: The optimized microemulsion (10% oil, 20% surfactant, 10% co-surfactant, and 0.5% betulin) exhibited droplet size of 152±3.4 nm, polydispersity index of 0.218, and zeta potential of −28.5 mV. *Ex vivo* studies revealed cumulative permeation of 50.2±3.0 µg/cm2 at 24 h with 24.7±2.3% retention in the skin. *In vivo*, betulin microemulsion (1%) significantly reduced paw edema and pro-inflammatory cytokines (interleukin-1 beta and tumor necrosis factor-alpha), and improved weight-bearing distribution compared to the MSU group (***p<0.001), demonstrating efficacy comparable to diclofenac emulgel. No dermal irritation was observed in guinea pigs.

Conclusion: Betulin microemulsion exhibited favorable physicochemical properties, high skin retention, and potent topical anti-inflammatory activity in gout, with excellent dermal safety. These findings support its potential as a safe and effective topical delivery system for the management of gouty arthritis.

Keywords: Betulin, Microemulsion, Gouty arthritis, Anti-inflammatory, Transdermal drug delivery.

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INTRODUCTION

Gouty arthritis is a painful inflammatory condition caused by the buildup of monosodium urate (MSU) crystals in the joints, leading to severe pain, swelling, and possible damage to joint tissues. The disease advances due to the activation of pro-inflammatory signaling pathways, particularly the NLRP3 (NOD-like receptor family, pyrin domain containing 3) inflammasome and subsequent cytokine production. Although non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids are routinely prescribed for symptom relief, prolonged use of these medications can lead to complications, including gastrointestinal disturbances, cardiovascular issues, and suppressed immune function [1]. Therefore, the pursuit of safer and more effective alternative therapies is gaining importance. One such candidate is betulin, a pentacyclic triterpenoid derived from the bark of birch trees, which has demonstrated anti-inflammatory, antioxidant, and pain-relieving potential. However, its limited aqueous solubility and poor systemic availability pose challenges for its clinical application. To overcome these drawbacks, nanotechnology-based delivery platforms like microemulsions are being explored. These stable colloidal systems, made up of oil, water, surfactants, and co-surfactants, enhance the solubility, stability, and permeation of lipophilic drugs such as betulin, thereby improving therapeutic efficacy [2].

In this study, a betulin-loaded microemulsion was developed and the main objective was to improve betulin's solubility, stability, and permeation for potential topical and systemic applications in gout management. The formulation was extensively characterized for its stability, droplet size, pH, viscosity, drug-excipient interactions, and skin permeability using *ex vivo* permeation studies [3]. Furthermore, the therapeutic efficacy of the microemulsion was evaluated in an MSU crystal-induced gouty arthritis model, assessing its impact on inflammation, pain reduction, cytokine modulation, and histopathological changes. This research aims to establish betulin-loaded microemulsion as a novel drug delivery system for enhancing anti-gout therapy, paving the way for its potential clinical applications [4].

MATERIALS AND METHODS

Materials

The materials used in this study include betulin (≥98% HPLC, Cat. No. B9756) purchased from Sigma-Aldrich, St. Louis, MO, USA. Caprylic/Capric Triglycerides (MCT oil), a pharmaceutical grade product, was sourced from Croda International (or equivalent) in Mumbai, India. Tween 80 (Polysorbate 80), with ≥99% purity (Cat. No. 822187), was obtained from Merck (Sigma), Darmstadt, Germany. Ethanol, with an analytical grade of 99.9%, was procured from Merck, Mumbai, India. Purified water was supplied by the Milli-Q System at Sandip University Lab, India. The monosodium urate crystals used in the study were prepared in-house and characterized microscopically. Additionally, ELISA kits for IL-1β and TNF-α were obtained from R&D Systems, Minneapolis, MN, USA, following the manufacturer's instructions (Table 1).

Table 1: List of materials, suppliers, and specifications used in the formulation and evaluation of betulin-loaded microemulsion gel for anti-gout therapy

Material	Supplier (company)	City, country	Purity/grade/catalog no.
Betulin	Sigma-Aldrich	St. Louis, MO, USA	≥98% (HPLC), Cat. No. B9756
Caprylic/Capric Triglycerides (MCT oil)	Croda International/equivalent	Mumbai, India (imported)	Pharmaceutical grade
Tween 80 (Polysorbate 80)	Merck (Sigma)	Darmstadt, Germany	≥99%, Cat. No. 822187
Ethanol	Merck	Mumbai, India	Analytical grade (99.9%)
Purified Water	Milli-Q System	Sandip Univ. Lab, India	Laboratory grade
Monosodium urate crystals	Prepared in-house (per method)	—	Characterized microscopically
ELISA Kits (IL-1β, TNF-α)	R&D Systems	Minneapolis, MN, USA	As per manufacturer's instructions

IL-1β: Interleukin-1 beta, TNF-α: Tumor necrosis factor-alpha, ELISA: Enzyme-linked immunosorbent assay, HPLC: High-performance liquid chromatography

Methods

The microemulsion was prepared by the phase inversion method with controlled stirring and thermal ramps to ensure reproducibility and uniform droplet formation. Briefly, the oil phase (caprylic/capric triglycerides) containing betulin (0.5% w/w) was stirred into the surfactant/co-surfactant blend (Tween 80: ethanol, Smix 2:1) using a digital magnetic stirrer set at 500 rpm. The oil was added dropwise at a rate of ≈0.5 mL·min^{-t}.[5] The mixture was heated from room temperature to the phase inversion temperature at a rate of 2°C·min^{-} and held at 65°C for 5 min to ensure complete equilibration. The system was then cooled to room temperature at 1.5°C·min^{-c} under continuous stirring (500 rpm). During cooling, the system became transparent, indicating microemulsion formation. All thermal ramps were performed in a temperature-controlled water bath (±0.5°C). These parameters were chosen because moderate stirring (500 rpm) and controlled thermal ramps favor small, uniform droplet size, and improved thermodynamic stability [6,7] (Table 2).

Characterization studies

Visual observation

After the gradual incorporation of water into the blend of oil, surfactant, and co-surfactant, the mixture was visually assessed at each step. Depending on their physical appearance, the resulting systems were categorized as micro-emulsions, emulsions, or gel-like structures [8].

Thermodynamic stability evaluation

To ensure the elimination of metastable or unstable formulations, a series of thermodynamic stability assessments were carried out [9].

Centrifugation analysis

The sample underwent centrifugation at 3500 rpm for 30 min to evaluate its resistance to phase separation and ensure structural integrity under mechanical stress [10].

Stress condition testing

To determine the robustness of the formulation under fluctuating temperature conditions, selected samples were subjected to alternate temperature cycles – stored at 4°C and 45°C for 48 h each across six cycles, followed by storage at 25°C and 21°C for 48 h over three cycles. After each cycle, the formulations were checked for any signs of phase separation, coalescence, or cracking [11].

pH Assessment

The pH value of the final formulation was recorded by immersing the electrode of a pre-calibrated digital pH meter (Model EQ-601, EquipTronics) directly into the sample dispersion [12].

Viscosity measurement

Brookfield viscometer was employed to measure the viscosity (Model DV-E, LVDVE), applied without prior dilution to maintain accuracy [13].

Table 2: Formula for preparation of microemulsion

Component	% (w/w)	Ingredient
Oil phase	10	Caprylic/capric triglycerides (MCT oil)
Surfactant	20	Tween 80
Co-surfactant	10	Ethanol
Water phase	59.5	Purified water
Phytochemical	0.5	Betulin

Drug-excipient interaction

The characterization of chemical interactions between the solid drug and excipient was done by Fourier-transform infrared spectroscopy (FTIR) [14].

Determination of particle size

The size of particle and size distribution of the drug-encapsulated micro-emulsion was evaluated with Horiba SZ-100 nanoparticle analyzer operated at 28°C. This device evaluates particle size by measuring variations in the intensity of light that is scattered, which is influenced by the Brownian motion of nanoparticles within a suspension. To ensure the reliability and reproducibility of results, each formulation was measured in triplicate [15].

Ex vivo permeation study protocol

This study utilizes excised animal skin mounted in a Franz diffusion cell to simulate *in vivo* conditions. The materials required for this study include excised porcine skin (dermatomed to ~0.5 mm thickness), a Franz diffusion cell with a receptor compartment volume of ~5–10 mL and a diffusion area of ~1–2 cm^2, phosphate buffered saline (PBS) (pH ~7.4) as the receptor medium, a magnetic stirrer with a stirring bar, an high-performance liquid chromatography- ultraviolet-visible (HPLC/UV-Vis) spectrophotometer for drug quantification, and a thermostatic water bath at 37°C±0.5°C. The procedure begins with the preparation of the skin sample, where excised porcine skin is cleaned to remove subcutaneous fat and equilibrated in PBS (pH 7.4) for 1 h at room temperature. The skin is then mounted onto the Franz diffusion cell, ensuring that the donor compartment at the epidermal side. The receptor compartment is filled with PBS or a suitable medium to uphold sink conditions, and the system is kept at a temperature of 37°C±0.5°C with on-going stirring. A known amount of the test formulation (e.g., 500 mg) is then applied to the donor compartment [16,17]

Samples are collected by withdrawing aliquots (0.5–1 mL) at predetermined intervals, such as 0, 1, 2, 4, 6, 8, 12, and 24 h, ensuring that the receptor compartment is replenished with fresh medium after each withdrawal. The collected samples are analyzed using HPLC or UV-Vis spectrophotometry, with a calibration curve prepared for betulin quantification. The cumulative amount permeated per unit area (μg/cm^2) and flux (J, μg/cm^2/h) are then calculated. The co-efficient of permeability (Kp) and steady-state flux (Jss) are determined to

evaluate the formulation's effectiveness. If skin retention is assessed, the remaining skin is homogenized, extracted with an appropriate solvent such as methanol, and analyzed for the retained betulin content.

Skin retention studies

At the end of the 24 h permeation experiment, the treated porcine skin samples were carefully removed from the Franz diffusion cells, rinsed with distilled water to remove surface formulation, blotted dry, and weighed. Each skin sample was homogenized in 5 mL of methanol using a tissue homogenizer for 5 min. The homogenates were centrifuged at 10,000 rpm for 15 min at 4°C, and the supernatant was collected, filtered through a 0.22 μm membrane filter, and analyzed for betulin content by UV-Vis spectrophotometry at 210 nm (or HPLC, if used). The retained drug amount was expressed as μg of betulin per g of skin tissue and calculated as a percentage of the total applied dose.

Guinea pig skin irritation test

The skin irritation test in guinea pigs is conducted to evaluate the potential of the formulation to cause dermal irritation and assess its safety for topical application. The test follows OECD 404 guidelines for acute dermal irritation/corrosion [18].

MATERIALS AND METHODS

Animals and ethical considerations

Healthy adult male and female guinea pigs (weighing 350–450 g) are selected for the study. The animals are kept in a regulated setting (22±2°C, 12-h light/dark cycle) where they have unrestricted access to food and water. Before the experiment, they are acclimatized for 1 week to ensure normal physiological conditions. All experimental procedures were conducted in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India. The study protocol was reviewed and approved by the Institutional Animal Ethics Committee (IAEC), School of Pharmaceutical Sciences, Sandip University, Nasik, under approval number: 2353/PO/Re/S/2025/CCSEA/IAEC/SOPS/03 [19].

Skin preparation and test sites

The dorsal area of each guinea pig is carefully shaved using an electric clipper 24 h before application to expose a 2.5 cm^2 test site. Care is taken to avoid any cuts or abrasions. Each animal has three sites marked [20]:

1. Test site – Application of the given formulation
2. Negative control site – Application of sterile saline solution
3. Positive control site – Application of 0.5% sodium lauryl sulfate solution to induce irritation.

Application of the formulation

A 0.5 mL aliquot of the formulation is applied directly onto the test site using a sterile cotton swab. The area is covered with a semi-occlusive dressing (gauze with adhesive tape) to ensure close contact with the skin. The dressing remains in place for 4 h, after which it is removed, and the skin is gently wiped with sterile saline to remove any residual formulation [21].

Observation and scoring

The test sites are examined for any visible signs of irritation at 24, 48, and 72 h post-application. The scoring is based on Draize dermal irritation criteria, which include assessments of erythema (redness), edema (swelling), and other skin reactions (dryness, scaling, necrosis, or ulceration) [22].

The irritation is scored as follows:

- Erythema scale
  - 0=No erythema
  - 1=Mild redness (hardly noticeable)
  - 2=Noticeable redness (clear redness)
  - 3=Intense redness (vivid red inflammation)
  - 4=Extreme redness (dark red with tissue death)
- Edema scale
  - 0=No edema
  - 1=Slight edema (barely perceptible)
  - 2=Moderate swelling (elevated skin)
  - 3=Severe swelling (swelling >1 mm)
  - 4=Extreme edema (massive swelling).

Anti-gout activity

Effect of microemulsion on MSU crystal-induced gouty arthritis in mice

MSU crystal synthesis

To synthesize MSU crystals, 4 g of uric acid was dissolved in 800 mL of distilled water, assisted by the addition of 9 mL of 0.5 M sodium hydroxide (NaOH). The mixture was heated, and the pH was adjusted to 8.9 at a temperature of 60°C. The solution was then allowed to sit overnight at a low temperature to promote crystallization. The resulting crystals were collected by decanting the excess liquid, followed by thorough washing and drying. The needle-like shape and dimensions of the crystals were confirmed through polarizing light microscopy. For subsequent use, the dried MSU crystals were mixed in 2.5% Tween 80 in PBS to achieve a final concentration of 80 mg/mL [23,24].

Induction of gouty arthritis using MSU crystals in mice

The study involved 7-week-old male mice, each weighing between 20 and 22 g. The mice were kept in a regulated environment at a temperature of 22±2°C, with a relative humidity of 55±10%, and were subjected to a 12-h light/dark cycle. During a 6-day acclimatization phase, the mice had free access to standard commercial chow (Dae-Han Bio Link) and tap water. After acclimatization, each mouse was housed individually and gradually habituated to the experimental procedures [25-27].

The animals were categorized into five groups, each containing five animals:

- Group I: Control group
- Group II: MSU crystal group: An MSU crystal suspension (4 mg/50 μL) was injected intra-dermally into the right paw
- Group III: Diclofenac diethylamine emulgel 1% with MSU crystal injection
- Group IV: 0.5% microemulsion with MSU crystal injection
- Group V: 1% microemulsion with MSU crystal injection.

The test formulations were administered once daily over a period of 3 days. On the 4^{th} day, following intra-articular injection of MSU crystals, the animals were anesthetized using sodium pentobarbital and subsequently euthanized. The right side hind paw was carefully dissected and was homogenized in ice-cold radioimmunoprecipitation assay (RIPA) buffer maintained at 4°C. The tissue homogenate was then subjected for 14,000 rpm for 15 min to centrigation at 4°C. The supernatant was harvested and stored at −70°C for downstream biochemical and molecular analyses.

Evaluation of inflammatory paw edema

At the conclusion of the experimental period, paw swelling – an indicator of inflammatory response – was quantified by measuring the paw thickness using a digital Vernier caliper. This method provided a simple and effective assessment of edema severity in the affected limb [28-30].

Evaluation of inflammatory pain

Following the induction of arthritis, an imbalance in the usual weight distribution between the hind limbs was noted, indicating behavior associated with pain. The transfer of weight from the inflamed paw to the unaffected (contralateral) paw served as an index of nociceptive response. This was assessed using a dynamic weight bearing (DWB) system, which recorded the individual load applied by each hind paw. The percentage distribution of weight on each paw was calculated using the formula:

(Weight on the right hind leg/[Weight on the right hind leg+Weight on the left hind leg]) × 100 [31-33].

Measurement of inflammatory cytokines

The concentrations of Interleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) were assessed utilizing enzyme-linked immunosorbent assay (ELISA) kits from R&D Systems (Minneapolis, MN, USA), following the instructions provided by the manufacturer [34-36].

Histological analysis

At the conclusion of the study, biopsy samples from the foot were gathered for histo-pathological analysis. The samples were stained with hematoxylin and eosin (H&E) and then inspected under a microscope [37-40].

Statistical analysis

Data were expressed as mean±standard error of the mean. Statistical comparisons were performed using one-way analysis of variance followed by Dunnett's *post hoc* test for multiple group comparisons, or unpaired Student's t-test for comparisons between two groups. All analyses were performed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA). p-value of below 0.001 was deemed statistically significant.

RESULTS

Visual inspection

The optimized formulation showed characteristics of a stable microemulsion with a clear to slightly opalescent appearance and no visible phase separation.

Thermodynamic stability studies

Centrifugation test

The formulation remained stable without any signs of phase separation, coalescence, or precipitation after centrifugation at 3500 rpm for 30 min. This confirms its physical stability under high-stress conditions.

Stress test

The optimized microemulsion formulation remained stable under extreme temperature conditions (4°C, 45°C, 25°C, and 21°C) throughout the test cycles. No coalescence, cracking, or phase separation was observed. These results indicate that the formulation is thermodynamically stable and can withstand temperature variations.

pH measurement

The pH of the optimized formulation was 6.2±0.1, making it suitable for topical or oral administration without causing irritation or instability.

Viscosity measurements

Microemulsion viscosity was analyzed by Brook field viscometer and was found to be 32.5±2.0 cP. This low viscosity confirms that the formulation retains its fluidic nature, making it ideal for drug delivery applications.

Drug-excipients interaction

The FTIR spectrum of pure betulin (Fig. 1a) exhibited a broad O–H stretching band in the ~3400–3250 cm^{-1} region, consistent with the presence of hydrogen-bonded hydroxyl groups at C-3 and C-28. Strong aliphatic C-H stretching bands were observed near 2958 and 2868 cm^{-1}. A medium-intensity band at ~1640–1660 cm^{-1} was assigned to the C=C stretching vibration of the exocyclic methylene (20(29)-ene) in the lupane skeleton; no absorption was observed in the ~1700–1750 cm^{-1} region, confirming the absence of carbonyl functionality. The C–O stretching vibration of the alcohol(s) appeared in the 1050–1150 cm^{-1} region, while bands at ~1450–1375 cm^{-1} correspond to CH_2/CH_2 bending. Characteristic out-of-plane =C–H vibrations of the exocyclic methylene were noted near 900–910 cm^{-1}. These assignments are in keeping with spectra reported for lupane-type triterpenes and indicate that betulin remains chemically unmodified.

In the FTIR of the betulin-loaded microemulsion (Fig. 1b), the principal betulin bands were preserved but showed minor shifts and changes in

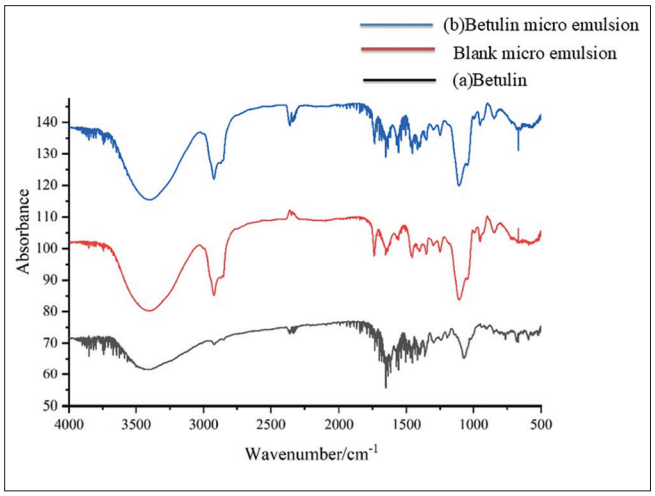

Fig. 1: Fourier-transform infrared spectroscopy spectra of (a) pure betulin and (b) betulin-loaded microemulsion. Major band assignments: broad O–H stretch ~3400–3250 cm^{-1} (hydroxyl groups), aliphatic C–H stretches ~2950–2850 cm^{-1}, C=C stretching (exocyclic methylene) ~1640–1660 cm^{-1}, C–O stretch ~1050–1150 cm^{-1}, and =C–H out-of-plane bending ~900–910 cm^{-1}. No carbonyl band (~1700–1750 cm^{-1}) was detected in pure betulin, indicating absence of oxidation or carbonyl impurities

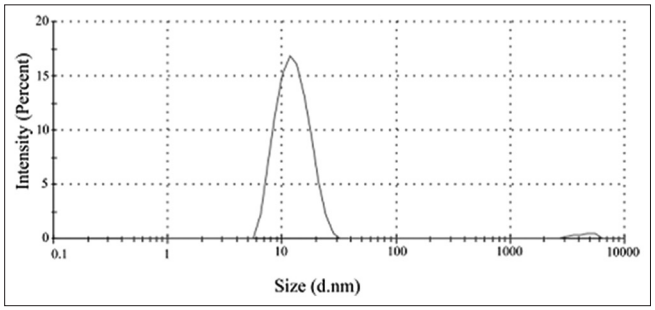

Fig. 2: Particle size of microemulusion by atomic force microscopy

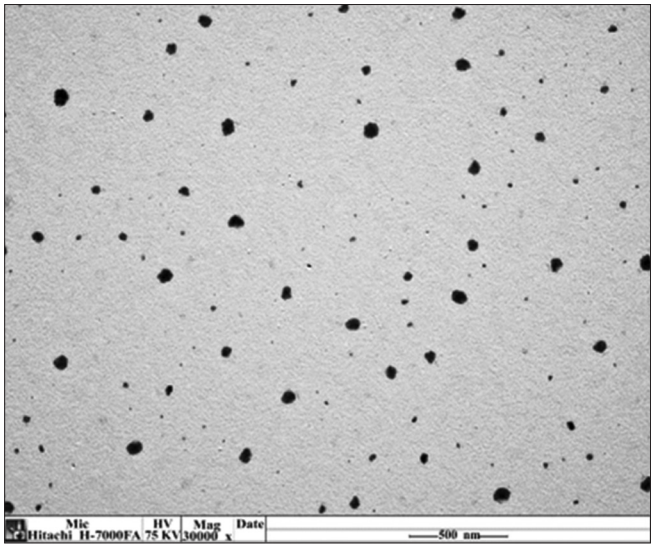

Fig. 3: Particle size of microemulusion by transmission electron microscopy

intensity (for example, the O–H band became slightly broader and the C–O band showed reduced intensity), consistent with physical solubilization/dispersion of betulin in the microemulsion rather than chemical modification (no new peaks indicative of covalent interaction were observed) (Fig. 1).

Particle size determination

The morphology and size characteristics of the microemulsion droplets were determined using atomic force microscopy (AFM) and transmission electron microscopy (TEM). Both techniques confirmed that the particles were spherical in shape, exhibited uniform size distribution, and were evenly dispersed. The mean droplet diameter of the microemulsion formulation was measured to be 15.95±0.77 nm based on triplicate analysis (n=3) (Figs. 2 and 3).

"*Ex vivo* permeation studies demonstrated a steady increase in cumulative betulin permeation, reaching 50.2±3.0 μg/cm^2 at 24 h with a flux of 3.2 μg/cm^2/h (Table 3). Skin retention analysis showed that 24.7±2.3% of the applied dose remained within the skin after 24 h, suggesting substantial local depot formation that may enhance topical efficacy."

Skin irritation studies

- Erythema: As light redness (0.5) observed at 24 h, fading by 48 h, with complete disappearance at 72 h.
- Edema: Minimal swelling (0.3 at 24 h), reducing at 48 h, and absent by 72 h.
- Other skin reactions: Mild dryness observed in one guinea pig, but no signs of necrosis, ulceration, or peeling (Table 4 and Fig. 4).

Based on results, all irritation scores are ≤1 and recover within 72 h, the formulation is classified as non-irritant.

Effect of betulin microemulsion on MSU crystal-induced paw edema

Thickness of paw was evaluated in both control and treated mice to assess the degree of edema. Injection of MSU crystals resulted in a significant increase in paw thickness compared to the control group, indicating inflammation. However, mice treated with betulin microemulsion (1%) and diclofenac diethylamine emulgel showed a noticeable reduction in paw thickness. These findings suggest that betulin microemulsion suppressed the parameter effectively (Table 5 and Fig. 5).

Table 3: *Ex vivo* permeation study (Franz diffusion cell)

Time (h)	Cumulative permeation (μg/cm^2)	Permeability coefficient (Kp, cm/h×10^{-3})	Flux (J, μg/cm^2/h)	Skin retention (% of applied dose)
0	0	–	–	–
1	2.3±0.5	0.15	2.3	–
2	5.1±0.7	0.20	2.5	–
4	10.8±1.2	0.22	2.7	–
6	15.5±1.5	0.23	2.8	–
8	22.3±1.8	0.24	2.9	–
12	35.6±2.1	0.25	3.0	–
24	50.2±3.0	0.27	3.2	24.7±2.3

Table 4: Observation of skin reactions (erythema and edema scores)

Time (hours post-application)	Erythema score (0–4) mean±SEM	Edema score (0–4) mean±SEM	Other reactions (scaling, dryness, necrosis)
24 h	0.5±0.2	0.3±0.2	Slight dryness in 1/5 animals
48 h	0.3±0.1	0.1±0.1	No visible irritation
72 h	0.1±0.1	0.0	Complete recovery

SEM: Standard error of the mean

Table 5: Betulin microemulsion effect on MSU crystal-induced paw edema

Group	Thickness of foot (mm)
Group I control group	3.2±0.12
Group II MSU crystal group	5.2±0.24*
Group III diclofenac diethylamine emulgel	3.6±0.36**
Group IV 0.5% microemulsion	4.9±0.32
Group V 1% microemulsion	3.9±0.45***

MSU: Monosodium urate. *Values are expressed as mean±standard error of the mean (n=5). Statistical significance was assessed by one-way analysis of variance followed by Dunnett's *post hoc* test comparing treatment groups (III-V) with the MSU crystal group (II). Symbols: *p<0.05, **p<0.01, and ***p<0.001 versus Group II

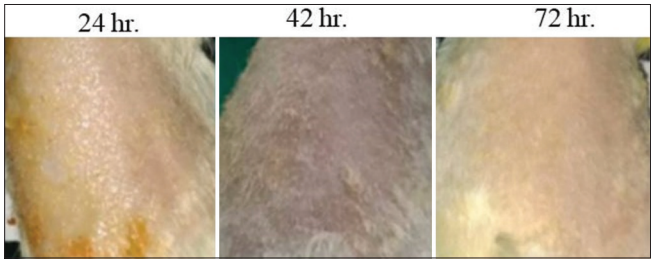

Fig. 4: Observation of acute skin reactions on guinea pig

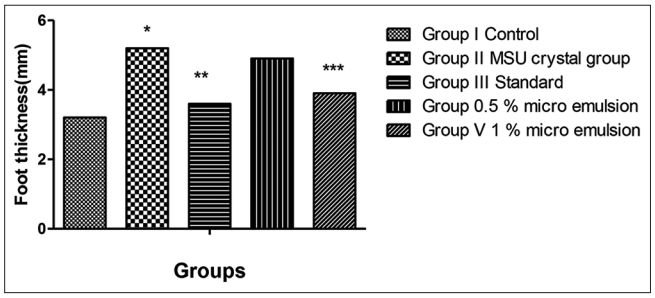

Fig. 5: Effect of betulin microemulsion on monosodium urate crystal-induced paw edema. Effect of betulin microemulsion on MSU crystal-induced paw edema. Values are mean±standard error of the mean (n=5). Statistical significance by one-way analysis of variance followed by Dunnett's test, comparing treatment groups with MSU group. *p<0.05, **p<0.01, ***p<0.001 versus Group II

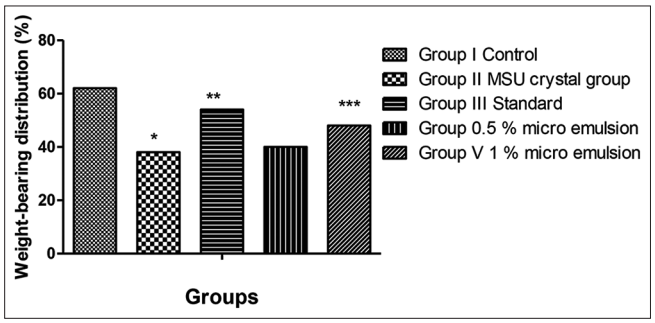

Fig. 6: Effect of betulin microemulsion on weight-bearing distribution (%). Effect of betulin microemulsion on weight-bearing distribution in monosodium urate crystal-induced gouty arthritis mice. Values are mean±standard error of the mean (n=5). *p<0.05, **p<0.01, ***p<0.001 versus Group II

Table 6: Effect of betulin microemulsion on hind paws weight-bearing distribution

Group	Weight-bearing distribution (%)
Group I control group	62±0.12
Group II MSU crystal group	38±0.16*
Group III diclofenac diethylamine emulgel	54±0.78**
Group IV 0.5% microemulsion	40±0.56
Group V 1% microemulsion	48±0.26***

*Values are expressed as mean±standard error of the mean (n=5). Statistical significance was assessed by one-way analysis of variance followed by Dunnett's *post hoc* test comparing treatment groups (III-V) with the MSU crystal group (II). Symbols: *p<0.05, **p<0.01, and ***p<0.001 versus Group II

Table 7: Betulin microemulsion effect on pro-inflammatory cytokines

Group	IL-1β (ng/mg)	TNF-α (ng/mg)
Group I Control group	1±0.1	0.7±0.15
Group II MSU crystal group	6.2±0.18*	1.8±0.79*
Group III diclofenac diethylamine emulgel	3.3±0.23**	1.2±0.32**
Group IV 0.5% microemulsion	4.2±0.42	1.5±0.45
Group V 1% microemulsion	3.6±0.89***	0.9±0.68***

*Values are expressed as mean±standard error of the mean (n=5). Statistical significance was assessed by one-way analysis of variance followed by Dunnett's *post hoc* test comparing treatment groups (III-V) with the MSU crystal group (II). Symbols: *p<0.05, **p<0.01, and ***p<0.001 vs. Group II

Betulin microemulsion effect on hind paws bearing weight distribution

The distribution ratio of weight between the right and left hind paws was utilized to assess the advancement of pain related to gouty arthritis. Mice injected with MSU crystals exhibited a significant reduction in weight-bearing on the affected limb compared to control mice, indicating inflammatory pain. However, treatment with betulin microemulsion and diclofenac diethylamine emulgel significantly restored the weight-bearing balance, as observed in the DWB measurements. These results demonstrate that betulin microemulsion effectively alleviated MSU crystal-induced pain in gouty arthritis (Table 6 and Fig. 6).

Betulin microemulsion effect on pro-inflammatory cytokines

The anti-inflammatory effects of betulin microemulsion were assessed by measuring the levels of TNF-α and IL-1β through ELISA after the injection of MSU crystals. Mice subjected to the MSU treatment exhibited a notable rise in both cytokines, reflecting a robust inflammatory response. Administration of betulin microemulsion significantly lowered the concentrations of TNF-α and IL-1β. Likewise, the diclofenac diethylamine emulgel also substantially reduced these pro-inflammatory cytokines in comparison to the MSU group. These results suggest that betulin microemulsion effectively suppresses critical inflammatory mediators that are involved in the initiation and progression of gouty arthritis (Table 7 and Fig. 7).

Histopathological examination

H&E staining of paw tissue from the gout model showed subcutaneous hyperplasia along with significant infiltration of inflammatory cells. Treatment with 1% betulin microemulsion significantly reduced inflammatory cell infiltration compared to the model group, although the effect was less pronounced than that observed with diclofenac diethylamine emulgel. The anti-inflammatory effect of the 1% microemulsion may be attributed to betulin-mediated suppression of NLRP3 activation of inflammasome and subsequent inhibition of the production of IL-1β (Fig. 8).

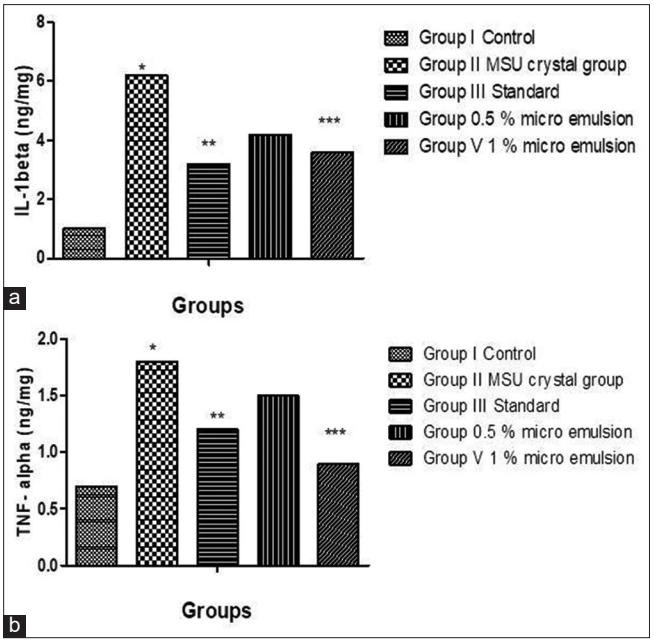

Fig. 7: (a and b) Effect of betulin microemulsion on pro-inflammatory cytokines. Effect of betulin microemulsion on pro-inflammatory cytokines (interleukin-1 beta, tumor necrosis factor-alpha-α). Data presented as mean±standard error of the mean (n=5). *p<0.05, **p<0.01, ***p<0.001 versus Group II

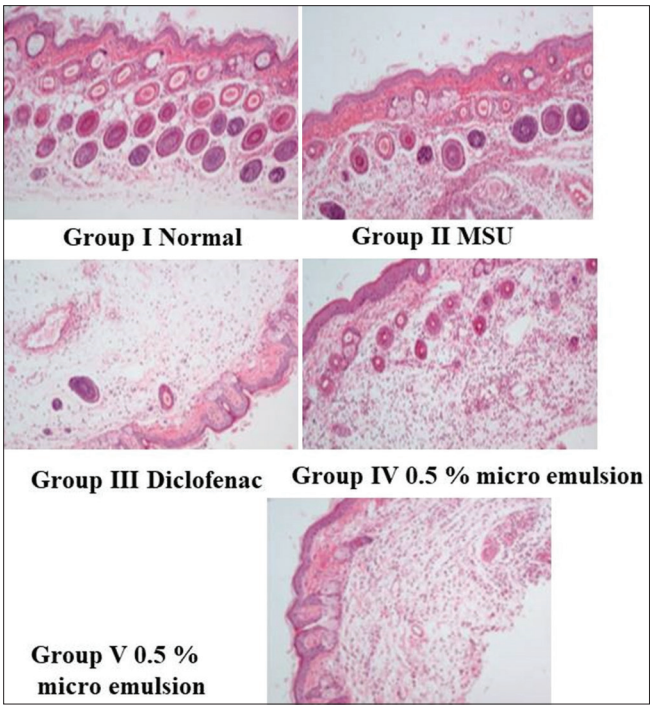

Fig. 8: Histopathological analysis of paw tissues in different groups showing inflammatory cell infiltration

DISCUSSION

The formulation of betulin-loaded microemulsion successfully improved the solubility and bioavailability of betulin, overcoming its inherent limitations. The optimized formulation demonstrated a stable oil-in-water microemulsion with a uniform droplet size of approximately 15.95±0.77 nm employing AFM and TEM. Stability studies, including centrifugation and thermal stress tests, confirmed

that the formulation remained stable under various storage conditions without phase separation, coalescence, or degradation. The *ex vivo* permeation study with Franz diffusion cell revealed a steady increase in betulin permeation, reaching approximately 50 μg/cm^2 after 24 h. The permeability coefficient (Kp) of 0.2–0.27 × 10^{-3} cm/h suggests a controlled and sustained drug release profile, making the formulation suitable for topical applications. Skin irritation studies in guinea pigs confirmed the formulation's biocompatibility, as minimal erythema and edema were observed, which resolved within 72 h, classifying it as non-irritant. The *in vivo* anti-gout study in an MSU crystal-induced gouty arthritis model demonstrated significant therapeutic efficacy. Mice treated with betulin microemulsion (1%) exhibited a notable reduction in paw edema, with foot thickness measurements significantly lower than the untreated MSU group. The weight-bearing distribution, a marker of inflammatory pain, improved considerably in the betulin-treated groups, comparable to diclofenac diethylamine emulgel. Furthermore, pro-inflammatory cytokine analysis revealed that betulin microemulsion significantly downregulated the expression of TNF-α and IL-1β, key mediators in gouty inflammation. Histopathological examination of paw tissues showed reduced inflammatory cell infiltration in the betulin-treated group, further confirming its anti-inflammatory potential. These findings suggest that the microemulsion formulation effectively enhances betulin's pharmacological activity and could serve as a promising alternative to conventional anti-gout therapies.

In summary, the research emphasizes the promise of a micro-emulsion containing betulin as a stable, biocompatible, and efficient treatment option for managing gout. Future studies should focus on clinical evaluations and long-term stability assessments to further validate its applicability in human subjects.

CONCLUSION

The successful formulation and characterization of a betulin-loaded microemulsion present a novel approach to enhancing the therapeutic efficacy of betulin in gouty arthritis. The optimized formulation demonstrated excellent stability, permeability, and biocompatibility, making it suitable for both topical and systemic applications. *In vivo* studies confirmed its potential to significantly reduce inflammation and pain in the MSU crystal-induced gouty arthritis, as evidenced by decreased paw edema, improved weight-bearing distribution, and down-regulation of pro-inflammatory cytokines such as TNF-α and IL-1β. The ability of the micro-emulsion to provide a controlled and sustained release of betulin further enhances its pharmacological benefits, ensuring prolonged therapeutic effects. In addition, the non-irritant nature of the formulation makes it a promising candidate for clinical development as a safer alternative to conventional NSAIDs and corticosteroids. Future research should focus on optimizing large-scale production, conducting long-term stability studies, and performing clinical trials to establish the efficacy and safety of betulin micro-emulsion in human patients. The findings from this study contribute to the growing interest in natural bioactive compounds and nanotechnology-driven drug delivery systems for therapeutic management of gouty arthritis and inflammatory diseases.

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AUTHORS' CONTRIBUTIONS

Conceptualization: NB; Methodology: SB; Data curation: SB: Formal analysis: NB; Writing - original draft: SB; Supervision: NB; and Writing – review and editing: NB and SB.

CONSENT FOR PUBLICATION

The authors declare no conflicts of interest.

AVAILABILITY OF DATA AND MATERIAL

All data generated during this study/systematic review are included in this published article since this data only includes information acquired from published studies (see references).

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