MS.ID-IJPBS-0063
International Journal of Pharmacy and Biological Sciences-IJPBS™ (2026) 16 (2): 76-85
Online ISSN: 2230-7605, Print ISSN: 2321-3272
Research Article | Pharmaceutical Sciences | OA Journal | MCI Approved | Index Copernicus

Pharmacological Evaluation of Iguratimod Loaded Nanostructured Lipid Carriers in Freund's Complete Adjuvant-Induced Rheumatoid Arthritis in Rats: Anti-Inflammatory, Biochemical, and Histopathological Insights

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Received: 10 Feb 2026 / Accepted: 20 Mar 2026/ Published online: 01 April 2026
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Abstract

Purpose: Rheumatoid arthritis (RA) is a chronic inflammatory disorder with limited treatment options due to drug-related toxicities and poor bioavailability. This study evaluated the in vivo anti-arthritic and anti-inflammatory efficacy of optimized Iguratimod-loaded nanostructured lipid carriers (IGU-NLCs) in Freund's complete adjuvant (FCA)-induced arthritis in Wistar rats. **Methods:** Acute oral toxicity was assessed per OECD-423 guidelines. Hyaluronidase inhibition assay was performed. Anti-inflammatory activity was evaluated using carrageenan-induced paw edema (1–6 h). RA was induced by intra-articular injection of FCA (0.1 ml) into the left ankle joint on day 0. Female Wistar rats (n=6/group) were divided into normal, control (FCA + normal saline), standard (Indomethacin 10 mg/kg i.p. or MHP 200 mg/kg p.o.), and test (optimized IGU-NLCs 200 mg/kg p.o.) groups. Paw edema, body weight, hematological parameters (Hb, ESR, WBC, RBC), biochemical markers (SGOT, SGPT, total protein, creatinine, uric acid, BUN), serum nitric oxide (NO), vascular permeability (Evans blue extravasation), behavioral parameters (ambulatory activity, rearing), and histopathology of ankle joints were evaluated over 35 days. **Results:** Acute toxicity study showed no mortality at 2000 mg/kg. IGU-NLCs inhibited hyaluronidase activity by 61.42% at 200 mg/ml. In carrageenan-induced paw edema, IGU-NLCs significantly reduced paw volume at 6 h (0.372 ± 0.074 ml vs. control 0.705 ± 0.079 ml, $p < 0.05$). In FCA-induced arthritis, IGU-NLCs significantly reduced paw edema from 2.721 ± 0.025 ml (control) to 1.202 ± 0.021 ml ($p < 0.001$) at day 21. Body weight loss in control (−30.51 g) was attenuated by IGU-NLCs (−19.0 g). Hematological parameters showed improvement. Biochemical parameters confirmed no hepatotoxicity or nephrotoxicity. Serum NO was reduced by 22.45% ($p < 0.05$). Vascular permeability was inhibited by 33.02% ($p < 0.05$). Behavioral studies showed partial recovery of ambulatory activity and rearing. Histopathology revealed mild protection (score 3) compared to severe damage in control (score 5). **Conclusion:** Iguratimod-loaded NLCs demonstrated significant anti-arthritic and anti-inflammatory activity in the FCA-induced rat model, with improved safety profile and partial restoration of behavioral and histological parameters, supporting their potential as an effective nanomedicine for rheumatoid arthritis.

Keywords

Iguratimod, nanostructured lipid carriers, Freund's complete adjuvant, rheumatoid arthritis, anti-inflammatory, histopathology

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1. INTRODUCTION

Rheumatoid arthritis (RA) is a long-lasting, systemic inflammatory condition primarily impacting the joints and surrounding tissues, with a global prevalence ranging from 0.3% to 1% and an estimated prevalence of about 0.75% in India (Smolen et al., 2016; Firestein and McInnes, 2017). The disease is characterized by synovial cell proliferation, cartilage degeneration, and bone erosion, driven by various pro-inflammatory substances released by macrophages, including reactive oxygen species and eicosanoids such as prostaglandins, leukotrienes, and cytokines (McInnes and Schett, 2007). Inflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-α) are crucial contributors to RA, with plasma IL-1 concentrations correlating with disease activity (Tanaka et al., 2013).

Iguratimod (IGU) is a small synthetic disease-modifying antirheumatic drug (csDMARD)that effectivelyinhibits expression of various inflammatory factors, inhibits B cells from producing immunoglobulins and auto-antibodies, downregulates T-cell-mediated cellular immunity, and accelerates bone formation (Tanaka et al., 2015; Li et al., 2019). Despite its therapeutic potential, IGU is associated with adverse effects including elevated transaminases, nausea, vomiting, abdominal pain, rashes, and diarrhea (Brunton et al., 2025). Additionally, its poor aqueous solubility (0.31 ± 0.76 mg/ml) and low oral bioavailability limit its clinical efficacy.

We previously developed and optimized Iguratimod-loaded nanostructured lipid carriers (IGU-NLCs) with a particle size of 143.7 nm, entrapment efficiency of 73.24 ± 1.23%, and sustained in vitro release of 83.18 ± 1.36% over 30 hours. The present study aimed to evaluate the in vivo anti-arthritic, anti-inflammatory, biochemical, behavioral, and histopathological efficacy of IGU-NLCs in Freund's complete adjuvant (FCA)-induced arthritis in Wistar rats, following acute oral toxicity assessment.

2. MATERIALS AND METHODS

2.1 Preparation of Iguratimod-Loaded NLCs

Optimized IGU-NLCs were prepared using the ultra-sonication method as described in Manuscript 1, with glyceryl monostearate: oleic acid (7:3), Tween 20 (1.5% w/v), total lipid concentration of 1.834% w/v, and sonication for 20 seconds. The formulation exhibited a particle size of 143.7 nm, PDI of 0.211, zeta potential of −19.1 mV, and drug entrapment of 73.24%.

2.2 Acute Oral Toxicity Study (OECD-423)

The acute oral toxicity study was conducted following OECD Guideline 423 (Organisation for Economic Co-operation and Development, 2001). Female Wistar rats (8–12 weeks, 150–200 g) were housed three per polypropylene cage within an animal house maintained under controlled experimental conditions (22 ± 3°C, 30–70% relative humidity, 12-hour light/dark cycle) with free access to standard laboratory diets and drinking water. The experimental protocol received approval from the Institutional Animal Ethics Committee (IAEC). Three animals per group received single oral doses of 5, 50, 300, and 2000 mg/kg body weight of IGU-NLCs via oral gavage. Animals were observed for 14 days for mortality, behavioral changes, body weight, and clinical signs (Ghosh, 2015).

2.3 Hyaluronidase Inhibition Assay

The hyaluronidase enzyme activity was analyzed through a spectrophotometric method incorporating hyaluronic acid (HA) precipitation using cetylpyridinium chloride (Tung, 1994). Enzyme levels measured at 800 U/ml were combined with HA substrate (0.40 mg/ml) and IGU-NLCs (200 mg/ml) and incubated for 1 hour at 37°C. The percentage of undigested HA remaining post-enzymatic activity was observed through absorbance readings at 415 nm (Venil et al., 2008).

2.4 Carrageenan-Induced Paw Edema in Rats

Anti-inflammatory activity was evaluated using the carrageenan-induced paw edema model (Winter et al., 1962). Wistar rats (n=6/group) were fasted overnight with adequate hydration. Paw volume was measured using a mercury plethysmometer. IGU-NLCs (200 mg/kg) or standard (indomethacin 10 mg/kg i.p.) were administered orally 30 minutes before subplantar injection of 0.1 ml of 1% carrageenan into the left hind paw. Paw volume was measured at 1, 2, 3-, 4-, 5-, and 6-hours post-injection. Percentage inhibition of paw edema was calculated using the formula:

Inhibition of paw edema = $\mathbf{[(V_c - V_t)/V_c] \times 100}$

where V_c is paw edema in control group rats and V_t is paw edema in drug-treated group rats.

2.5 Freund's Complete Adjuvant (FCA)-Induced Arthritis

2.5.1 Animal grouping and induction of arthritis

Female Wistar rats (n=6/group) were randomly assigned to five groups (Chou et al., 2009):

- **Normal group:** Normal saline
- **Control group:** Normal saline + FCA
- **Standard-I group:** Indomethacin (10 mg/kg i.p.) + FCA
- **Standard-II group:** MHP (200 mg/kg p.o.) + FCA

- **Test group:** Optimized IGU-NLCs (200 mg/kg p.o.) + FCA

On day 0, under thiopentone sodium anesthesia (40 mg/kg i.p.), 0.1 ml of FCA (0.05% *Mycobacterium butyricum* in mineral oil) was injected into the left ankle joint (Sagar et al., 2012).

2.5.2 Measurement of rat paw edema

Hind paw volumes were measured using a paleothermometer before inoculation (day 0) and then on days 7, 14, and 21. Results were expressed as the increase in volume relative to the original day 0 measurement.

2.5.3 Measurement of body weight

Body weights were recorded weekly from day 0 to day 35 using a single-pan weighing balance.

2.5.4 Measurement of haematological profile

On day 35, blood was collected from the retro-orbital plexus under anaesthesia into EDTA tubes. Haemoglobin levels were assessed using Sahli's Hellige hemometer (expressed as g%), erythrocyte sedimentation rate (ESR) was measured using Westergren pipettes (mm/h), and red blood cells (RBCs) and white blood cells (WBCs) were counted (Kumar et al., 2021).

2.5.5 Measurement of biochemical profile

Blood samples were collected from the retro-orbital plexus on day 35, and serum was separated by centrifugation at 3000 RPM for 10 minutes (Burtis et al., 2018). Serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), total protein, creatinine, uric acid, and blood urea nitrogen (BUN) were estimated using commercial kits following the manufacturer's protocols.

2.5.6 Nitric oxide synthesis estimation

Serum nitric oxide (NO) concentrations were measured by separating sera from each animal group. Sodium nitroprusside (50 mM) was suspended in standard phosphate buffer solutions with serum samples and incubated at 25°C for 5 hours. The incubation solution was diluted with Griess reagents, and chromophore absorption formed by diazotization of nitrite with sulphanilamide and coupling with naphthyl ethylene diamine was read at 515 nm using a UV-visible spectrophotometer (Burtis et al., 2018).

2.5.7 Assessment of vascular permeability

Evans blue dye (1% in saline, 0.2 ml/100 g body weight) was administered intravenously into the jugular vein of anesthetized rats. After 35 hours, anterior and posterior synovial capsules of the ankles were dissected, and small tissue pieces from four ankle joints were pooled and weighed. Evans blue was extracted by cutting capsules into smaller pieces and mixing with acetone containing Na_2SO_4 (7:3 ratio), shaken intermittently for 24 hours at room temperature, centrifuged for 10 minutes at 2000 rpm, and the supernatant was measured for absorbance at 620 nm using a UV-visible spectrophotometer (Kumar et al., 2021).

2.5.8 Behavioural observations (Open-field test)

For behavioural assessment, all animals were subjected to the open-field test prior to arthritis induction and subsequently on days 3, 14, 21, 28, and 35 following FCA inoculation. Each rat was placed at the centre or in one of the four corners of the open-field apparatus and allowed to explore freely for 5 minutes. The following parameters were recorded (Kumar et al., 2021):

- **Ambulatory activity:** Number of grid lines crossed by the rat (horizontal locomotion)
- **Rearing:** Frequency with which the rat stands on its hind limbs

2.5.9 Histopathological examination of joints

On day 21, animals were sacrificed, and ankle joints were excised and preserved in 10% formalin for histological analysis (Chou et al., 2009). Sections were stained with haematoxylin and eosin and examined microscopically for inflammation, bone erosion, cartilage degeneration, and pannus formation. Arthritic scoring was performed using the following criteria (Sagar et al., 2012): normal paw = 0; mild swelling and erythema of digits = 1; moderate swelling and erythema of digits = 2; severe swelling and erythema = 3; gross deformity with loss of limb function = 4.

2.6 Statistical Analysis

Data were expressed as mean ± standard error of the mean (SEM) from six rats per group. Comparisons between the control and normal groups, and between drug-treated groups and the control group, were performed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. A p-value of less than 0.05 was deemed significant. Statistical significance levels were indicated as: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

3. RESULTS

3.1 Acute Oral Toxicity Study

The acute toxicity study revealed that optimized Iguratimod-loaded NLCs produced no mortality at 2000 mg/kg body weight in Wistar female rats (**Table 1**). The result revealed that optimized IGU-NLCs had no mortality at 2000 mg/kg. Therefore, 2000 mg/kg dose was considered as the LD50 cutoff (a safe dose), so 1/10th of that was selected (200 mg/kg) for all in vivo experiments. As per OECD-423 guidelines, the dose is said to be "unclassified" under the toxicity scale. The acute toxicity study showed no toxic effects, as evidenced by the absence of signs of

toxicity or mortality during the 14-day observation period. Additionally, no weight losses, alterations in pellet consumption, or macroscopic alterations in the viscera of treated animals were detected (**Table 2**).

Table 1- Acute Toxicity Study of Optimized Iguratimod loaded NLCS

S/No.	No. of Animals	Dose mg/kg Optimized Iguratimod loaded NLCS	No. of death of animals
1	3	5	0
2	3	50	0
3	3	300	0
4	3	2000	0

Table 2- Results for acute toxicity study

Parameters	1 hr	2 hr	3hr	4 hr	8hr	24 hr	8th day	14th day
Appearance	N	N	N	N	N	N	N	N
Activity	P	P	P	P	P	P	P	P
Reaction to stimulus	N	N	N	N	N	N	N	N
Sound	++	++	++	++	++	++	++	++
Touch	++	++	++	++	++	++	++	++
Light	++	++	++	++	++	++	++	++
Lacrimation	A	A	A	A	A	A	A	A
Salivation	A	A	A	A	A	A	A	A
Licking of Paw	A	A	A	A	A	A	A	A

N = Normal; A = Absent; P = Present; + = minimum; ++ = Present medium

3.2 Hyaluronidase Inhibitory Activity

The optimized Iguratimod-loaded NLCs inhibited hyaluronidase activity in a dose-dependent manner. As depicted in **Table 3**, IGU-NLCs exhibited 61.42% enzyme inhibition at a concentration of 200 mg/ml. The hyaluronidase (HAase) enzyme controls hyaluronic acid (HA) metabolism, and its inhibition helps preserve joint lubrication, thereby preventing arthritic conditions.

Table 3: Hyaluronidase inhibition by Optimized Iguratimod loaded NLCS

Treatments	Optimized Iguratimod loaded NLCS			
	HA*	HA+ Enzyme	EA	% EI
NLCS 200	1.205	0.465	61.42	0.48
STD	0.981	0.538	45.16	40.84

3.3 Carrageenan-Induced Paw Edema in Rats

The effect of IGU-NLCs on carrageenan-induced paw edema in albino rats is presented in **Table 4**. IGU-NLCs (200 mg/kg) demonstrated significant anti-inflammatory effects ($p < 0.05$) when assessed against the control group. The standard drug (indomethacin 10 mg/kg) showed greater inhibition (0.062 ± 0.030 ml at 6 h, $p < 0.001$), while IGU-NLCs reduced paw edema to 0.372 ± 0.074 ml at 6 h (control: 0.705 ± 0.079 ml, $p < 0.05$).

Table 4: Effects of Optimized Iguratimod loaded NLCS on carrageenan induced rat paw edema

Treatment Groups (n=6)	Paw edema (ml)					
	1st hour	2nd hours	3rd hours	4th hours	5th hours	6th hours
Normal	0.116±0.041	0.066±0.056	0.031±0.078	0.031±0.067	0.026±0.070	0.015±0.073
Control	0.551±0.053	0.581±0.065	0.612±0.076	0.689±0.089	0.714±0.086	0.705±0.079
STD	0.251±0.032	0.245±0.035	0.232±0.033[b]	0.187±0.031[a]	0.142±0.034[a]	0.062±0.030[a]
NLCS	0.331±0.56	0.365±0.059[b]	0.431±0.064[a]	0.465±0.067[a]	0.382±0.065[b]	0.372±0.074[c]

3.4 FCA-Induced Rat Paw Edema

Table 5 presents the effect of IGU-NLCs on FCA-induced changes in paw volume. FCA-induced paw edema in rats showed a significant ($p < 0.001$) progressive increase in edema within the control group compared to the normal group. The control group paw volume increased from 0.03 ± 0.024 ml at day 0 to 2.721 ± 0.025 ml at day 21. The group

treated with IGU-NLCs exhibited a considerable ($p < 0.01$) reduction in paw edema by day 7, with an even more pronounced reduction ($p < 0.001$) observed between days 14 and 21 (1.202 ± 0.021 ml at day 21) when compared to both standard-I and MHP treatment groups.

Table 5 Effect of Optimized Iguratimod loaded NLCS on FCA-induced changes in paw

Treatment Groups	0 day	7th day	14th day	21st day
Normal	0.045±0.011	0.48±0.009	0.06±0.014	0.047±0.016
Control	0.03±0.024	2.395±0.018	2.615±0.024	2.721±0.025
STD	0.017±0.018	1.663±0.017	1.421±0.012	0.98±0.015a
NLCS -200	0.031±0.022	1.698±0.019	1.389±0.028	1.202±0.021a

3.5 Changes in Body Weight

Table 6 shows the effect of IGU-NLCs on FCA-induced changes in body weight. The weights across all groups were nearly the same from days 0 to 7. However, during disease progression, the control group's body weight decreased between days 14 and 35 (from 172.01 ± 0.039 g at day 0 to 141.5 ± 0.032 g at day 35). The normal group exhibited a consistent increase in body weight. IGU-NLCs attenuated body weight loss (from 164.5 ± 0.022 g at day 0 to 145.5 ± 0.047 g at day 35, loss of 19.0 g) compared to the control group (loss of 30.51 g).

Table 6: Effect of Optimized Iguratimod loaded NLCS on FCA-induced changes in body weight

Treatment Groups	0 day	7th day	14th day	21st day	28th day	35th day
Normal	157.9±0.013	161.2±0.08	161.4±0.06	166.2±0.09	166.2±0.04	167.1±0.06
Control	172.01±0.039	168.5±0.027	158.9±0.036	152.9±0.029	145.6±0.026	141.5±0.032
STD	167.7±0.21	162.6±0.011	161.2±0.013	163.6±0.017c	166.2±0.019a	166.9±0.022a
NLCS -200	164.5±0.022	158.4±0.045	156.2±0.034	154.5±0.046c	150.1±0.039b	145.5±0.047a

3.6 Hematological Profiles

Table 7 presents the effect of IGU-NLCs on FCA-induced changes in hematology profiles. FCA-induced arthritic rats exhibited a minor increase in total WBC count (control: 11.94 ± 0.029 $\times 10^3/mm^3$ vs. normal: 10.45 ± 0.011 $\times 10^3/mm^3$) and a decrease in RBC (control: 6.861 ± 0.033 $\times 10^6/mm^3$ vs. normal: 7.016 ± 0.08 $\times 10^6/mm^3$) after 35 days. The control group demonstrated a significant ($p < 0.001$) rise in ESR (12.4 ± 0.021 mm/h vs. normal: 3.822 ± 0.012 mm/h) and a reduction in hemoglobin levels (11.45 ± 0.023 g/dl vs. normal: 15.1 ± 0.09 g/dl). Administration of IGU-NLCs resulted in improvements in RBC (6.432 ± 0.033 $\times 10^6/mm^3$), WBC (11.79 ± 0.041 $\times 10^3/mm^3$), hemoglobin (11.21 ± 0.045 g/dl), and ESR (9.223 ± 0.056 mm/h) relative to the control group.

Table 7: Effect of Optimized Iguratimod loaded NLCS on FCA-induced changes in hematology profiles

Treatment Groups	Hemoglobin g/dl	ESR mm/h	WBC $\times 10^3/mm^3$	RBC $\times 10^6/mm^3$
Normal	15.1±0.09	3.822±0.012	10.45±0.011	7.016±0.08
Control	11.45±0.023	12.4±0.021	11.94±0.029	6.861±0.033
STD	13.52±0.011	5.749±0.031	10.62±0.019	6.799±0.023
NLCS -200	11.21±0.045	9.223±0.056	11.79±0.041	6.432±0.033

3.7 Biochemical Profiles

The results of the biochemical profiles are presented in **Table 8**. No abnormalities were observed. In the FCA-injected group, there was a slight reduction in SGOT (control: 56.57 ± 0.045 IU/L vs. normal: 57.35 ± 0.024 IU/L) and total proteins (control: 6.73 ± 0.046 g% vs. normal: 6.8 ± 0.017 g%), along with a minor increase in SGPT (control: 65.38 ± 0.025 IU/L vs. normal: 62.1 ± 0.012 IU/L) and creatinine (control: 0.78 ± 0.032 mg/dl vs. normal: 0.74 ± 0.021 mg/dl). Uric acid and BUN levels did not show any significant changes. Treatment with IGU-NLCs resulted in SGOT (56.64 ± 0.023 IU/L), SGPT (64.5 ± 0.056 IU/L), total protein (7.18 ± 0.028 g%), creatinine (0.74 ± 1.011 mg/dl), uric acid (5.578 ± 0.022 mg/dl), and BUN (41.81 ± 0.67 mg/dl). These findings confirm that no toxicity was detected in either the liver or kidneys.

Table 8: Effect of Optimized Iguratimod loaded NLCS on FCA-induced changes in biochemical parameters

Treatment Groups	SGOT (IU/L, Mean ± SD)	SGPT (IU/L, Mean ± SD)	Total Proteins (g%, Mean ± SD)	Creatinine (mg/dL, Mean ± SD)	Uric Acid (mg/dL, Mean ± SD)	Urea Nitrogen (mg/dL, Mean ± SD)
Normal	57.35 ± 0.024	62.10 ± 0.012	6.80 ± 0.017	0.74 ± 0.021	5.34 ± 0.011	42.18 ± 0.025
Control	56.57 ± 0.045	65.38 ± 0.025	6.73 ± 0.046	0.78 ± 0.032	5.37 ± 0.022	42.19 ± 0.016
STD	57.91 ± 0.011	62.71 ± 0.015	6.86 ± 0.034	0.77 ± 0.056	5.59 ± 0.023	47.08 ± 0.031
NLCS-200	56.64 ± 0.023	64.50 ± 0.056	7.18 ± 0.028	0.74 ± 0.011*	5.58 ± 0.022	41.81 ± 0.067

3.8 Nitric Oxide Level

Serum nitric oxide (NO) concentrations are presented in **Table 9**. NO was notably ($p < 0.001$) increased to approximately double the levels observed in the control group (9.71 ± 0.024 µM) when compared to the normal group (4.81 ± 0.011 µM). IGU-NLCs significantly decreased NO levels to 7.53 ± 0.021 µM (22.45% inhibition, $p < 0.05$) in arthritic rats relative to the control group. The standard achieved 45.82% inhibition ($p < 0.001$).

Table 9: Effect of Optimized Iguratimod loaded NLCS on FCA-induced serum nitric oxide synthesis% Inhibition at day 35

Treatment Groups	Nitric Oxide (Mean ± SD)	% Inhibition (Day 35)
Normal	4.81 ± 0.011	-
Control	9.71 ± 0.024	0
STD	5.26 ± 0.032	45.82[a]
NLCS-200	7.53 ± 0.021	22.45[c]

3.9 Vascular Permeability

The findings regarding vascular permeability are presented in **Table 10**. There was a notable increase ($p < 0.001$) in Evans blue extravasation in the FCA-injected ankle joints of the control group (169.2 ± 1.022 µg/g) when contrasted with the normal group (43.48 ± 0.043 µg/g). IGU-NLCs significantly reduced Evans blue extravasation to 113.32 ± 1.021 µg/g (33.02% inhibition, $p < 0.05$) compared to the control group. The standard group showed 68.02% inhibition ($p < 0.001$).

Table 10: Effect of Optimized Iguratimod loaded NLCS on FCA-induced vascular permeability % Inhibition at day 35

Treatment Groups	Evans Blue Concentration (µg/g, Mean ± SD)	% Inhibition of Infiltration
Normal	43.48 ± 0.043	-
Control	169.20 ± 1.022	0
STD	54.11 ± 1.012	68.02[a]
NLCS-200	113.32 ± 1.021	33.02[c]

3.10 Behavior Study

3.10.1 Ambulatory activity

Table 11 presents the effect of IGU-NLCs on FCA-induced changes in ambulatory behavior. A reduction in ambulatory activity was noted in the normal group at baseline, followed by a significant increase ($p < 0.01$) from days 7 to 14, which returned close to baseline levels by day 35. The control group experienced a marked decrease ($p < 0.001$) in ambulatory activity from day 7 through to the conclusion of the experiment (from 55.04 ± 0.017 at day 0 to 14.3 ± 0.023 at day 35). IGU-NLCs exhibited decreased ambulatory activity during the first 7 days but showed an increase from days 14 to 28, ultimately reaching 56.33 ± 0.056 by day 35.

Table 11: Effect of Optimized Iguratimod loaded NLCS on FCA-induced change in ambulatory behavior in rats

Treatment Groups	0 day	7th day	14th day	21st day	28th day	35th day
Normal	43.3±0.012	86.12±0.023	99.01±0.034	78.02±0.022	69.01±0.028	54.6±0.011
Control	55.04±0.017	43.06±0.032	34.09±0.044	22.06±0.031	18.6±0.054	14.3±0.023
STD	69.02±0.025	64.81±0.019	62.29±0.022	63.7±0.013	64.5±0.033	66.79±0.021a
NLCS -200	61.03±1.023	52.25±0.032	49.35±0.077	53.3±0.082	55.87±0.048	56.33±0.056a

3.10.2 Rearing

Table 12 presents the effect of IGU-NLCs on FCA-induced changes in rearing behavior. Rearing was observed to decline across all groups by day 7. The control group exhibited a significant reduction ($p < 0.001$) in rearing throughout the study duration (from 12.15 ± 0.045 at day 0 to 1.15 ± 0.013 at day 35). IGU-NLCs showed gradual recovery between days 14 and 28, with values reaching 6.65 ± 0.029 by day 35.

Table 12: Optimized Iguratimod loaded NLCS on FCA-induced change in Rearing behavior in rats

Treatment Groups	0 day	7th day	14th day	21st day	28th day	35th day
Normal	16.16±0.021	6.51±0.22	9.68±0.011	10.23±0.012	12.69±0.032	14.02±0.011
Control	12.15±0.045	2.48±1.012	2.23±0.059	1.48±0.034	1.35±0.021	1.15±0.013
STD	15.6±0.021	6.01±0.029	9.48±0.034	10.03±0.021	10.65±0.024	11.32±0.028[a]
NLCS -200	15.65±0.019	8.81±1.022	5.09±1.032	5.31±0.024	6.15±0.039	6.65±0.029[a]

3.11 Histopathological Examination of Joints

Figure 1 presents the histopathological images, and **Tables 13** and **14** summarize the observations and scoring. Tissue sections from the control joints of arthritic rats revealed pathological changes associated with arthritis when contrasted with normal joints. The control group exhibited marked joint damage (score 5), characterized by severe inflammatory infiltration, defects in cortical and trabecular bone, and severe loss of cartilage. The standard group showed marked protection (score 1), with minimal inflammatory infiltration, small areas of bone resorption, and minimal cartilage loss. IGU-NLCs-treated rats exhibited mild protection (score 3), with mild inflammatory infiltration, resorption of trabecular and cortical bone, mild loss of cartilage and collagen disruption, and pannus formation with marked cartilage destruction.

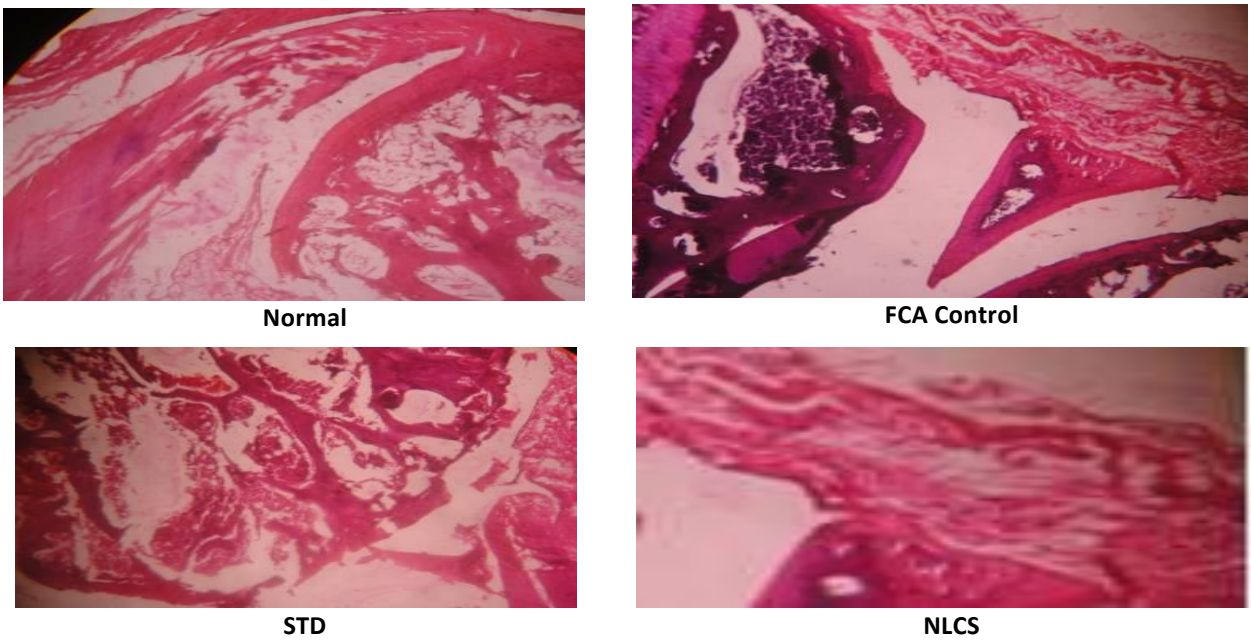

Figure 1: Effect of Optimized Iguratimod loaded NLCS on histology of FCA-induced.

Table 13: Effect of Optimized Iguratimod loaded NLCS on histology of FCA-induced

Treatment Groups	Observation
Normal	-
Control	Marked damaged
STD	Marked protection
NLCS -200	Mild protection

Table 14: Histopathology studies of Optimized Iguratimod loaded NLCS

Groups	Score	Inflammation	Bone erosion	Cartilage Degeneration	Groups
				Remote From Pannus	Adjacent to Pannus
Normal	0	Normal	Normal	Normal	Normal
Control	5	Severe Infiltration	Defects in the cortical, Trabecular bone loss	Severe loss of cartilage	NA
STD	1	Minimal infiltration	Small areas of resorption in trabecular, cortical bone	Minimal cartilage loss	Pannus formation with superficial cartilage destruction
NLCS - 200	3	Mild infiltration	Resorption of trabecular, cortical bone	Mild loss of cartilage and collagen disruption	Pannus formation with marked cartilage destruction

4. DISCUSSION

The present study evaluated the in vivo anti-arthritic and anti-inflammatory efficacy of optimized Iguratimod-loaded NLCs (200 mg/kg) in FCA-induced arthritis in Wistar rats. FCA-induced arthritis is a well-established model that closely mimics human RA, characterized by T-cell activation, pro-inflammatory cytokine release, synovial hyperplasia, pannus formation, and progressive joint destruction (Chou et al., 2009; Sagar et al., 2012).

The acute oral toxicity study confirmed the safety of IGU-NLCs up to 2000 mg/kg, with no mortality or adverse effects, consistent with previous reports on Iguratimod safety (Miyazaki et al., 2016). The absence of hepatotoxicity (normal SGOT/SGPT) and nephrotoxicity (normal creatinine, BUN, uric acid) in the 35-day study further supports the biocompatibility of the NLC formulation.

Hyaluronidase inhibition (61.42%) is mechanistically relevant because HAase degrades hyaluronic acid in synovial fluid, reducing joint lubrication and contributing to arthritis progression (Tung, 1994). The inhibition of HAase by IGU-NLCs helps preserve the viscosity and lubricating properties of synovial fluid, thereby protecting joints from degradation.

The carrageenan-induced paw edema model showed that IGU-NLCs significantly reduced acute inflammation at 6h, confirming their anti-inflammatory activity. The FCA-induced chronic arthritis model demonstrated progressive edema in control rats (peak 2.721 ml at day 21), while IGU-NLCs reduced edema by 55.8% at day 21 (1.202 ml). This sustained efficacy is attributed to the controlled release of Iguratimod from NLCs (83.18% over 30 h in vitro), providing prolonged therapeutic levels at the inflamed joint. These findings are consistent with Ma et al. (2019), who reported that nanoiguratimod-loaded hydrogels significantly inhibited the proliferation, migration, and invasion of RA-FLS in a dose-dependent manner and provided improved bioavailability and a longer half-life compared to oral raw iguratimod.

Body weight changes are an important indicator of disease severity in RA (Roubenoff, 2008). Control rats lost 30.5 g over 35 days due to chronic inflammation and cachexia. IGU-NLCs significantly attenuated this weight loss (19.0 g loss), suggesting reduced systemic inflammatory burden.

The elevated ESR in control (12.4 mm/h) indicates active inflammation, which was reduced by IGU-NLCs to 9.22 mm/h (25.6% reduction). This improvement in hematological parameters is consistent with the anti-inflammatory action of Iguratimod, which downregulates the expression of several inflammatory factors (IL-1, IL-6, IL-8, TNF-α, NF-κB) (Li et al., 2019).

Serum nitric oxide levels increased 2-fold in arthritic controls (9.71 vs. 4.81 µM in normal). NO is a key mediator of inflammation, contributing to vasodilation, edema, and joint damage (Sharma et al., 2002). IGU-NLCs reduced NO by 22.45%, indicating modulation of the iNOS pathway. This is consistent with previous reports that Iguratimod suppresses the production of inflammatory mediators.

Vascular permeability, measured by Evans blue extravasation, increased nearly 4-fold in control (169.2 µg/g vs. normal 43.48 µg/g). IGU-NLCs reduced extravasation by 33.02%, indicating stabilization of the synovial microvasculature and reduced plasma protein leakage into the joint space. This anti-permeability effect contributes to the reduction of joint swelling and edema.

Behavioral parameters (ambulatory activity and rearing) are objective measures of pain, mobility, and quality of life in arthritic animals (Kumar et al., 2021).

Control rats showed severe motor deficits (81% reduction in ambulation, 91% reduction in rearing by day 35). IGU-NLCs partially restored ambulation (92% of normal activity) and rearing (47% of normal activity), indicating improved pain relief and joint mobility. This improvement in behavioral parameters is likely due to the sustained release of Iguratimod from NLCs, providing continuous anti-inflammatory and analgesic effects.

Histopathological examination confirmed the protective effects of IGU-NLCs. Control joints showed severe inflammation, trabecular and cortical bone defects, and complete cartilage loss (score 5). IGU-NLCs reduced these changes to moderate severity (score 3), with mild inflammatory infiltration, resorption of trabecular bone, and pannus formation with marked cartilage destruction. The standard drug showed minimal changes (score 1). The superior histoprotection by the standard may be due to the higher potency of indomethacin, but at the cost of higher gastrointestinal toxicity. IGU-NLCs offer a safer alternative with acceptable efficacy, as also reported by Madhavi and Shiva Kumar (2024), who developed IPNs of carboxymethyl tamarind gum and cyclodextrin nanosponges for iguratimod oral formulations with improved bioavailability.

The sustained release profile of IGU-NLCs (in vitro: 83.18% at 30 h) likely translates into prolonged plasma concentrations and targeted accumulation in inflamed joints via the enhanced permeation and retention (EPR) effect, as reported by Tao et al. (2024) for IGU nanodrugs fabricated by high-gravity nanoprecipitation, which demonstrated improved dissolution rates and efficacy in inhibiting synovial fibroblast proliferation, migration, and invasion.

5. CONCLUSION

Iguratimod-loaded nanostructured lipid carriers (200 mg/kg p.o.) demonstrated significant anti-arthritic and anti-inflammatory activity in FCA-induced arthritis in rats, as evidenced by:

- 55.8% reduction in paw edema at day 21 ($p < 0.001$)
- Attenuation of body weight loss (19.0 g loss vs. 30.5 g loss in control)
- Improvement in hematological parameters (ESR reduced by 25.6%)
- No hepatotoxicity or nephrotoxicity
- 22.45% inhibition of serum nitric oxide ($p < 0.05$)
- 33.02% inhibition of vascular permeability ($p < 0.05$)
- Partial restoration of ambulatory and rearing behaviors
- Moderate histoprotection (score 3 vs. control score 5)

The NLC formulation successfully addressed the poor solubility and bioavailability limitations of Iguratimod, translating into enhanced in vivo efficacy with a favorable safety profile. Future studies should focus on detailed pharmacokinetic evaluations, targeted ligand functionalization for site-specific delivery, and clinical translation.

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