In-vitro and *In-vivo* Evaluation of Antiarthritic, Anti-inflammatory and Analgesic Effect of Nanoemulsion loaded with C*rinum latifolium* leaves extract

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ABSTRACT

This study explores the therapeutic potential of a nanoemulsion formulated with Crinum latifolium leaf extract for its anti-arthritic, anti-inflammatory, and analgesic effects. Stability and particle size distribution were also characterized. In-vivo evaluations included CFA-induced arthritis, carrageenan-induced paw edema for inflammation assessment, and Eddy's hotplate test for analgesic effects. Invitro study of nanoemulsion showed potent antioxidant activity and inhibited hyaluronidase enzyme by 75.05%, indicating its role in preventing joint degradation. At a 1 mg/kg dose in the CFA-induced arthritis model, the nanoemulsion significantly reduced paw edema by 64.28%, comparable to standard treatment. Body weight analysis showed an increase from 187.83 ± 2.89 g to 230.32 ± 9.33 g, suggesting overall health improvement. Hematological parameters were normalized, with a marked reduction in ESR from 8.40 ± 0.33 mm/h to 1.76 ± 0.30 mm/h, indicating reduced inflammation. Histopathological examination confirmed reduced synovial hyperplasia, inflammatory cell infiltration, and cartilage damage. Additionally, the nanoemulsion enhanced pain tolerance, showing a 45.45% increase in pain threshold in Eddy's hotplate test. These findings highlight the nanoemulsion's therapeutic potential for arthritis management, warranting further research into its clinical application.

1. Introduction

Arthritis encompasses a spectrum of joint disorders, including conditions such as rheumatoid arthritis (RA) and osteoarthritis (OA), which can affect single or multiple joints. This group of diseases can manifest in individuals regardless of gender, age, or ethnic background. Common clinical manifestations include joint inflammation, tenderness, and reduced mobility [1]. The severity of arthritis varies widely, ranging from mild discomfort to debilitating conditions that significantly impair locomotion and daily functioning. In severe cases, arthritis can lead to irreversible structural damage to joints and degradation of ligamentous tissue. RA, specifically, is characterized as a systemic autoimmune disorder in which the body's immune system erroneously targets its own tissues. This condition has emerged as a global health concern affecting numerous healthy individuals [2]. In the Indian context, the prevalence of RA is particularly striking, with an estimated 15% of the population—approximately 180 million individuals—affected by this

condition. Notably, the incidence of RA surpasses that of other major non-communicable diseases such as cancer and diabetes. Research indicates that arthritis ranks as the second most prevalent cause of disability worldwide, contributing significantly to the global burden of functional impairment [3]. The prevalence of rheumatoid arthritis (RA) exhibits an age-dependent progression, with peak incidence observed in individuals aged 35 to 50 years. Global epidemiological data suggest an estimated incidence rate of 3 cases per 10,000 individuals [4]. Current conventional therapeutic approaches for RA include analgesics, non-steroidal anti-inflammatory drugs (NSAIDs), disease-modifying anti-rheumatic drugs (DMARDs), and corticosteroids. However, these pharmacological interventions are often associated with adverse effects, including gastrointestinal disturbances, ulceration, and hemorrhage [5]. The global market for herbal-based therapies has experienced substantial growth, with annual revenues reaching billions of dollars in Western Europe and China. This increasing preference for herbal medicines is often attributed to the limitations and side effects associated with conventional treatments [6]. Herbal medicinal products have demonstrated multifaceted therapeutic potential in addressing various disorders, offering an alternative or complementary approach to mainstream medical interventions. This growing interest in plant-based remedies reflects a broader trend towards exploring natural therapeutic options in healthcare management [7].

The plant crinum latifolium is already reported for its antimicrobial, anti-inflammatory, anti-tumor, anthelmintic activity and also responsible for degranulation of mast cells [8].

2. Materials and Methods

2.1 Materials

Cinnamon oil, tween 80 and span 20 was purchased from Merck, USA. All other ingredients were of analytical grade and purchased from Hi media, Mumbai. Milli pore water was used throughout the experiment.

2.2 Methods

2.2.1 Collection and identification of plant material

The fresh leaves of *C. latifolium* were collected from Alwar, Rajasthan's local region and were taxonomically authenticated by Dr Sunita Garg, Chief Scientist and Head, RHMD, CSIR-NIScPR, Delhi (authentication number- NIScPR/RHMD/Consult/2022/4193-94 on 30.09.2022). The sample specimen was deposited in the herbarium of the institute-wide voucher no. 4193-94.

2.2.2 Extraction of plant material

The leaves were manually plucked off the plant and cleared of any trash. After that, the leaves were air-dried using mechanically graded aluminum foil, and they were ultimately stored for 14 days at room temperature [9]. From which 400 grams of the ground material were removed and extracted using 1600 milliliters of 80% methanol in a clean, appropriate glass container with a flat bottom. The sample was shaken at regular intervals over this period. The mixture was then run through Markin cloth to remove as much as possible. After passing it through the Whatman filter paper, it was placed in a rotary evaporator to evaporate excess methanol. After that, the methanolic extract was then placed in a water bath. After a certain period, the extract converted into a brownish black color residue, properly preserved at 4º C temperature.

2.2.3 Phyto-chemical analysis of *C. latifolium* leaves extract

Following tests were performed for *C. latifolium* leaves extract to determine the presence of phytochemicals:

2.2.3.1 Test for Carbohydrates

Carbohydrate detection was performed using the Molisch test. A test sample was prepared by dissolving 1 g of the desiccated methanolic extract of *C. latifolium* in 10 ml of distilled water. 2 ml of the prepared sample with an equal volume of alpha-naphthol solution was combined. Subsequently, concentrated sulfuric acid was carefully introduced along the inner wall of the test tube, allowing it to form a distinct layer [10].

The formation of a purple-violet colored ring at the interface between the aqueous and acid layers was observed. This chromogenic reaction is indicative of the presence of carbohydrates in the sample. The appearance of this characteristic-colored boundary serves as a positive result in the Molisch test, confirming the presence of carbohydrate moieties in the methanolic extract of *C. latifolium*. This colorimetric assay relies on the acid-catalyzed dehydration of carbohydrates to form furfural derivatives, which then react with alpha-naphthol to produce the observed chromophore. The test is widely employed as a preliminary screening method for carbohydrate detection in phytochemical analyses.

2.2.3.2 Test for glycosides

A test solution was prepared by solubilizing 1 g of the lyophilized methanolic extract of *C. latifolium* in 10 ml of deionized water. 1 ml of this prepared sample was added in 3 ml of anthrone reagent. The mixture was homogenized with careful attention to maintain the integrity of the reaction [11].

The formation of a green-colored complex was observed following the addition of the anthrone reagent. This chromogenic response is characteristic of the presence of glycosidic compounds in the sample. The development of this distinctive green coloration serves as a positive indicator in this assay, confirming the presence of glycosides in the methanolic extract of *C. latifolium*.

This colorimetric test relies on the reaction between the anthrone reagent and the sugar moieties of glycosides under acidic conditions. The resulting chromophore produces the observed green coloration, allowing for the qualitative detection of glycosidic structures in phytochemical analyses.

2.2.3.3 Test for polysaccharides

The sample was formulated by solubilizing 1 gram of desiccated methanolic extract of *C. latifolium* in 10 ml of deionized water. Subsequently, 2 drops of iodine reagent were introduced to a 1 ml of the prepared solution. The formation of a blue chromatic shift in the mixture served as a positive indicator for the presence of polysaccharide compounds [12].

2.2.3.4 Test for free amino acids

A test solution was prepared by reconstituting 1 gram of lyophilized methanolic extract of *C. latifolium* in 10 milliliters of distilled water. An aliquot of 1 milliliter of this solution was then combined with 5 microliters of ninhydrin reagent. The mixture was subjected to thermal treatment at 100°C for 120 seconds. The development of a purple color in the reaction medium was indicative of the presence of free amino acids in the sample [13].

2.2.3.5 Bradford's test

An aqueous solution was prepared by solubilizing 1 gram of methanolic extract of *C. latifolium* in 10 milliliters of deionized water. A 500 ml of this sample was subsequently combined with 3 ml of Dragendorff's reagent. The emergence of a blue chromatic shift in the reaction mixture served as a positive indicator for the presence of proteinaceous compounds [14].

2.2.3.6 Test for alkaloids

An aqueous solution was prepared by solubilizing 1 gram of lyophilized methanolic extract of *C. latifolium* in 10 milliliters of deionized water. 3 ml of sample was combined with Dragendorff's

reagent and homogenized. The mixture was then heated to its boiling point and maintained at that temperature for 300 seconds. The development of a dark brown or orange color in the reaction medium was indicative of alkaloid present. 1ml of the sample solution was mixed with Mayer's reagent and gently homogenized. This mixture was subsequently subjected to thermal treatment at its boiling point for 300 seconds. The formation of a white or pale-yellow precipitate served as a positive indicator for alkaloid compounds [15].

2.2.3.7 Test for flavonoids

An aqueous solution was prepared by reconstituting 1 gram of desiccated lyophilized methanolic extract of *C. latifolium* in 10 milliliters of distilled water. 1 milliliter aliquot of this solution was then combined with 2 milliliters of concentrated sulfuric acid (H_2SO_4) and thoroughly homogenized. The emergence of a yellow color in the reaction medium indicated the presence of flavonoid compounds [16].

2.2.3.8 Tests for tannins

Ferric Chloride Assay: A test solution was prepared by solubilizing 1 gram of lyophilized methanolic extract of *C. latifolium* in 10 milliliters of deionized water. An aliquot of 2 milliliters of this reconstituted sample was combined with a 5% w/v ferric chloride (FeCl3) solution and thoroughly homogenized. The development of a deep blue-black color in the reaction medium confirmed the presence of tannin compounds [17].

2.2.3.9 Test for saponin

An aqueous solution was prepared by solubilizing 1 gram of methanolic extract of *C. latifolium* in 10 milliliters of deionized water. The sample was combined with ethanolic potassium hydroxide (KOH) and heated to its boiling point for 60 seconds, followed by rapid cooling. The mixture was then acidified with 1 milliliter of concentrated hydrochloric acid (HCl). A portion of the acidified solution was diluted with 10 milliliters of water. A 5% w/v sodium hydroxide (NaOH) solution was added dropwise to the diluted mixture. The formation of a transparent, soap-like substance in the reaction medium served as a positive indicator for the presence of saponin compounds [18].

2.2.4 Preparation of nano-emulsion from plant extract

The nano-emulsion formulation was prepared by emulsification followed by homogenization method. The oil was taken in 60 w/w, Smix (tween 80: span 20) taken in a ratio of 1:1 at a concentration of 40 w/w, sonication speed used was 1200 rpm, and sonication time was 15 minutes. These parameters were optimized and selected in our previous studies [19]. The solubility of the drug signifies the amount of drug that are to be incorporated into oil phase of the NE formulation. First, a precise quantity of lyophilized extract was dissolved into the oily phase and subjected to sonication at 60˚C until complete dissolution of the drug was achieved. Second, the aqueous phase was formulated by dissolving a known concentration (2%) of tween 80 into water. Add this aqueous surfactant phase drop by drop to oily phase and stirred it vigorously under continuous stirring using mechanical stirrer until a clear NE was obtained. The resultant nano-emulsion underwent homogenization at 10,000 rpm for 10 minutes and was subsequently cooled to room temperature [19].

2.2.5 *In vitro* Studies

2.2.5.1 Total antioxidant capacity

2.2.5.1.1 DPPH Method

The total antioxidant capacity of nanoemulsion loaded with extract of *C. latifolium* was determined for its 1mg concentration by DPPH method. The spectrophotometer was used to measure the DPPH scavenging activity. The stock solution (6 mg in 100 mL methanol) was produced to give an initial absorbance of 1.5 mL in 1.5 mL methanol. After 15 minutes, there was a decrease in

absorbance in the presence of sample extract at various concentrations (10-100g/ml). 1.5 ml of DPPH and 1.5 ml of varying concentrations of the test sample were placed in a succession of volumetric flasks, and the final volume was adjusted to 3 ml using methanol. Three test samples were collected and processed in the same way. Finally, the average was calculated. After 15 minutes at 517 nm, the absorbance of DPPH with varied concentrations showed a final reduction [20].

Calculation of % reduction= Control Absorbance -Test absorbance/Control Absorbance x 100

2.2.5.1.2 Superoxide radical scavenging assay

The scavenging ability of extracts against superoxide radicals (O2) was determined using the NBT reagent technique. This approach relies on the autooxidation of hydroxylamine hydrochloride in the presence of NBT, which is then reduced to nitrite, to generate O2. In the presence of EDTA, the nitrite ion produced a colour with a maximum wavelength of 560 nm. Sodium carbonate (1 mL, 50 mM), NBT (0.4 mL, 24 mM), and EDTA (0.2 mL, 0.1 mM) solutions were added to extracts or vitamin C (1 mL, 10-100 µg/mL) test samples, and absorbance was measured at max 560 nm immediately [21].

Calculation of % Reduction =Control Absorbance -Test absorbance/Control Absorbance x 100

2.2.5.2 Hyaluronidase inhibition activity

The hyaluronidase inhibition assay was conducted in a medium comprising 3-5 U hyaluronidase, 100 µL of 20 mM sodium phosphate buffer (pH 7.0), 77 mM sodium chloride, and 0.01% bovine serum albumin. The assay mixture was pre-incubated with 50 µg concentration of *C. latifolium* loaded nanoemulsion in the test compound for 15 minutes at 37°C. The reaction was initiated by the addition of 100 µL hyaluronic acid and 0.03% of 300 mM sodium phosphate (pH 5.35) to the pre-incubation mixture, followed by a 45-minute incubation at 37°C. Subsequently, undigested hyaluronic acid was precipitated using 1 mL of acid albumin solution (0.1% bovine serum albumin in 24 mM sodium acetate and 79 mM acetic acid, pH 3.75). After a 10-minute equilibration period at room temperature, the absorbance of the reaction mixture was measured spectrophotometrically at 600 nm. The absorbance in the absence of enzyme served as the reference for maximum inhibition. The inhibitory efficacy of nanoemulsion loaded with extract of *C. latifolium* extract was quantified as the percentage ratio of absorbance in the presence of the test compound relative to the absorbance in the absence of enzyme. Enzymatic activity was assessed via a control experiment conducted in parallel, wherein the enzyme was pre-incubated with 5 µL DMSO, followed by the aforementioned assay procedures. Indomethacin was employed as a reference standard in this experimental protocol [22].

2.2.6 *In Vivo* Studies

2.2.6.1 Carrageenan-induced paw edema acute inflammatory model

The experimental animal i.e rats (Rattus norvegicus) were administered nanoemulsion loaded with extract of *C. latifolium*, via oral gavage 60 minutes prior to subplantar injection of 0.2 mL of 1% (w/v) carrageenan suspension into their right hind paw. The time of injection was designated as t = 0 h. Baseline paw thickness measurements were obtained using a vernier caliper immediately before carrageenan administration. Subsequent measurements were recorded at hourly intervals post-induction, from t = 1 h to t = 5 h. Paw edema was quantified as the difference in thickness between each time point (t) and the baseline (0 h), expressed as a mean increase in paw thickness (cm). The anti-inflammatory efficacy of both the standard drug and nanoemulsion loaded with extract of *C. latifolium* was calculated as a percentage inhibition of edema formation using the calculated formula ¼ [1- (Ct – C0) treated group/(Ct – C0) control group] x 100%, where C0 is

mean paw thickness measured at time 0 h and Ct is the mean paw thickness measured at particular time point [23].

2.2.6.2 Effects on hematological parameters in adjuvant induced arthritic rat model

Blood samples were obtained via cardiac puncture and collected in EDTA-containing vacutainers to prevent coagulation. Hematological parameters were assessed using an automated hematology analyzer (Sysmex XT-1800i). the samples were evaluated for ESR, Hemoglobin, erythrocytes count, leukocyte count and platelets count [24].

2.2.6.3 Acute toxicity study

The toxicity potential of the test compound was evaluated according to OECD guideline 423[25].

2.2.6.4 *In vivo* antiarthritic activity (CFA induced arthritis)

Adult albino Wistar rats (Rattus norvegicus) with body weight ranging from 150 to 200 g were used in the protocol. The rats were randomized into four experimental Groups (n=6 per Group) as follows:

1. Negative control group: Blank nanoemulsion
2. Positive control group: Received subplantar injection of 0.1 mL Complete Freund's Adjuvant (CFA) emulsion in the right hind paw
3. Experimental group: Treated with nanoemulsion loaded with *C. latifolium* extract as per the body weight (1mg/kg).
4. Reference group: Administered indomethacin at a dose of 10 mg/kg body weight

Inflammatory arthritis was induced in group 2-4 via subplantar injection of 0.1 mL CFA emulsion into the right hind paw. This model was employed to evaluate the potential anti-arthritic efficacy of the nanoemulsion loaded with extract of *C. latifolium*, comparing outcomes against both untreated arthritic group and a standard non-steroidal anti-inflammatory drug (NSAID) treatment. The negative control group served to establish baseline parameters and account for any nanoemulsion-related effects [26].

2.2.6.5 Assessment of inflammation and arthritis

The nanoemulsion loaded with extract of *C. latifolium* was administered orally (p.o.) once daily, beginning on the day of arthritis induction and continuing for 14 days. Following parameters were assessed:

1. **Paw Volume:** Mercury plethysmograph was used to measure changes in paw volume, which serves as an indicator of inflammatory response. Measurements were taken on days 0 (baseline), 7, 14, and 21 after arthritis induction. The development and potential reduction of edema was tracked over time.
2. **Body Weight:** Using a digital weighing balance, the body weight of each rat on the same schedule - days 0, 7, 14, and 21 post-induction was recorded. Changes in body weight reflects the overall health status of the animals and the systemic effects of both the induced arthritis and the treatments.

The use of parameters measurements like paw volume and body weight helped to evaluate the efficacy of the treatment in a systematic and quantifiable manner, allowing for statistical analysis and comparison between groups.

2.2.6.6 Analgesic effect of plant extract loaded nano emulsion.

The experimental rats were randomized into four groups, each comprising six animals. All animals underwent a 2-hour fasting period prior to the experiment's initiation. Preliminary screening was conducted using Eddy's hot plate maintained at 55 °C ± 0.1 °C. Animals exhibiting latency times exceeding 15 seconds were to be excluded from the study. The groups received the following treatments via different routes of administration as follows:

1. Negative control group: Administered oral nanoemulsion without extract.
2. Positive control group: Received no treatment but checked for the maximum pain response only.
3. Experimental group: Treated with oral nanoemulsion loaded with extract of C. latifolium as per the body weight (1mg/kg).
4. Reference group: Administered tramadol i.p at a dose of 20 mg/kg body weight.

Thirty minutes post-administration, each animal was placed on Eddy's hot plate. The latency time is defined as the duration the animal remained on the hot plate without exhibiting nociceptive behaviors (hind limb licking, flicking, or jumping), was measured and recorded for a maximum of 60 seconds. The percentage analgesia was calculated by following Equation.

Percentage Analgesia = Initial reading – final reading / initial reading x 100 [27].

2.2.6.7 Histopathological evaluation

Histopathological evaluation of rat paw specimens was evaluated at day 28 post-induction demonstrated disease progression in the arthritic control group and subsequent groups over the course of the study. The evaluation parameters were investigated for reduction in rheumatoid arthritis (RA) manifestations, including synovial hyperplasia (pannus formation), inflammatory cell infiltration, and bone erosion in the Freund's Complete Adjuvant (FCA)-induced arthritic rat model via histopathological examination [28].

2.2.7 Statistical Analysis

All data were expressed as mean ± standard error of the mean (SEM) for each parameter investigated. Statistical analysis was performed using one-way analysis of variance (ANOVA) via Graph Pad INSTAT software. Post-hoc comparisons were conducted using Dunnett's test to assess differences between treatment groups and controls. A p-value < 0.05 was established as the threshold for statistical significance [29]. This approach allowed for robust evaluation of treatment effects while controlling for multiple comparisons across experimental groups.

3 Results

3.1 Phytochemical analysis

The result of qualitative Phytochemical screening of methanolic extracts of C latifolium are represented in Table 1.

Table 1: Phytochemical screening of methanolic extracts of C latifolium

Tests	Inference
Carbohydrates	+
Glycosides	+
Polysaccharides	−
Free amino acids	+
Bradford test	+
Tests for alkaloids	+
Dragendroff’s test	+
Mayer’s test	+
Tests for steroids	−
Triterpenoids	−
Tests for flavonoids	+
Tests for Tannins	+
FeCl3 test	+
Dilute HNO3 test	−

Test For Lipid	–
Test for Oils	–
Test for saponins	+

(+) positive, (−) negative

3.2 Total antioxidant activity

3.2.1 DPPH Method:

Table 2: Superoxide radical scavenging activity of extract loaded nanoemulsion with reference to ascorbic acid

S.no.	Concentration(µg/ml)	%Inhibition	
		Ascorbic acid	Extract loaded Nanoemulsion
1	10	37.39	22.23
2	20	52.13	29.44
3	40	67.78	58.14
4	60	69.91	65.65
5	80	83.74	75.76
6	100	85.56	82.12
IC50		20.03	44.35

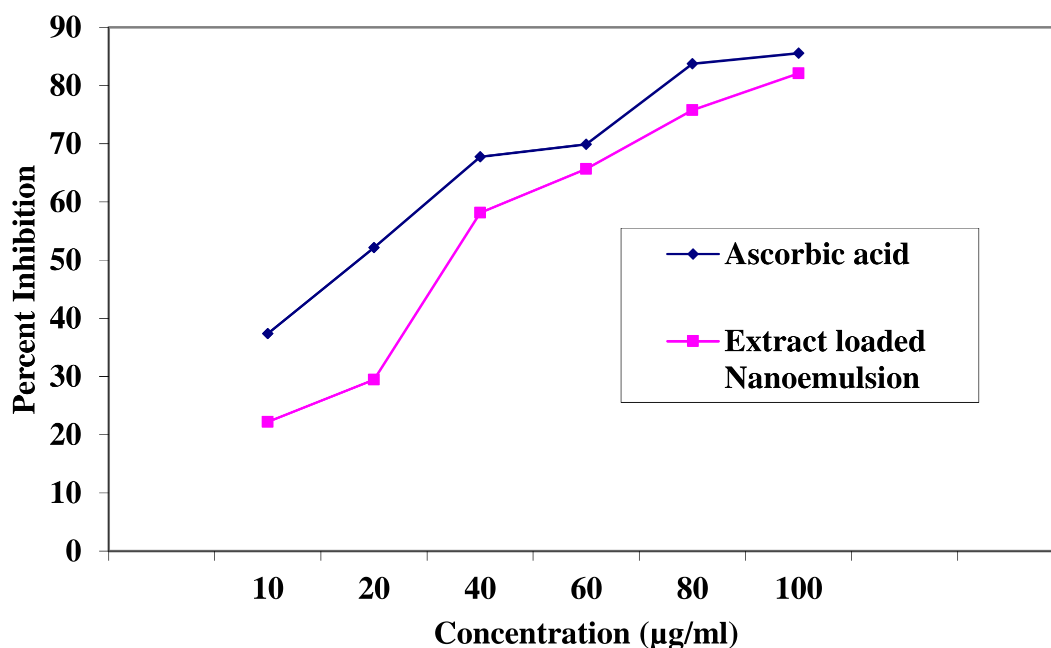


Figure 1: Percent inhibition activity of ascorbic acid and extract loaded nanoemulsion with respect to concentration

3.2.2 Superoxide radical scavenging assay:

Table 3: Superoxide radical scavenging activity of extract loaded nanoemulsion with reference to ascorbic acid

Concentration	Sample (%)	Indomathacin (%)
1 mg/kg	75.05	87.14

S.No.	Concentration(µg/ml)	%Inhibition)	
		Ascorbic Acid	Extract loaded Nanoemulsion
1	10	41.49	18.65
2	20	51.06	23.43
3	40	60.79	47.45
4	60	67.48	61.22
5	80	71.88	64.21
6	100	79.33	75.52
IC50Value		20.91	60.94

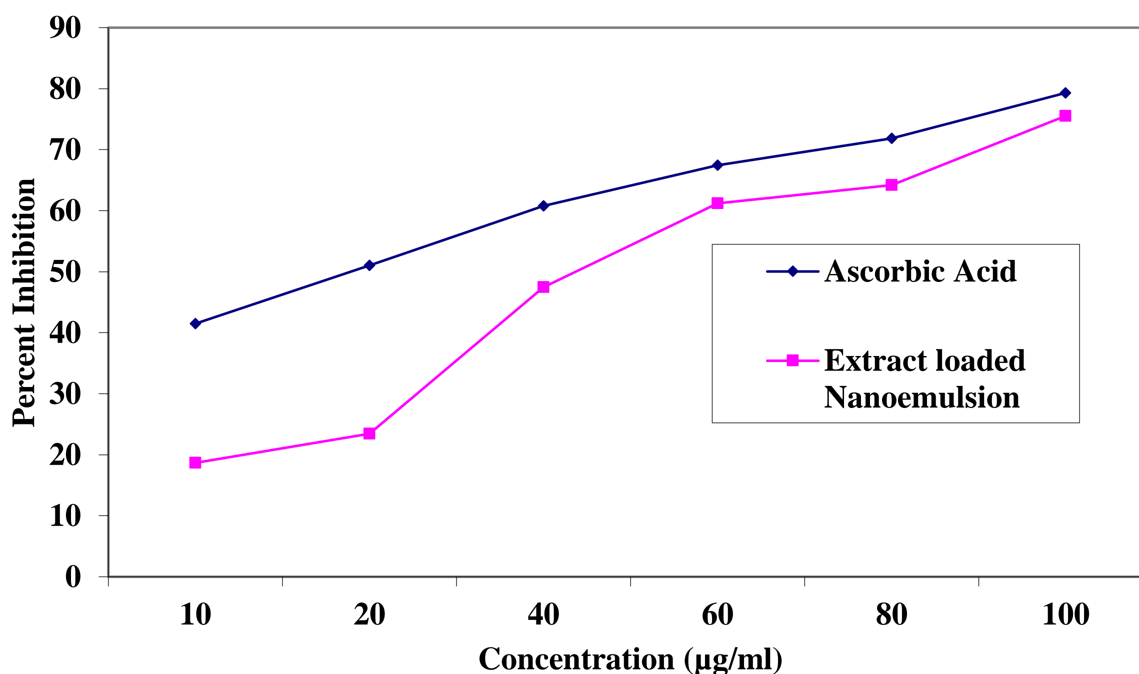


Figure 2: Percent inhibition activity of ascorbic acid and extract loaded Nanoemulsion with respect to concentration

3.3 Effect on hyaluronidase enzyme inhibition activity

The hyaluronidase inhibitory potential of nanoemulsion loaded with extract of *C. latifolium* was evaluated (Table 4). At 1 mg/kg, the extract demonstrated 75.05% inhibition of hyaluronidase activity. While the % inhibition of hyaluronidase activity of standard drug was found to be 87.14. These findings suggest that nanoemulsion loaded (1mg/kg of extract) with extract of *C. latifolium* exhibits significant anti-inflammatory properties through its hyaluronidase inhibitory action. This hyaluronidase inhibition mechanism may contribute substantially to the overall anti-inflammatory effects of that nanoemulsion loaded with extract of *C. latifolium*, warranting further investigation into its potential therapeutic applications.

Table 4: Hyaluronidase inhibitory potential of *C. latifolium* loaded nanoemulsion

3.4 Acute toxicity study

In an acute toxicity study, the methanolic extract of *C. latifolium* was tested on animals at doses as high as 2000 mg/kg and was found to be non-toxic. Throughout the 14-day observation period, there were no signs of toxicity, such as changes in fur color, behavior, or patterns of writhing, lethargy, urination, or eating habits.

3.5 Effect of nanoemulsion on articular inflammation of experimental animal.

The data from this study demonstrate a strong correlation between the severity of articular inflammation and the magnitude of weight loss in the experimental animals (Table 5). Following adjuvant administration, the arthritic rat model exhibited a biphasic weight change pattern. The initial phase, occurring within the first week post-injection, was characterized by a pronounced decrease in body weight. This acute weight loss was succeeded by a second phase of minimal weight gain over the ensuing weeks.

Groups	Treatment	Dose (mg/kg)	Body weight of rats (in grams)			
			0th Day	7th Day	14th Day	21st Day
I	Normal control	Blank Nanoemulsion	180.70±1.09	172.33±0.6	178.39±2.01	180.15±1.98
II	CFA	0.1mL	180.71±1.98	181.73±2.09[a]	18313±1.20[a]	184.23±1.99[a]
III	CFA +Nanoemulsion (1mg/kg of loaded extract)	0.1mL+1mg/kg	187.83±2.89	190.42±1.60*	206.39±8.33*,[B]	230.32±9.33**,[B]
IV	CFA +indomathacin	0.1mL+15 mg/kg	195.18±2.86	218.39±3.33**,[a]	228.23±8.13**,[a]	240.63±13.87**,[a]

Table 5: Estimation of severity of articular inflammation and the magnitude of weight loss in the experimental animals

3.6 Effect on Haematological parameters

Hematological parameters, including erythrocyte and leukocyte counts, hemoglobin concentration, erythrocyte sedimentation rate (ESR), and platelet count, were significantly elevated in the arthritic control group compared to the treatment groups ($p < 0.05$) (Table 6). Administration of nanoemulsion loaded extract of *C. latifolium*, resulted in a significant improvement and normalization of hematological parameters. The treated groups exhibited hematological values that were comparable to those of the non-arthritic control group.

Table 6: Comparison of various hematological parameters of arthritic control group with the treatment groups (p < 0.05)

Parameters	Negative control	Positive control	Standard	Test Compound (1mg/kg)
Hb (g/dL)	**13.40 ± 0.67**	**8.64 ± 0.40***	**11.29 ± 0.31****	**15.02 ± 0.29****
RBC (106/Ul)	**5.19 ± 0.23**	**1.96 ± 0.22***	**4.23 ± 0.27****	**5.64 ± 0.25***
WBC (103/Ul)	**7.48 ± 0.40**	**16.70 ± 0.47***	**13.81 ± 0.27****	**13.63 ± 0.41****
platelets (103/Ul)	**772 ± 21**	**1467 ± 19***	**842 ± 36****	**1072 ± 42***
ESR (mm/h)	**2.83 ± 0.27**	**8.40 ± 0.33***	**5.79 ± 0.33****	**1.76 ± 0.30****

** represents p<0.025, * represents p<0.05

3.7 *In vivo* anti-inflammatory activity (carrageenan-induced paw edema)

The *in-vivo* anti-inflammatory effects of nanoemulsion loaded with extract of *C. latifolium* was assessed using the carrageenan -induced paw edema model in rats (Table 7). The results indicated that the nanoemulsion loaded with extract of *C. latifolium* was found to be more effective at reducing inflammation compared to negative and positive control groups. However, the standard drug ibuprofen demonstrated more pronounced anti-inflammatory activity than the nanoemulsion loaded with an extract of *C. latifolium*. Overall, the nanoemulsion loaded with an extract of *C. latifolium* significantly reduced paw inflammation when compared to the control group.

Table 7: Effect of nanoemulsion loaded with extract of *C. latifolium* on carrageenan induced paw edema in rats

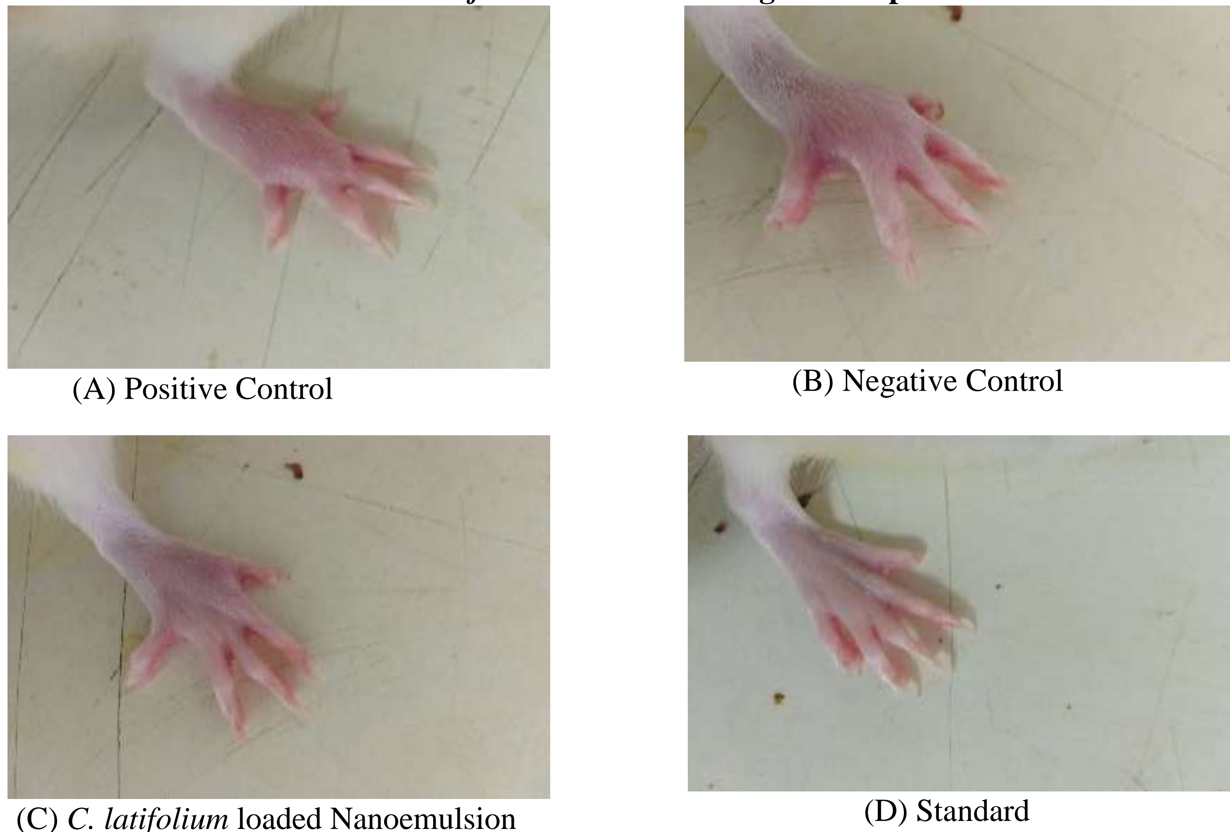
Time (h)	Negative Control (Blank Nanoemulsion)		Positive Control (Carrageenan only)		Nanoemulsion loaded with *C. latifolium* extract 1mg/kg)		Standard	
	Initial volume (ml)	Final volume (ml)	Initial volume (ml)	Final volume (ml)	Initial volume (ml)	Final volume (ml)	Initial volume (ml)	Final volume (ml)
0	**8.09 ± 1.01**	**8.93 ± 2.25**	**8.23 ± 0.26**	**8.98 ± 1.59**	**9.76 ± 1.23**	**10.23 ± 2.01**	**9.12 ± 0.22**	**9.48 ± 1.42****
1	**8.09 ± 0.70**	**09.23 ± 1.27**	**8.23 ± 1.83**	**9.86 ± 1.90**	**9.76 ± 0.49**	**10.31 ± 1.58**	**9.12 ± 0.39**	**9.08 ± 1.59*****
2	**8.09 ± 1.28**	**10.26 ± 2.03**	**8.23 ± 1.25**	**10.62 ± 1.65***	**9.76 ± 1.98**	**10.71 ± 1.14****	**9.12 ± 1.37**	**9.61 ± 1.37*****
4	**8.09 ± 1.20**	**10.30 ± 1.93**	**8.23 ± 0.35**	**10.78 ± 1.75***	**9.76 ± 1.96**	**10.67 ± 2.12****	**9.12 ± 2.01**	**10.01 ± 2.33*****
6	**8.09 ± 1.52**	**10.38 ± 1.11**	**8.23 ± 1.78**	**11.10 ± 1.67****	**9.76 ± 1.47**	**10.71 ± 2.32****	**9.12 ± 2.02**	**10.10 ± 1.38*****

Data are represented as mean ± SEM (*n* = 5), significantly different at *** represents 0.0025, ** represents p<0.025, * represents p<0.05 in comparison to control group. Standard = ibuprofen

3.8 *In vivo* antiarthritic activity (CFA induced arthritis)

Arthritis in the animals was induced by injecting CFA directly into the joint. The impact of *C. latifolium* extract on CFA-induced paw edema in rats is presented in Table 8 and Figure 3. The findings showed that the extract significantly reduced arthritis by 64.28% compared to the control group. However, the standard treatment group achieved the greatest reduction in arthritis.

Figure 3: Effect of *C. latifolium* extract on CFA induced paw edema in rats. Figure A represents positive control, Figure B represents negative control, Figure C represents emulsion loaded with *C. latifolium* extract and Figure D represents standard.



(A) Positive Control (B) Negative Control

(C) *C. latifolium* loaded Nanoemulsion (D) Standard

Table 8: Effect of *latifolium* extract on CFA induced paw edema in rats

Time (Day)	Negative Control (nanoemulsion without extract)		Positive Control (CFA only)		Nanoemulsion loaded with *C. latifolium* extract 1mg/kg)		Standard	
	Initial volume (ml)	Final volume (ml)	Initial volume (ml)	Final volume (ml)	Initial volume (ml)	Final volume (ml)	Initial volume (ml)	Final volume (ml)
0	9.33 ± 0.28	9.46 ± 1.34	9.35 ± 0.37	9.46 ± 0.44	9.56 ± 1.33	9.93 ± 1.06	9.48 ± 1.29	9.50 ± 1.38
7	9.42 ± 1.29	10.54 ± 0.25	10.06 ± 1.31	11.83 ± 1.68	10.56 ± 0.22	10.79 ± 1.02***	10.46 ± 0.56	11.11 ± 2.33***
14	9.33 ± 0.39	10.43 ± 1.37	10.22 ± 0.78	11.23 ± 1.28	10.56 ± 0.98	11.15 ± 0.65***	10.46 ± 1.17	11.15 ± 0.37***
21	10.22 ± 1.47	10.42 ± 1.45	10.28 ± 0.25	11.13 ± 1.31**	10.56 ± 1.33	11.18 ± 0.33***	10.46 ± 1.98	11.18 ± 1.28***

Data are represented as mean ± SEM (n = 5), significantly different at *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ in comparison to control group. Standard = indomethacin.

3.9 Effect of plant extract loaded nanoemulsion on analgesic activity

The analgesic model was performed using Eddy's hotplate method. The nanoemulsion loaded with plant extract showed 45.45% increase in pain threshold which was less than standard group but much increased than the negative and positive control groups which were 6.25 and 4.28% respectively (Table 9). The %age increase in pain threshold was found to be 68.42 % in the case of the standard group. The responses shown by the experimental animal i.e paw licking, jumping, and vigorous running were taken as end points and parameters for the evaluation of the final result of this activity.

Table 9: Effect of plant extract loaded nanoemulsion on percent increase in pain threshold and reaction time on a hot plate at 0 and 90 minutes

Name of Groups	Reaction time on a hot plate		% increase in pain threshold
	Zero time	90 min after administration	
Negative Control (Only nanoemulsion)	6.4 ± 0.1	6.8 ± 1.6	6.25 ± 7.6
Positive Control (No treatment)	7 ± 0.2	7.3 ± 0.7	4.28 ± 2.9
Test Group (Nanoemulsion loaded with plant extract 1mg/kg)	6.6 ± 0.4	9.6 ± 0.2	45.45 ± 1.1
Standard Group (Aspirin)	5.7 ± 0.2	9.6 ± 0.9	68.42 ± 8

3.10 Histopathological evaluation

The histopathological analysis revealed distinct features across various experimental groups. Figure 4(1A) and (1B) present typical ankle joint images from the normal control group at 40X and 100X magnification, respectively, where the double arrow delineates the space between cartilage and bone, which is noticeably increased in the diseased control group, indicating joint degeneration and sclerotic bone containing osteocytes, suggestive of active bone remodeling. In the negative control group, Figure 4(2A) shows an enlarged joint space alongside granuloma formation, characterized by epithelioid macrophages, as well as inflammatory cell infiltration, synovial hyperplasia, and articular cartilage degeneration, while Figure 4(2B) marks fibro-collagenous tissue, indicative of tissue remodeling and fibrotic alterations. The standard group Figure 4(3A) & (3B) exhibits a decrease in the articular space, with reduced granulomatous tissue observed at 100X magnification, and areas of moderate inflammation accompanied by thickened, congested blood vessels, as highlighted by the two-headed arrow and arrowhead, respectively. Figure 4(4A) demonstrates moderate inflammation with granulomatous tissue and chronic infiltrates alongside congested, dilated blood vessels, whereas Figure 4(4B) shows bony trabeculae surrounded by moderate chronic infiltrates with no evidence of pannus formation or cartilage damage, as indicated by the circle, and a reduced articular space, reflecting joint space narrowing.

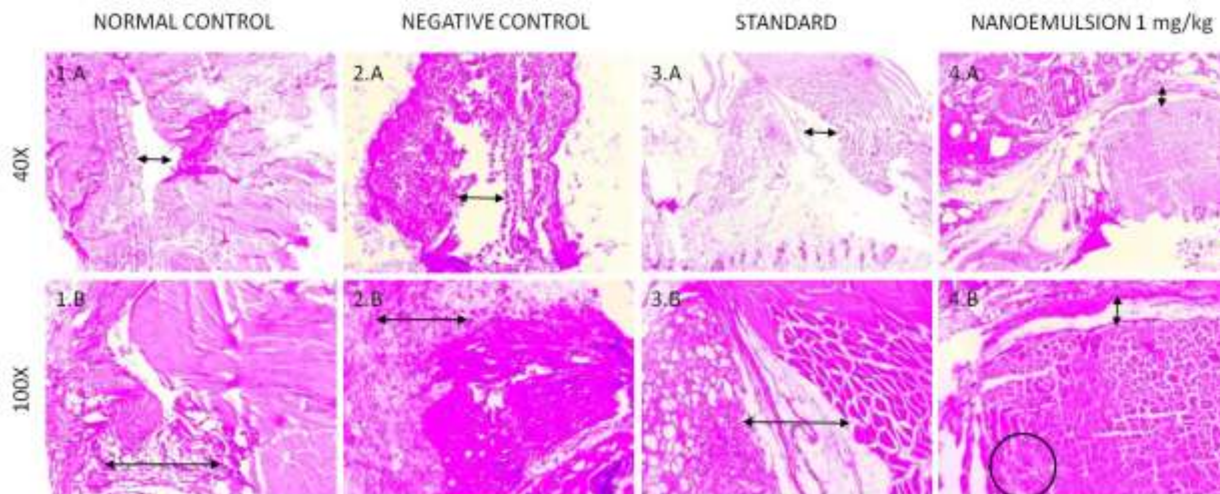


Figure 4: Histopathological analysis of distinct features across various experimental groups

4 Discussion

The present study was conducted for formulation of nanoemulsion loaded with extract of *C. latifolium* and evaluating the *in-vitro* and *in-vivo* anti-inflammatory and antiarthritic activity. First, the phytochemical analysis of *C. latifolium* revealed the presence of several bioactive compounds, including carbohydrates, glycosides, and alkaloids, which are known for plant's anti-inflammatory and antioxidant effects, which are crucial in managing the inflammatory processes associated with RA. Second, the nanoemulsion formulation loaded with *C. latifolium* plant extract was optimized in our previous studies and formulated accordingly to investigate its therapeutic potential.

The *in vitro* studies highlighted the antioxidant capacity and hyaluronidase inhibition activity of the nanoemulsion formulation of *C. latifolium*, suggesting its potential to mitigate oxidative stress and enzymatic degradation of connective tissues in RA. The phenolic compounds and flavonoids were believed to be responsible for the antioxidant activities observed. The *in vivo* studies further supported these findings, showing significant anti-inflammatory effects in the carrageenan-induced paw edema model and the complete Freund's adjuvant (CFA)-induced arthritis model. Arthritis in the animals was induced by intra-articular injection of complete Freund's adjuvant (CFA). The results showed that the test compound significantly reduced paw edema at 6 hours, achieving a reduction of 65.33%, compared to 60.08% for the standard drug ibuprofen at the same time point. The reduction in paw volume and improvement in body weight was found in treated groups. These results of the anti-arthritic activity revealed that the test compound exhibited significant anti-arthritic efficacy.

5 Conclusion

The study clearly demonstrates that *C. latifolium* extract loaded Nanoemulsion exhibits dose-dependent antioxidant and hyaluronidase inhibition activities, as well as significant anti-arthritic and anti-inflammatory effects. The study's results are particularly relevant where the prevalence of RA is notably high. The shift towards herbal-based therapies, driven by the adverse effects associated with conventional RA treatments, further highlights the importance of exploring plant-based alternatives like *C. latifolium*. The nanoemulsion formulation could offer a more effective and safer therapeutic option for RA patients, potentially reducing the reliance on conventional drugs that often come with significant side effects. The scope of this research is limited to preclinical models, and further clinical trials are necessary to establish the efficacy and safety of *C. latifolium* in human subjects. Such studies could pave the way for developing it into a

commercially viable dosage form, benefiting overall social well-being. Additionally, the study could be expanded to explore the molecular mechanisms underlying the plant's therapeutic effects, providing a more comprehensive understanding of its role in RA management.

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