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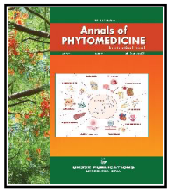

Original Article : Open Access

Formulation and evaluation of naringenin niosomes for topical drug delivery and antirheumatic activity

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Abstract

Naringenin (NAR) is recognized for its anti-inflammatory and antiarthritic activities but has the limitation of low bioavailability. The purpose of the present study was to formulate niosomes for naringenin for topical application in rheumatoid arthritis condition. Niosomes were prepared by thin film hydration and ethanol injection methods employing span 60 as a non-ionic surfactant and variable factors among the formulations being span 60 and cholesterol concentrations. Optimized formulation was selected based on the parameters the particle size and the EE%. The optimized formulation was from ethanol injection method batch with particle size 160.2 ± 12.1 nm and EE% 83.82 ± 1.4. Furthermore, NRG niogel was prepared by incorporating the optimized NRG niosomes into carbopol 934 hydrogel and was evaluated for pH, viscosity, and *in vitro* drug release. The release data was interpreted in the kinetic models to find the best fitting and revealed that it follows the first order. *Ex vivo* permeation studies were conducted for NRG niogel and compared with the permeation pattern of normal NRG gel, to evidence the sustained release of NRG from the niogel for a prolonged period of time. Finally, *in vivo* antiarthritic activity was studied on FCA-induced arthritic rat models and observed that arthritic score and pro-inflammatory cytokines levels have elevated in disease control, and in the treatment groups, they have gradually decreased. NRG niogel treated group has shown a significant recovery signs of cartilage regeneration and the synovial membrane anatomy similar to that of normal control group, in the histopathology study. Our findings suggest that a topical delivery of NRG using a niosomal hydrogel delivery system could find a prospective application in the management of rheumatoid arthritis.

1. Introduction

Rheumatoid arthritis (RA), an inflammatory joint disorder that is systemic and autoimmune, leading to significant disability and morbidity globally, and most common in adults (Carmona *et al.,* 2010) with an annual incidence of approximately 1%. It is characterized by synovial hyperplasia, symmetrical swelling in multiple small joints leading to joint dysfunction (Subramoniam *et al.,* 2013) plagues chronically with remission and flare-ups and in some cases, can also affect internal organs, inflicting long-term complications. The inflammation results in debilitating pain, stiffness, and erosion of bone (Michelle *et al.,* 2011). In children younger than 16, it is referred to as juvenile RA, where the rheumatoid arthritis factor is not detected (Bullock *et al.,* 2018). The proinflammatory molecules, including reactive oxygen species (ROS), cytokines, prostaglandins, and leukotrienes unequivocally contribute to the etiology of this disease (Snedegard, 1985; Henderson *et al*., 1987; Subramoniam, 2016). RA therapy aims at improving joint function and preventing joint damage which usually is tailored to individual patient needs, taking into account their overall well-being (Najam, 2016).

The conventional treatment modalities for RA, including TNF-α antagonists and anti-inflammatory drugs-NSAIDs, DMARDs, and biological DMARDs have shown some success, but with limitations like toxicity, high cost, and adverse effects (Curtis *et al.*, 2011). Hence, there is an ensuing search to find the safest lead molecules with antiarthritic potential derived from natural sources (Subramoniam, 2016).

A sizable portion of the global population prefers using herbal medicines due to their perceived lower occurrence of side effects and the benefit of being economical (Padmini *et al.,* 2016). These medications integrate diversity among all the formally acknowledged healthcare systems (Iqbal, 2013; Pushpangadan, 2013; Subramoniam, 2014; Udupa, 2016), thus allowing one to contemplate on complementary and alternative medicine (CAM) to destine, along with the other contemporary inclusions. A meticulous and thoughtful approach by humanity is required for the effective extraction and application of the potent therapeutic properties found in medicinal plants to ensure optimal efficacy (Das *et al.,* 2019).

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Folklore medicine has revealed many potential abilities of flavonoids including anti-inflammatory properties (Lee *et al.,* 2009). Flavonoids are primarily found in plants, mainly natural pigments and they comprise a basic phenolic structure (Diwan *et al.,* 2016; Tungmunnitum *et al.,* 2018*;* Samuelsen, 2000).

Naringenin chemically is 4,5,7-trihydroxyflavonone, a flavonoid compound found in the fruits of citraceae family, and berries, synthesized from the aromatic amino acid phenylalanine. It exists in various conjugated forms with differing pharmacokinetic properties, and glycosylated forms where an inactive glycone form is known as "naringin" (Shakeel *et al.,* 2017). Naringenin has garnered recent attention due to studies suggesting that many of its biological properties have medicinal importance (Patel *et al.,* 2014; Rani *et al.,*2016). The antioxidant activity-associated therapeutic effects of naringenin are linked to its ability to scavenge the free radicals produced during various metabolic processes (Mir *et al.,* 2015). Naringenin primarily acts by inhibiting specific cytochrome P450 isoforms, targeting CYP1A2 (Arafah *et al.,* 2020) and CYP3A4 in humans (Fuhr *et al.,*1995; Kanazeet *et al.,* 2007; Fuhr *et al.,* 1994).

Naringenin has low water solubility, up on GI absorption undergoes rapid metabolism in the liver, and is turned into glucuronide products, leading to limited presence in the bloodstream (Joshi *et al.,* 2018). Despite being quickly absorbed, by passive diffusion and active transport; its bioavailability remains low because of the extensive metabolism in the intestines and limited membrane transportation (Bhia *et al.,* 2021). Researchers presently are utilizing various methods to intricate inclusion techniques to develop multiple viable formulations of naringenin, all aimed at improving its bioavailability thereby enhancing its potential clinical applications (Rivoira *et al.,* 2020). Naringenin is recognized with a sensitivity to environmental conditions that can lead to photodegradation and thereby loss of therapeutic efficacy, it is recommended to be protected from light, therefore, to preserve its effectiveness (Patel*.,* 2018).

Niosomes, is a recently developed vesicular system with diverse applications, a bi-layered structure formed by non-ionic amphiphilic surfactant and a lipid component, primarily cholesterol. They surpass liposomes in stability and can encapsulate both the hydrophilic and the lipophilic molecules more effectively. Niosomes enable a controlled as well as a sustained drug release, extending therapeutic benefits; thereof, mitigating the side effects through a precise disease site-targeted drug delivery by surface functionalization, eventually minimizing off-target effects, and ultimately enhancing drug effectiveness (Rauf *et al.,* 2022). Moreover, niosomes demonstrate outstanding biocompatibility, thereby reducing the likelihood of toxic or immunogenic reactions. Additionally, they confer stability upon encapsulated drugs, extending shelf-life while preserving therapeutic efficacy. Moreover, niosomes have the potential to mitigate the toxicity of specific drugs, enabling higher therapeutic dosages without compromising patient safety. Their versatility allows for customization to various administration routes, encompassing oral, topical, or intravenous, thus rendering them applicable across a broad range of medical settings (Bharani *et al.,* 2018). Niosomes provide a physical barrier that shields photosensitive drugs from UV light, preventing photodegradation. This is particularly important for drugs that degrade quickly when exposed to light, ensuring that they retain their therapeutic properties. Niosomes, when topically applied, fulfill various functions such as acting as a solubilization matrix, providing sustained release of dermally active substances, enhancing penetration, and regulating drug absorption (Parashar *et al.,* 2018). They have the potential to reduce the adverse effects of drugs, extend drug circulation, and offer sustained release for substances with low therapeutic indices and poor water solubility (Bhia *et al.,* 2021). Niosomes, the novel delivery systems represent a cost-effective and scalable approach to drug formulation, thereby promisingly reduce healthcare expenses (Said Elbahr *et al.,* 2022; Dehghan *et al.,* 2023). Reports summarize that niosomes with a size lesser than 200 nm can penetrate easily through the epidermis, occur systematically, and get accumulated in the synovial joint with the assistance of the EPR effect (Enhanced permeation and retention) (Sutapa *et al.,* 2014). Hence, we hypothesized that a noninvasive, *i.e.*, the topical delivery of a therapeutic agent via a nanovesicular delivery system could improve the therapeutic efficiency of the drug for a prolonged period through a sustained release.

The present study was aimed at the fabrication and characterization of naringenin loaded niosomal hydrogel topical formulation and investigating its antiarthritic potential in FCA induced arthritic rat models against the plain drug-loaded hydrogel in the same route.

2. Materials and Methods

2.1 Chemicals and reagents

Naringenin and cholesterol were purchased from SRL Pvt Ltd, Maharastra, India. Span 60 was obtained from Jeevika Specialities Pvt Ltd, Maharastra, India. The procurements of pluronic F127, and FCA-Freund's Adjuvant Complete and Incomplete, were done from Merck, Sigma-Aldrich. ELISA kits were bought from Invitrogen, Thermofisher scientific, USA. All other chemicals, reagents, and solvents utilized in the study were of high quality analytical grade.

Male Wistar rats used for the evaluation of antiarthritic activity were purchased from VAB Biosciences, Hyderabad, Telangana. The protocol of animal experiments was accepted by IAEC in protocol number: IAEC/SVCP/2022/10.

2.2 Preparation of naringenin niosomal vesicles

Naringenin loaded niosomes were fabricated employing two methods. Niosomal formulations fabricated by the Thin film hydration method were coded TH (1-8) and the formulations from the Ethanol Injection method, were coded as EI (1-8) (Gupta *et al*., 2010; Parthibarajan *et al*., 2013)

2.2.1. Thin film hydration

Accurately weighed amounts of span 60, cholesterol, and drug were dissolved into solvent ethanol in a round bottom flask. Then the solvent was evaporated using a rotary flash evaporator (M/S Ika RV 10) under reduced pressure, temperature of 50°C, and rotation speed of 80 rpm, to result in the formation of a dry film on the walls of the flask. Further, the film was hydrated with 10 ml of Pluronic F 127 aqueous solution for 1 h at room temperature (Pando *et al.*, 2013).

2.2.2 Ethanol injection method

Weighed amounts of span 60, cholesterol, and drug as per the formulation scheme were dissolved into solvent ethanol and injected slowly, at an injection flow rate of 1 ml/min, into 1% pluronic F127

aqueous solution while continuously stirring over a magnetic stirrer at 800 rpm with heating mantle at 65-70°C, *i.e.,* the temperature above the transition temperature that results in the formation of niosomes on evaporation of the organic solvent.

2.3 Optimisation of formulation variables

The critical parameter variables in the formulation are elements that affect and alter the formulation properties and thus need to be suitably adjusted to attain the desired quality attributes. In the niosomes preparation, formulation ingredients composition was found to be a critical parameter and the quality attributes include size, polydispersity index, and entrapment efficiency of the nanovesicles.

2.3.1 Characterization of fabricated niosomes

The particle size assessment and the PDI of formulated vesicles and their zeta potential were performed using dynamic light scattering and zeta sizer (Nano ZS90, Malvern instruments, UK) after appropriate dilutions with deionized water.

2.3.2 Entrapment efficiency

EE% was calculated by indirect estimation method, wherein the supernatant unentrapped NRG is estimated, and trapped NRG was calculated by subtracting the amount of drug in the supernatant from the initial amount used for fabrication of niosomes (Poonam, *et al*., 2017). The HPLC method was developed and validated for the quantification of NRG, and furthermore for the determination of EE% in the formulation. Quantification of NRG with RP-HPLC, method was developed to plot a calibration curve of NRG. An isocratic elution, a mobile phase of methanol and water (50:50), at a detection wavelength of 291 nm with a PDA detector, were the conditions of the method developed. Isocratic elution and a flow rate of 1 ml/min in C18 column were used in the method (Tsai *et al*., 2015).

%Entrapment efficiency = [(Total NRG–Free NRG in Supernatant)/ (Total NRG)] × 100

Table 1: Ingredient composition of niosome formulations

Thin film hydration method	Ingredients		Ether injection method
Formulation code	Span 60 (mg)	Cholesterol (mg)	Formulation code
TF1	20	10	EI1
TF2	30	10	EI2
TF3	40	10	EI3
TF4	50	10	EI4
TF5	20	15	EI5
TF6	30	15	EI6
TF7	40	15	EI7
TF8	50	15	EI8

Note: Each niosome formulation contained 6 mg of naringenin, 1% of pluronic F127 in 10 ml of hydration volume.

2.3.3 Surface morphology analysis

SEM (scanning electron microscopy) was performed to understand the morphology of formed vesicles, using 200 ESEM (FEI, USA) at 25 ± 2°C.

2.3.4 *In vitro* drug release study on Naringenin niosomes

In vitro drug release studies were performed using the dialysis sac method (Hu *et al*, 1999). Niosomes suspension was placed in the dialysis bag of nitrocellulose membrane of molecular weight cut off range 12-14 k Da, Himedia, India., and was immersed in 50 ml of pH 5.5 phosphate buffer saline at 37 ± 0.5°C. At predetermined time intervals, 1 ml of sample was withdrawn from this testing unit for HPLC analysis, for quantitative estimation of drug release, and replenished with the same quantity of fresh medium. Samples drawn were analyzed using HPLC and the percentage drug release was calculated at different time points. Obtained data was correlated with established drug release kinetics models to demonstrate the mechanism of NRG release.

2.3.5 Stability studies

Physical and chemical stability of NRG niosomes was assessed at different conditions of storage, 2-8°C, 25 ± 2°C, and 45 ± 2°C, in a humidity chamber, according to ICH guidelines, for a period of three months. Changes in the particle size and EE% were observed each month to estimate the stability of the preparation.

2.4 Preparation of NRG niosomes entrapped carbopol hydrogel (NRG niogel) and characterization

Niosomes were concentrated by centrifuging at 20,000 rpm for 20 min and discarding the supernatant portion with unentrapped NRG, sediment was resuspended into water. Carbopol 934, the gelling agent, was dispersed in water along with the niosomal concentrate and left to stir for 6 h on a magnetic stirrer at room temperature to prepare the carbopol hydrogel. The resultant formulation was then neutralized using 0.5% triethanol amine and proceeded for characterization (Rapalli *et al*., 2021).

2.4.1 pH determination

pH of NRG niogel was measured using a digital pH meter, model CPH 102; after an appropriate dilution of a fixed quantity of gel in distilled water.

2.4.2 Viscosity

The viscosity of the hydrogel was measured by using a Brookfield viscometer with spindle number 64.

2.4.3 Spreadability

Spreadability evaluation was carried out by placing a preweighed quantity of NRG niogel in between the two glass slides and recording the diameter spread of gel with the addition of weights over the upper slide, as a function of the spread area to the mass applied.

2.4.4 Drug content

0.5 g of NRG niogel was dispersed into 5 ml of ethanol, by sonicating and later centrifuged to extract the freed NRG to supernatant which was analyzed for drug content, using the HPLC method.

2.5 *In vivo* antiarthritic activity

Rheumatoid arthritis animal models were developed by induction of arthritis using Freund's complete and incomplete adjuvant factor in male Wistar rats. One group out of the four groups of animals with 6 animals in each group, taken for the study was acting, normal group (group-1) (NC) and one was disease control (group-2) (DC). The other two were treatment groups. Disease induction was done in three groups of animals by injecting 0.1 ml of FCA into the sub-plantar region of the right hind paw and incomplete Freund's factor on the seventh day into the same paw as the booster dose. The normal control group was left unimmunized. Arthritis was confirmed on visual observations and measurement of inflammation, joint and paw swelling, and by estimation of pro-inflammatory cytokines like TNF-α and IL-6 whose levels increase will indicate an established RA condition. Further, the study proceeded with bis-in-die topical treatment of two groups of animals with NRG gel (group-3) and NRG niogel (group-4) for 14 days starting from the 14th day of disease inoculation with FCA. Arthritic score, paw volumes, and joint stiffness were regularly assessed throughout the study period (Kumar *et al*., 2006).

2.5.1 Cytokine's assessment using ELISA

The serum samples collected on the 28th day of initialization of the animal study were analyzed for TNF-α, and IL-6, using Duoset ELISA kit, following the manufacturer's recommendations. The absorbance of the end colour developed was measured using ELISA reader at 450 nm.

2.5.2 Histopathological analysis

On the 29th day of the study, animals were sacrificed to collect the hind paws for histology analysis (Kumar *et al*., 2006). Hematoxylin and eosin-stained tissue sections were observed under 100x of fluorescent optical microscopy.

2.6 Statistical data analysis

Experiments were conducted in triplicates and data interpretation, through a two-tailed student t-test using mean ± SEM, to derive probability value. p-value < 0.05 is considered statistical significance.

3. Results

3.1.1. Preparation and characterization of NRG niosomes

Niosomes prepared by two different methods-TFH method and EIM have produced vesicles of size ranging between 148.7 ± 12.3 nm and 243 ± 24.2 nm and the PDI, as low as 0.08 ± 0.02 to the highest value 0.43 ± 0.11. It is observed that, with the increase in span 60 concentration, the size of the particles has increased. At low concentrations of surfactant, the particles were small, as there are very few surfactant molecules to form vesicles, they form smaller vesicles, are unstable, and have poor encapsulation efficiency. An increase in cholesterol concentration has decreased the vesicle size but increased after a point. That was observed in both methods of preparation. Span and cholesterol concentrations have to be optimized to get smaller vesicles with good entrapment efficiency and uniform dispersion represented by lower PDI values. The vesicles formed have shown a globular shape and particles showed good dispersion with lesser fusion and aggregation. EI6 is the optimized formulation, from the set of formulations prepared, has an EE% of 83.82 ± 1.4 % and a particle size of 160.2 ± 12.1 nm. The zeta potential of the preparation was -32.66 ± 0.85 mV. A zeta potential > ± 30 mV is considered to be stable. The negative zeta value also prevents the aggregation for the reason of repulsive forces between like charged vesicles.

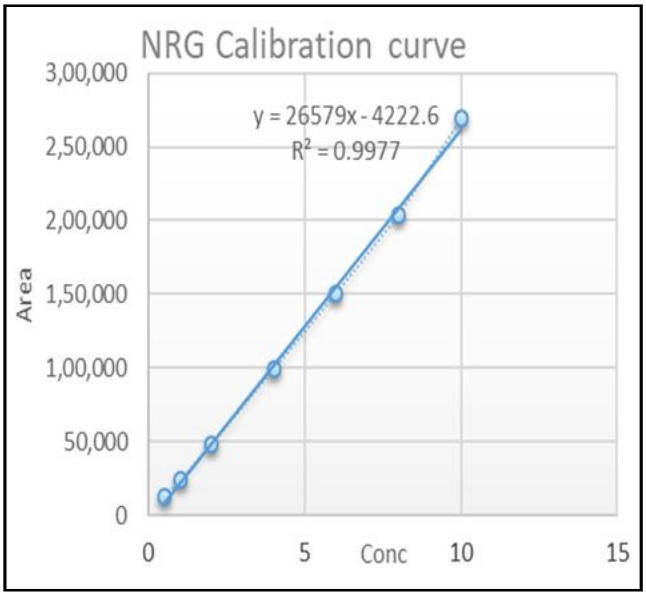

Figure 1: NRG calibration curve.

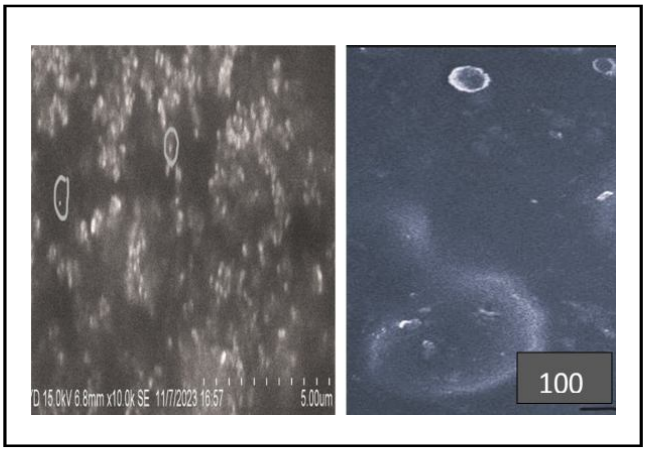

Figure 2: SEM image of optimized niosomes.

PDI is polydispersity index, a representation of homogeneity in the preparation and lower PDI values indicate a uniform distribution of particles throughout the preparation. PDI values varied within the formulations in the method batch and in between the batches and were in correspondence with the particle size. It was observed that,

with an increase in particle size, the PDI increased but at a certain concentration ratio of span and cholesterol it decreased to increase again. The span to cholesterol ratio, at which the formulation has shown a low dispersity index, bears a smaller particle size, and more entrapment efficacy was considered as an optimal concentration and the processing conditions were termed as optimal conditions.

EIM is a simple and scalable technique that has yielded vesicles with reasonably good EE and so the optimized formulation was from the EIM batch. Quantitative analysis of NRG has been performed by HPLC method. A calibration curve was plotted to obtain the slope value. The retention time was 5.8 with mobile phase 50:50 of methanol: water at 291nm.

Table 2: Evaluation parameters of NRG loaded niosomes

F code	Particle size (nm)	PDI	EE%	F code	Particle size (nm)	PDI	EE%
TF1	156.9 ± 13.7	0.16 ± 0.02	55.43 ± 4.32	EI1	148.7 ± 12.3	0.08 ± 0.02	52.11 ± 7.84
TF2	173.3 ± 8.3	0.21 ± 0.05	72.76 ± 3.78	EI2	159 ± 8.9	0.23 ± 0.04	67.09 ± 1.3
TF3	216.9 ± 11.5	0.32 ± 0.09	74.67 ± 5.06	EI3	205.9 ± 19.5	0.35 ± 0.02	78.25 ± 3.63
TF4	241.4 ± 22.07	0.41 ± 0.12	61.23 ± 7.37	EI4	230.6 ± 23.1	0.43 ± 0.11	69.73 ± 6.89
TF5	157.3 ± 6.2	0.27 ± 0.008	60.89 ± 2.07	EI5	136.4 ± 10.7	0.25 ± 0.02	54.11 ± 3.9
TF6	169 ± 5.4	0.182 ± 0.01	82.43 ± 4.83	EI6	160.2 ± 12.1	0.183 ± 0.03	83.82 ± 1.4
TF7	176.1 ± 19.1	0.24 ± 0.03	79.83 ± 5.51	EI7	164.8 ± 11.6	0.243 ± 0.05	77.62 ± 4.32
TF8	243.2 ± 24.2	0.38 ± 0.15	71.32 ± 3.33	EI8	238 ± 20.2	0.43 ± 0.02	68.31 ± 3.73

Note: Data are Mean ± SEM; n=3

3.1.2 Determination of EE%

The amount of unentrapped free drug in the supernatant was estimated, and the amount of NRG encapsulated in niosomes can thus be calculated by the indirect method of estimation. It was observed that both the surfactant and cholesterol concentrations influence the EE of the formulation. Table 2 represents the values of critical parameters analyzed for deriving into optimal formulation.

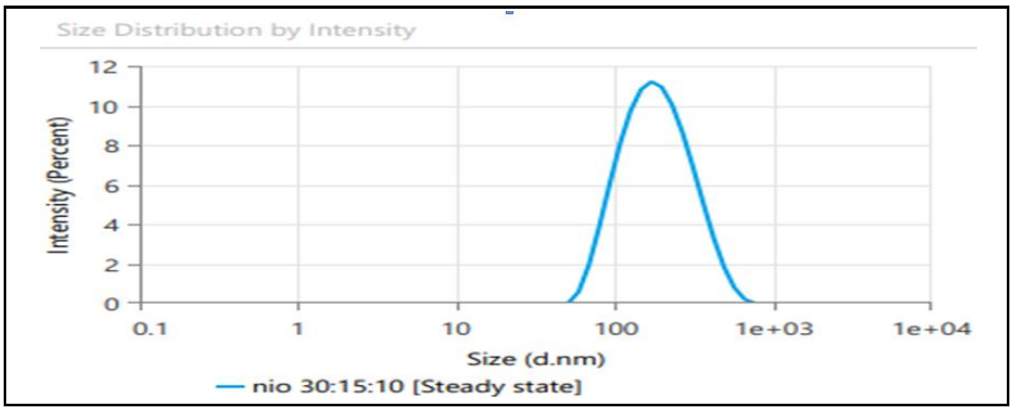

Figure 3: Particle size and PDI of optimised niosomes.

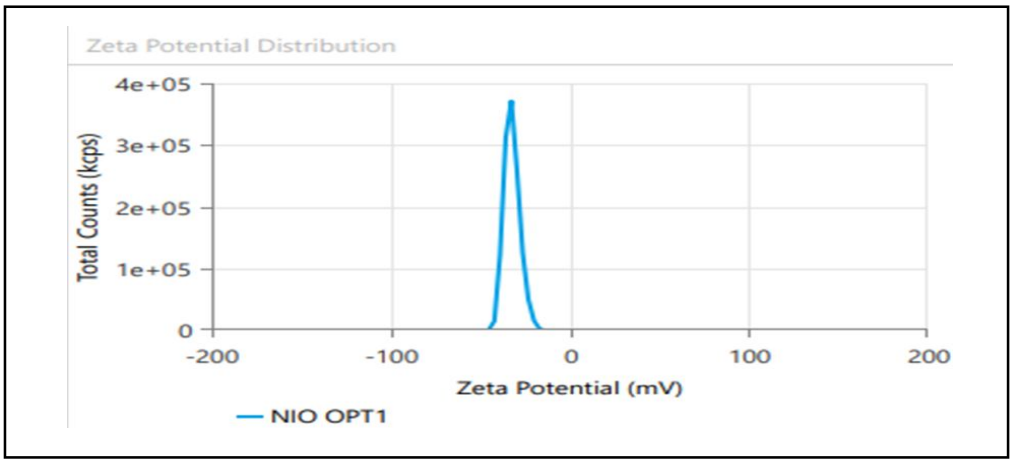

Figure 4: Zeta potential report of optimised niosomes.

Table 3: R^2 values of kinetic models

Kinetics model	R^2 value
Zero order	0.639
First order	0.889
Higuchi	0.822
Koresmeyer peppas	0.885

SEM images of the optimised formulation depicted in Figure3 have revealed a spherical structure of the vesicles. Moreover, the vesicle size analysed using a particle size analyzer and the vesicle size results of SEM analysis, were in good agreement.

3.1.3. *In vitro* drug release study

The study was conducted using a dialysis sac method. Release samples collected at fixed time intervals for 24 h were assayed for drug content using HPLC and cumulative drug release data was interpreted in kinetic models. Drug release from NRG niosomes was compared with the release pattern from NRG saturated solution. The cumulative drug release (CDR) % after 24 h for the NRG solution was 75.2 ± 2.4 % and NRG niosomes have shown a release of 88.3 ± 1.6 %. A significant increase in the %CDR of NRG niosomes over NRG solution has thereby illustrated the statement that niosomes can enhance the permeation capacity of the drugs. A release kinetic study of NRG niosomes conducted in four models, the zero-order, first-order, Higuchi, and Korsmeyer peppas indicated, the first-order model to be the best fit, as this has the highest R^2 value. The kinetic model graphs are depicted in Figure 5

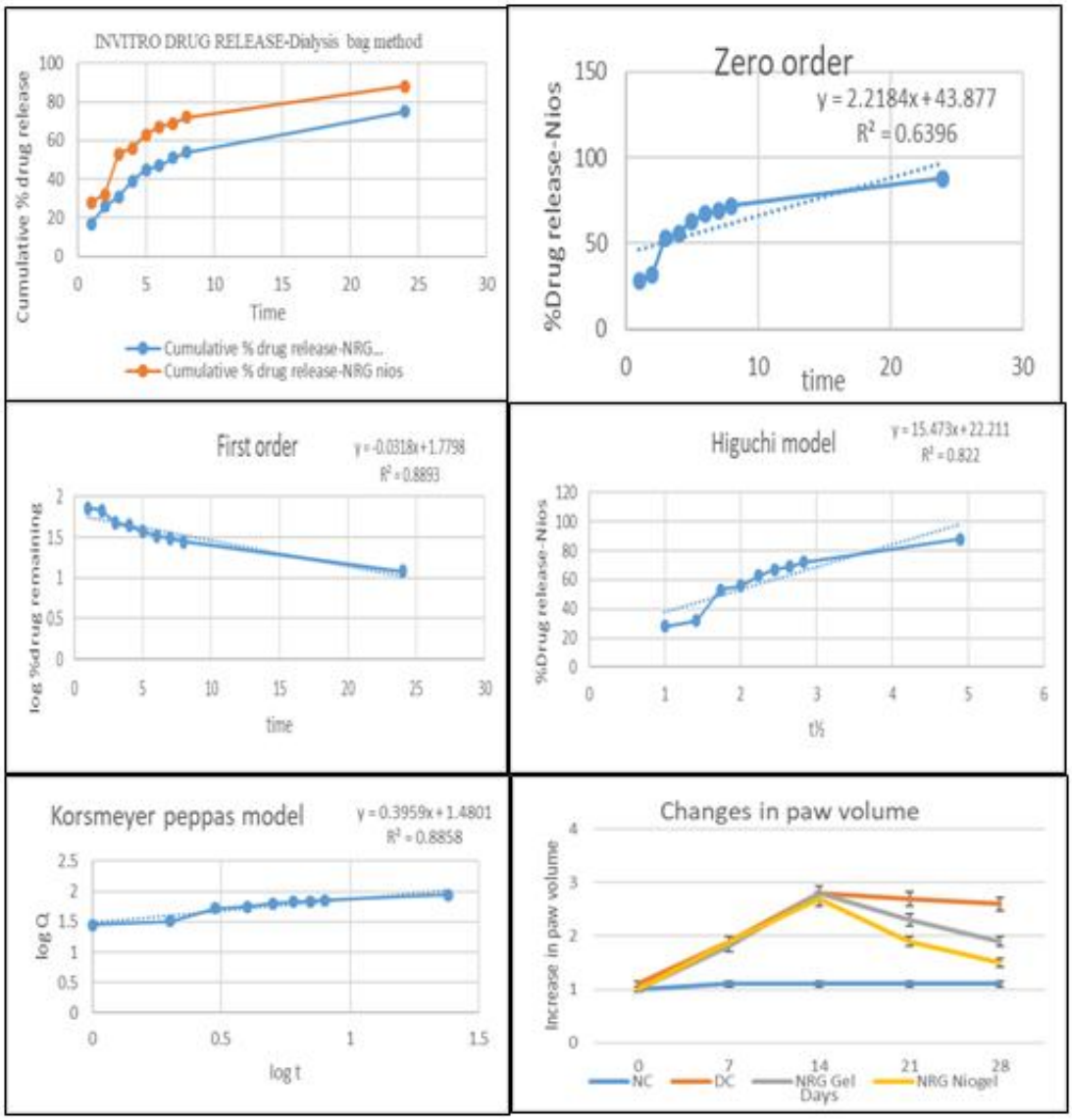

Figure 5: *In vitro* drug release graph showing %CDR of NRG saturated solution and NRG niosomes suspension and kinetic model plots with drug release data of NRG niosomes suspensions; the chart showing the changes in paw volume in the groups of *in vivo* antiarthritic activity.

3.2 Preparation and characterization of NRG niogel

NRG niogel was prepared by incorporating the optimized formulation in to 0.75 % carbopol 934, and the prepared hydrogel was analyzed for physical parameters. Prepared NRG niogel was white homogenous with uniform consistency and pH was found to be 5.2 ± 0.02.

3.2.1. Viscosity, spreadability, and drug content

The viscosity of the NRG niogel was recorded using a Brookfield viscometer and the value was found to be 18340 ± 2.37 cp.

Spreadability was measured to understand the application effort required for the topical formulation. Gels with higher spreadability value and good consistency that can be applied with minimum shear stress are desirable for preparations that need to be applied over larger areas as a thin film. The spreadability for prepared hydrogel was found to be 11.31 ± 0.12 g.cm/sec. Drug content in the gel was quantitatively estimated using HPLC. By letting the drug be released into the methanol solvent that was added, and estimating the freed drug in the centrifuge supernatant, the content can be estimated, and it was found to be 0.52 ± 0.016 mg of NRG in 0.5 gms of NRG niogel.

3.2.2. Stability testing

Stability studies were conducted at predetermined conditions of temperature and relative humidity for a definite period. The results have shown no significant changes in the assessed parameters, the particle size and EE%, at low temperature. Sedimentation was observed along with an increase in particle size, which could be because of particle aggregation and also a decrease in EE%, sooner after one month of storage at 45 ± 2°C and later, on storage at 25 ± 2°C. Physical instability and microbial mould growth were observed when stored at temperatures other than cool temperature, appealing a requirement of the addition of stabilizer and preservative.

3.3 *In vivo* antiarthritic activity

Arthritis was induced using FCA as adjuvant-induced arthritis is the best-known simulating model of human RA. The day of arthritis induction with FCA complete was termed day "0" and a booster dose, incomplete Freund's factor was given on the 7th day. Treatment started from day 14 after established arthritis was confirmed and up to day 28, with the topical application of NRG-free drug gel and NRG niogel. The arthritic score was assessed, with various parameters like paw volume, joint inflammation, joint stiffness, and immobility, at regular intervals during the study. The disease control (DC) group and the treatment groups have shown an increase in the paw volume after the FCA inoculation. In treatment groups, the paw inflammation gradually reduced upon treatment with NRG gel and NRG niogel, separately to the groups, and in the DC group, it was sustained throughout the study period. Changes in the paw volume are depicted in the chart in Figure 5

3.3.1 Cytokine's assessment

Assessment of cytokine levels in the serum can give an understanding of the disease condition. There will be an accumulation of pro-inflammatory factors and infiltration of leucocytes at the synovial joint during RA condition. Serum cytokine levels were estimated using ELISA. The IL-6, and TNF-α levels in DC and treated groups were compared to levels in NC. It was observed that treated groups had lower levels of cytokines than DC, the level that indicates the disease severity, and the nearer it comes to the values of normal indicates the effectiveness of treatment. Significance values showing the comparison of levels between NC and DC were indicated with *p<0.05; **p<0.01; ***p<0.001. Comparison between DC and TG (Treatment Groups) were indicated as $^{\&}p$<0.05; $^{\&\&}p$<0.001; $^{\&\&\&}p$<0.0001. IL-6, and TNF-α levels have shown a superior reduction in TG in the order NRG Niogel>NRG gel enabling the comprehension of NRG's effectiveness in niosomal topical administration for the management of RA.

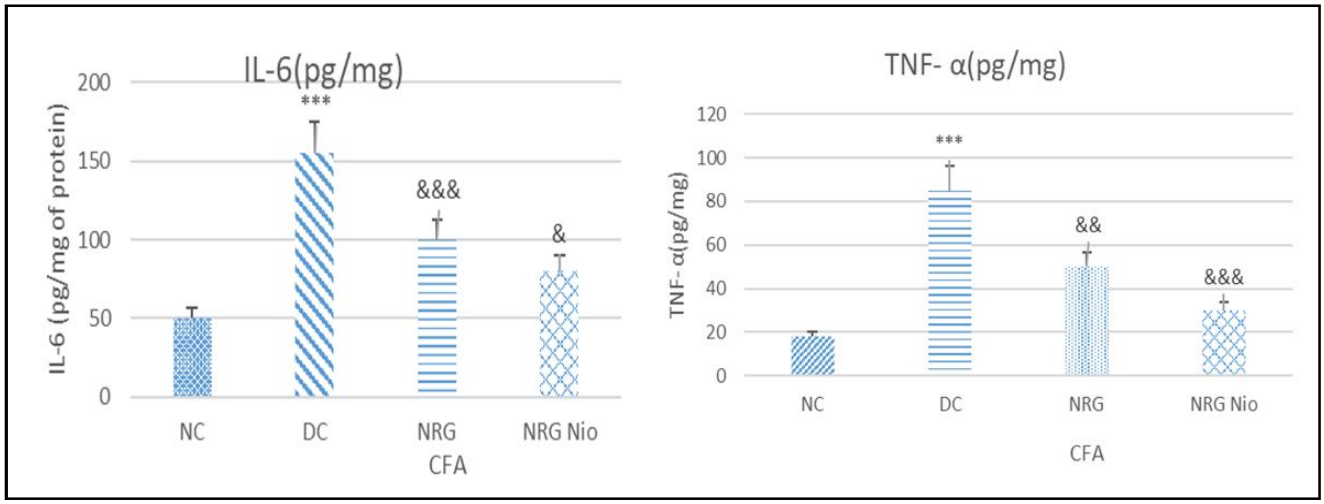

Figure 6: Effect of NRG gel and NRG niogel on cytokine levels. All values were mean ± SEM (n=3).

3.3.2 Histopathological examination

Synovial joint and bone tissue was extracted from sacrificed animal at the end of study period and a histopathological study was conducted by microtome sectioning and H&E staining for microscopic observations which has revealed normal bone anatomy in NC and DC showed synovial hyperplasia, a profuse infiltration of inflammatory cells to the joint site and severe cartilage degeneration, major hallmarks showing RA. In the treated groups, group-3 exhibited signs of reversal, reduction in joint gaps, cell infiltration, and a rejuvenation sign as cell proliferation. In group-4, these signs were much prominent indicating the treatment efficiency.

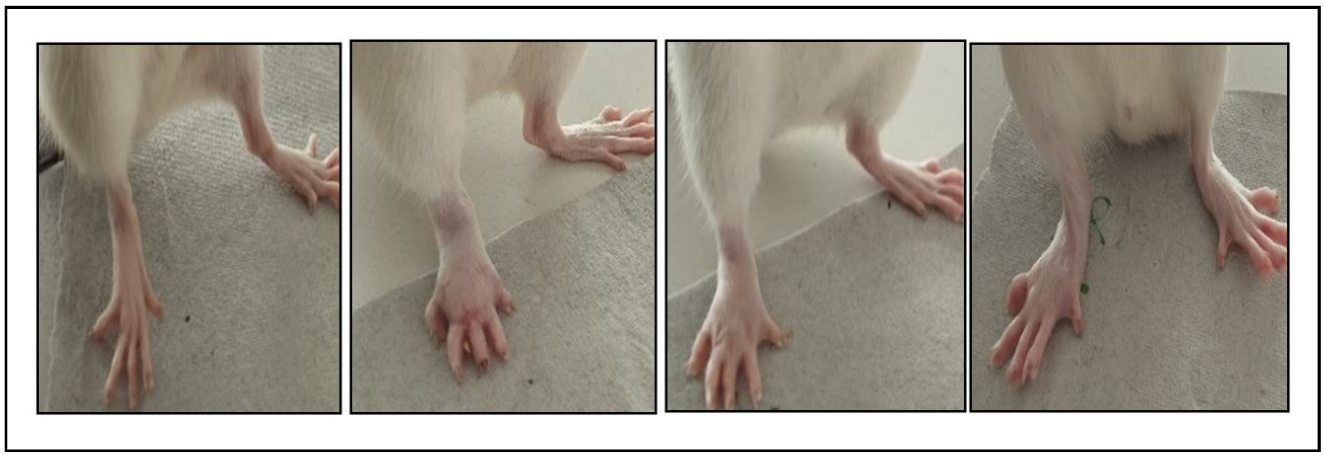

Figure 7: Images of sham control animal paw vs disease control showing inflamed paw and treated groups, with free NRG gel and NRG niogel.

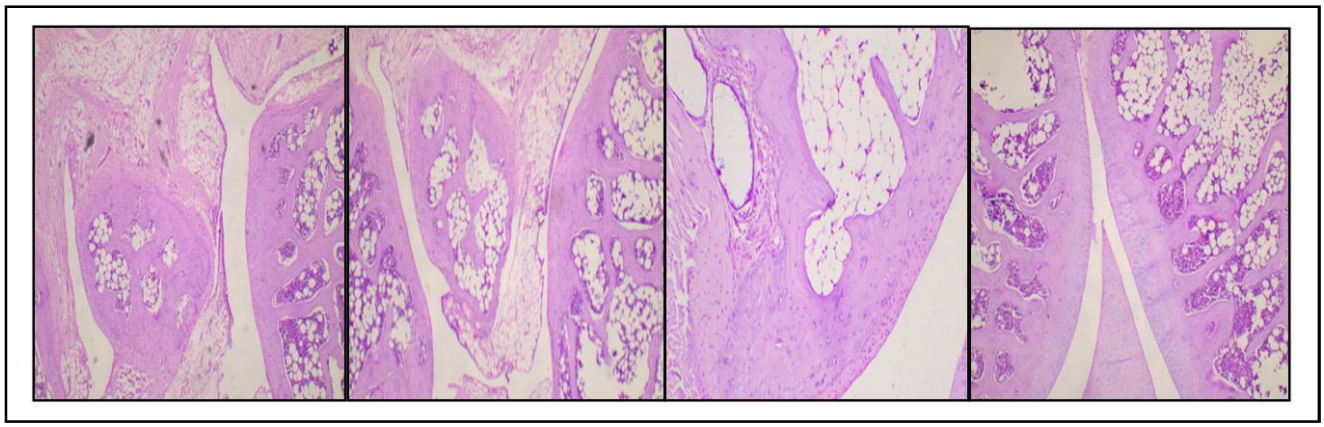

Figure 8: Images of H&E stained histopathological analysis of knee joint of normal control, disease control, free NRG gel treated and NRG niogel treated rat model.

4. Discussion

Naringenin, a potential antiarthritic drug, has a limitation of oral bioavailability because of its low aqueous solubility and rapid metabolism by liver microsomal enzymes. Successful attempts were made to improve its therapeutic efficacy by formulating in to nanocrystals and evaluated the effectiveness in arthritis management (Guangshuai *et al*., 2021). The present study was conducted to formulate and evaluate NRG niosomes for topical administration. The niosomes fabrication includes a pre-requisite step, to plan the formulation components.

Ethanol is comparatively safer than methanol or chloroform or any other organic solvent used to solubilize the surfactant, lipid, and drug (Smix) and the Smix in the study, as is soluble in ethanol, the solvent selected for both the methods employed for the fabrication of niosomes of NRG is ethanol. Span 60 is observed to have a high transition temperature (Tc), and HLB value of around 4.7 which is most favourable for forming rigid and stable niosomes that are less prone to leakage and fusion and have greater EE. Span 60 exhibits good compatibility with cholesterol, the other component in the niosome preparation, and also shows an enhanced skin permeability which is a requirement for the topical application. Pluronic F127, used at a concentration of 1% of hydration volume, acts as a copolymer and helps to improve niosomes formation and thereby the yield and also enhances the stability, EE, and drug permeability through the skin. Cholesterol contributes to the structural integrity and stabilizes the vesicular bilayer of nonionic surfactant by intercalating between the molecules, imparts rigidity thereby reduces fluidity and diffusibility of vesicles and hence modulates entrapment efficacy and drug loading capacity.

Niosomes were prepared by two methods, TFH and EIM, ethanol injection is a modified ether injection method. The smaller size of niosomes formed by EIM may be due to the reason that when an organic phase is introduced into a counter phase, instantly breaks down into smaller particles to minimize the surface interactions between the phases, thus acting as a compulsive force, whereas the vesicles formed in the TFH method are by solid dispersion wherein the thin film formed by evaporation of organic solvent over the inner wall of the round bottom flask will disperse into the hydration volumes passively during the second step of TFH and may require a further step of sonication to reduce the vesicles to nano size. The results find support from the previous research publication on the preparation and evaluation of niosomes (Negi *et al*., 2017). An increase in cholesterol concentration has decreased the vesicle size which can be for a reason that the rigidity imparted has created more compact vesicles with lesser curvature. Cholesterol imparts rigidity, minimizes

the leakiness, and hence increases retention of the drug, but excess cholesterol would take away the drug's place in the surfactant bilayer and thereby reduces EE. The negative charge on zeta potential can be due to the greater adsorption of hydroxyl ions on the outer core of the vesicles. The vesicles formed were spherical and were showing lamellarity. In niosomes, inside of the lipid bilayer is an aqueous core, where the hydrophilic molecules find their place and lipophilic one's get embedded in to lipid membrane. As cholesterol forms an integral component in to surfactant bilayer, excess amount of cholesterol occupies the major slots in the membrane, leaving less scope for the drug for entrapment.

The drug release data from *in vitro* studies of NRG niosomes and NRG solution have shown a demarcation, and the enhanced release effects can be attributed to that quality of niosomes, which are effectively designed for better penetration ability and deformable vesicular structures that effectively release the drug entrapped at the effective sites. Niosomes embedded in to the hydrogel have shown a prolonged and sustained release in *ex vivo* studies explaining the retention of drug in different skin layers and acting as a depot for slow and extended drug activity.

IL-6 and TNF-α are pro-inflammatory cytokines, the elevation of these factors indicates the severity of the disease. The higher the significance of the difference in the levels of this factor, the more effective the treatment modality. The results obtained were in correlation with the histopathology study. A re-established microenvironment of the synovium in the treatment groups, more significantly in NRG niogel and to a lesser extent in NRG niosomes. The infiltration of inflammatory cells, degradation of cartilage, are among the hallmarks of the disease and the rejuvenation was indicated by reformed synovial membrane and cartilage and reduction in the synovial gap. These results assure that NRG niogel provides a promising strategy for rheumatoid arthritis treatment.

5. Conclusion

The study displays the effectiveness of naringenin via topical route in RA management. Niosomes fabricated with NRG has improved the treatment efficiency by enhancing the permeation and thereby its availability at target site in a sustained fashion for a prolonged period, maybe for a reason that the skin layer acts as a depot. Niosomal systems known for their biocompatibility, serve as efficient nanocarriers for the drug to be transported across the skin layers and hence improve the therapeutic outcomes. This was supported by *in vivo* studies as there was a significant improvement in RA condition in the NRG niogel treated group. In conclusion, Naringenin, a phytocompound, in a nanovesicular topical delivery system proves effective in RA treatment, hence finds scope as NRG niogel formulation for being a suitable translational strategy.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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