Development of nanostructured lipid carriers loaded caffeic acid topical cream for prevention of inflammation in wistar rat model

Prateeksha Prakash Kamath1,†, Rutu Rajeevan1,†, Swastika Maity2, Yogendra Nayak2, Reema Narayan1, Chetan Hasmukh Mehta1, Varalakshmi Velagacherla1, Anjaneyulu Konuri1, Usha Yogendra Nayak1,‡

1Department of Pharmaceutics, Manipal College of Pharmaceutical Sciences, Manipal Academy of Higher Education, Manipal, Karnataka 576104, India. 2Department of Pharmacology, Manipal College of Pharmaceutical Sciences, Manipal Academy of Higher Education, Manipal, Karnataka 576104, India. 3Department of Anatomy, Manipal-TATA Medical College, Jamshedpur, Manipal Academy of Higher Education, Manipal, India.

ARTICLE INFO
Received on: 09/06/2022
Accepted on: 15/09/2022
Available Online: XX

Key words: Caffeic acid, nanostructured lipid carriers, topical cream, anti-inflammatory, skin irritation.

ABSTRACT
Inflammation is a defensive response against invasion by foreign bodies, which need to be treated with safer molecules such as phytomolecules like caffeic acid (CA). This work aimed to formulate and evaluate topical cream with CA-loaded nanostructured lipid carriers (NLCs) for anti-inflammatory action. CA-loaded NLCs were formulated by solvent diffusion method followed by preparation of NLCs loaded cream which was evaluated and characterized for various parameters including in-vitro release behavior and in-vivo acute anti-inflammatory effect in carrageenan-induced rat paw edema wistar rats model. CA-loaded NLCs showed good results for particle size, polydispersity index (PDI), zeta potential, and encapsulation efficiency, while 0.5% w/w cream had a significant anti-inflammatory effect when tested for the acute study. Furthermore, skin-irritation study was carried out on wistar rats and it was observed that the formulated creams did not produce any signs of irritation, edema, and erythema compared to the positive control group (0.9% v/v formalin group). Therefore, formulated NLCs loaded cream can be used as a safe and efficient acute anti-inflammatory agent and considered an alternative treatment strategy to existing formulations.

INTRODUCTION
Inflammation is a defensive response against invasion by foreign bodies like bacteria, parasites, viruses, or mechanical skin injury (Medzhitov, 2008). Increased vascular permeability, accelerated blood flow, and nerve fiber sensitization is associated with swelling, redness, and pain (Cavaillon, 2021). Nonsteroidal anti-inflammatory drugs (NSAIDs) are the approved class of anti-inflammatory agents, blocks cyclooxygenases (COX) enzyme, and inhibit prostaglandin and thromboxane production, to reduces pain, fever, and inflammation (Botz et al., 2017). However, the oral administration produces severe ulcers and hence topical formulations of NSAIDs were tried in the past, especially for joint pain and inflammation (Baranowski et al., 2018). Furthermore, considering the adverse effects of currently used NSAIDs, natural phytomolecules from edible plants and plant products prove to be a safer option. Most of natural compounds are inhibiting COX as well as the nuclear factor inflammatory pathway. However, phytomolecules cannot be patented and will not be of interest in pharmaceutical industries; hence, most of their applications are limited to cosmeceutical and nutraceutical products (de Lima Cherubim et al., 2020; Vamanu, 2019). Some of the potent natural compounds with anti-inflammatory activity show poor solubility, instability, reduced skin permeation, and low skin retention time.

Caffeic acid (CA) is a phytomolecule proven to show anti-inflammatory and antinociceptive effects upon oral administration (Mehrotra et al., 2011). The anti-inflammatory activity of CA is attributed to inhibition of nitric oxide, prostaglandin E2, and significantly decreased tumor necrosis factor-alpha (TNF-α) release. Overall, the anti-inflammatory properties of CA is result
from the inhibition of induced nitric oxide, COX-2, and TNF-α expression through the down-regulation of NF-κB binding activity. However, the topical anti-inflammatory effect of CA is not explored and CA incorporated in cream lacks substantial evidence (Spagnol et al., 2017). Hence, it was attempted to study the topical effect of CA in the form of cream and study its formulation stabilities. Further to increase the efficacy, with good hydration effect and improved skin penetration, the CA was loaded in nanostructured lipid carriers (NLCs) and incorporated into cream.

Lipid-based drug delivery systems are gaining popularity in recent years as they are very promising. Liposomal CA was formulated to enhance its permeation as a photoprotective agent (Katuwavila et al., 2016). Lipid nanoparticles are potentially used over the liposomes to have better control over the drug release (Puri et al., 2009). The first generation solid lipid nanoparticles (SLNs) have increased biocompatibility, scale-up, and protect the active ingredients from chemical degeneration. Hallan et al. (2021) formulated SLNs and ethosomes of CA for skin delivery. However, they had certain limitations like large particle size (>200 nm) and low drug loading ranging from 1% to 11%. In general, SLNs have drug expulsion during storage. Moreover, the study was limited to in vitro permeation of native SLNs or ethosomes without using any other semisolid carrier (Hallan et al., 2021). In comparison to SLNs and ethosomes, NLCs have several advantages such as a distorted crystal structure that prevents drug expulsion on storage, low toxicity, stable physical and chemical properties, and organized targeting. NLCs have higher drug loading potential due to the presence of liquid oil droplets in a solid matrix and in addition to this, it also enables strong immobilization of therapeutic drugs and prevents particle coalescence. NLCs will have less particle size which can help in faster permeation of the nanoparticles and thus faster effect can be achieved. NLCs provide prolonged action without causing irritation to the skin. It is also used extensively in dermal and cosmetic products as it produces skin hydration, smoothness, occlusion, and skin penetration (Ravaomanarivo et al., 2014). Therefore, CA loaded NLCs were prepared in the present study by varying the formulation parameters and incorporated into cream. Incorporating NLCs in the cream will provide prolonged retention time for the NLCs to permeate across the skin. The formulated NLCs loaded cream were subjected for various in vitro characterization and in vivo anti-inflammatory activity.

MATERIALS AND METHODS

Materials

The caffeic acid (CA) and Tween 80 were purchased from Merck Life Science Pvt. Ltd., Mumbai, India. Compritol® 888 ATO was procured from Gattefosse India Pvt. Ltd., Mumbai, India. Poloxamer 407 and Oleic acid were procured from Sigma Aldrich, Mumbai, India. Kolliream® OD was obtained as a gift sample from BASF India Ltd., Navi Mumbai, India. All other chemicals and solvents were of analytical grade.

Methods

Preparation of NLCs

Compritol and oleic acid were accurately weighed in a beaker and heated in the water bath (Equitron Pvt. Ltd, Mumbai) at 80 ± 2°C. CA was dissolved into the 1 ml acetone and ethanol (50:50) and added to the above-molten mixture. The aqueous phase was prepared by mixing poloxamer 407 and tween 80 in the ratio of 1:1 in 9.7 ml of water and heated up to 80°C in a water bath. The aqueous phase was added to the lipidic phase, and the mixture was probe sonicated (Sonic & Materials Inc., USA) for 10 minutes at 40% amplitude and pulse of 10 seconds (on) and 2 seconds (off).

After sonication, the prepared formulation was kept for magnetic stirring (Remi equipment Ltd, Bangalore) for 24 hours to remove the organic solvent (Aliashgarh et al., 2016). The composition for the NLCs preparation is shown in Table 1. The prepared NLCs formulation was subjected for lyophilization without cryoprotectant which included two steps, pre-prozen using deep freezer at −75°C to −80°C for 12 hours followed by freeze drying (Christ Freezedryer Alpha 2 LD plus) at −48°C at 10 mtorr pressure for 48 hours (Abdelwahid et al., 2006; Gao et al., 2007; Sahu et al., 2016).

Evaluation and characterization of NLCs

Particle size, PDI and zeta potential

The NLCs formulation was suitably diluted with milli-Q water and analyzed for its particle size, PDI and zeta potential by dynamic light scattering technique using Malvern Zetasizer Nano ZS (Malvern Instruments, UK) at 25°C ± 1°C. The analysis was performed in triplicates and results are shown as mean and standard deviation (Mehta et al., 2019).

Entrapment efficacy

Encapsulation efficiency was analyzed by using the Sephadex G-50 gel chromatographic separation method. The prepared Sephadex column (2.5 and 1 cm, length and internal diameter, respectively) was used for the separation of free drug from the encapsulated drug. The formulation was passed through the Sephadex column, and the filtered formulation was suitably diluted and used for the drug analysis by UV spectrophotometer (UV-Visible spectrophotometer, UV1601PC, Shimadzu, Japan) at 286.5 nm. The same procedure was followed for blank formulation and checked its absorbance to examine the presence of any lipid interference (Managuli et al., 2019).

Table 1. Composition and Characterization of NLCs

<table>
<thead>
<tr>
<th>Trial No.</th>
<th>CA (mg)</th>
<th>Compritol (mg)</th>
<th>Oleic acid (mg)</th>
<th>Poloxamer 407 (mg)</th>
<th>Tween 80 (mg)</th>
<th>Particle size (nm)</th>
<th>PDI</th>
<th>Zeta potential (mV)</th>
<th>Encapsulation efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>105</td>
<td>45</td>
<td>100</td>
<td>100</td>
<td>116.1 ± 1.2</td>
<td>0.374 ± 0.02</td>
<td>−15.20 ± 2.3</td>
<td>66.23 ± 1.63%</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>105</td>
<td>45</td>
<td>100</td>
<td>100</td>
<td>174.0 ± 2.6</td>
<td>0.28 ± 0.001</td>
<td>−5.55 ± 1.60</td>
<td>52.14 ± 2.30%</td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>105</td>
<td>45</td>
<td>100</td>
<td>100</td>
<td>214.4 ± 1.9</td>
<td>0.435 ± 0.03</td>
<td>−2.28 ± 1.06</td>
<td>46.38 ± 2.45%</td>
</tr>
</tbody>
</table>
**Surface morphology**

The morphology of the NLCs was determined by Atomic Force Microscopy (AFM) using the tapping mode method and transmission electron microscopy (TEM). In case of AFM study, a drop of each NLCs dispersion was added on a coverslip and allowed to dry in a vacuum oven at 35°C till it forms the thin and dried film. The dried thin film was analyzed using AFM with required modification (Bruker Innova, Bruker corporations, MA) (Lima et al., 2020). TEM was used for analysing the morphology of the NLCs by negative staining of thin film of NLCs dispersion with 0.2% w/v sodium phosphotungstate solution and tissue paper was used for wiping the excess solution. The sample was air dried and analyzed at 1,000,000× (Instrument -Jeol/JEM 2100) of 200 kV operating voltage (Mehta et al., 2021).

**Solid-state characterization**

**Fourier transform infrared spectroscopy (FTIR)**

FTIR spectroscopy of pure drug (CA), lyophilized blank and CA loaded NLCs were performed by using FTIR spectrophotometer equipped with an ATR accessory (Bruker ALPHA II FTIR spectrophotometer). The procedure includes the sample was placed on the ATR crystal, and little pressure was applied from the top to keep the sample in contact with the crystal. The spectra was analyzed in the range of 4,000–500 cm⁻¹ with an accumulation of 36 scans (Santos et al., 2018).

**Differential scanning calorimetry (DSC)**

DSC was performed for the lyophilized NLCs using DSC-60 Plus, Shimadzu Corporation, Japan. The samples were hermetically sealed in aluminium pans and were exposed to heat in the presence of nitrogen with a flow rate of 10 ml/minute at 100°C/minute heating rate from 0°C to 300°C, and the thermograms were recorded (Mehta et al., 2019).

**Preparation of cream**

**Preparation of CA loaded cream**

The lipid phase comprising of beeswax (0.283 g), Kollicream® OD (0.815 g) and borax (0.216 g) were weighed accurately, added to a china dish and heated up to 70°C. The CA was added to the molten lipidic phase and stirred properly to get mixed. The distilled water (0.5 ml), the aqueous phase was also heated up to 70°C and added slowly into the lipidic phase and stirred till it formed the required consistency. As the efficacy of topical CA is not proven earlier, three potential dosages for the topical CA cream 0.5% w/w, 1.0% w/w, and 1.5% w/w were prepared and compared.

**Preparation of CA encapsulated NLCs loaded cream**

The lipid phase comprising of beeswax (0.283 g), Kollicream® OD (0.815 g) and borax (0.216 g) were weighed accurately, added to a china dish and heated up to 70°C. The distilled water (0.5 ml), the aqueous phase, was also heated up to 70°C and added slowly into the lipidic phase. Before reaching room temperature, the lyophilized NLCs were added and stirred till they formed the homogeneous cream-like consistency (Remi equipment Ltd, Bangalore) (Khan et al., 2020).

**Evaluation and characterization of CA loaded and ca encapsulated NLCs loaded cream**

**Homogeneity and appearance**

For any product homogeneity and its appearance is the primary requirement to market the same. Hence, the formulated cream was inspected for color, clarity, presence of particles by visualization method. Preparation and evaluation of polyherbal cosmetic cream (Aswal et al., 2013; Gupta et al., 2015).

**pH**

The suspension form of a cream was prepared by dispersing the 1 g of cream in 10 ml of distilled water to check the pH of the cream using pH meter (Systronics μ pH System 361). (Purushothamrao et al., 2010).

**Viscosity**

Viscosity was analyzed by using Brookefield viscometer (LVDP-II+P, Brookefield Engineering Laboratories, MA). The cream was kept in the viscosity measurement pan, and the spindle (spindle no. 96) was rotated at different RPM based on the requirement. The viscosity was measured at room temperature. The experiments were performed in triplicates (Khan et al., 2014).

**Spreadability**

The spreadability of the cream was checked by the glass or petri dish method. In this method, an accurately weighed quantity of cream was kept on the centre of a prepared circle on the petri dish, and then another petri dish was kept on the first, followed by pressing it with 500 g of weight for 5 minutes. The increase in the area of the circle was measured, and spreadability was calculated (Kumar et al., 2011).

**Centrifugation test**

The 2 g of cream was filled in ependrops and centrifuged (Sigma Labort Centrifuge GmbH, Germany) for 10 minutes at 5,000 rpm at room temperature and evaluated any signs of phase separation, which indicates the cream stability.

**Drug content and content uniformity**

Three different samples of 200 mg were taken from different parts of the cream formulation and added into three Eppendorf. 2 ml of methanol was added and kept for bath sonication (Ultrasonic bath sonicator, Oscar micro clean-103, Growell Instruments, Bangalore) for 2 hours. The sonicated solution was centrifuged at 10,000 rpm for 10 minutes, and the supernatant was separated. The supernatant was analyzed for the presence of a drug in cream loaded with drug and NLCs using UV spectrophotometer (UV-Visible spectrophotometer, UV1601PC, Shimadzu, Japan) at 286.5 nm. The analysis was carried out in triplicates (n = 3) (Aswal et al., 2013; Gupta et al., 2015).

**In vitro drug diffusion studies**

The in vitro drug release studies were performed using Franz diffusion apparatus (Orchid Scientifics, Maharashtra, India). The dialysis membrane (Sigma Aldrich, 12 KDa) was soaked overnight and used for release studies. The membrane was sandwiched between the donor and receptor compartment. The cream (0.5 g) was kept in the donor compartment, whereas the receptor compartment was filled with pH 5.5 phosphate buffer under magnetic stirring at 800 rpm. 1 ml of sample was removed at
various time intervals and same volume of buffer was replenished to maintain the sink condition. The samples were analyzed using UV spectrophotometer (UV-Visible spectrophotometer, UV1601PC, Shimadzu, Japan) at 286.5 nm (Sathe et al., 2019).

**Accelerated stability studies**

Accelerated stability studies were performed for pure drug (CA) loaded cream as well as the CA encapsulated NLCs loaded cream as per the guidelines of ICH. The samples were packed in glass vials, sealed with a rubber closure, and crimped with aluminum caps. The packed and sealed samples were stored at 25 ± 2°C/60 ± 5% RH and 40 ± 2°C/75 ± 5% RH for 1 month. The stored stability samples were removed at different time intervals and analyzed for appearance, viscosity, spreadability, pH, and drug content (Asif Dosani et al., 2011; Yadav et al., 2014).

**Anti-inflammatory activity in wistar rats**

**Procurement of rats**

The protocol for the study was approved by the Institutional Animal Ethics Committee. Female wistar rats weighing 160–210 g were selected for this study. The rats were procured from Central Animal Research Facility and were housed three rats per cage, water and food ad libitum with 12 hours day and night light exposure. The rats were acclimatized for 7 days before the conduction of the study. Based on the literature and the preliminary studies conducted, three formulations of CA loaded cream with 0.5% w/w, 1.0% w/w, and 1.5% w/w CA and NLCs loaded with CA 0.5% were selected for the acute inflammation study.

**Carrageenan induced inflammation in wistar rats**

In this model, carrageenan was used to induce paw edema in rats and compared with the normal rats which was injected with saline at the right hind paw plantar region. The group of rats injected saline act as normal control (NC). The group of rats injected only carrageenan (50 µl of 1% w/v, s.c., plantar) act as disease control (DC). The rats of the treatment group (T) were injected carrageenan to the right hind paw 30 minutes after the topical application of prepared formulations. The standard control (SC) group of rats applied diclofenac 1% w/w gel (Chaudhari and Baviskar, 2020). The groups T1, T2, and T3 rats applied topically 0.5% w/w, 1.0% w/w, 1.5% w/w CA cream, and the NLCs loaded CA (0.5%) cream at 2 cm² area on the right hind paw, respectively. The rats in the NC group are applied only cream base. The paw volume was recorded using a plethysmometer for each 0, 30, 60 and 120 minutes after injection of carrageenan. The percentage inhibition paw volume was calculated by using the equation, % inhibition of paw volume = [(Vc − Vf) / Vc] * 100; where Vc represents the average paw volume of control and Vf represents the average paw volume of the test (Ranegra et al., 2019). After the estimation of the paw inflammation and edema, the rats were sacrificed by euthanizing methods as per the guidelines of Euthanasia of Animals 2020 by using an overdose of ketamine and their right hind paw dorsal skin was isolated from the body and fixed in 10% formal saline and embedded in paraffin for histopathological analysis.

**Skin irritation test in wistar rats**

If there is no serious reaction observed during the conduction of acute inflammatory test of the topical agent; furthermore, irritation test will be conducted to confirm the safety of the topical cream (Padol et al., 2011).

In this study, 0.9% formalin was used as skin irritant for female wistar rats. The groupings of rats and the treatment was the same as that of considered in the previous study. However, in this study, the NLCs and the cream was applied at 1 cm² area on the shaved back skin of the rat where formalin was injected after 30 minutes (Elimian and Eze, 2019). The rats were observed over 7 days for signs of edema and erythema. Draize evaluation of dermal reactions was used for scoring the rats. The scoring for edema was done as 0, 1, 2, 3, and 4 as none, well defined, moderate and severe, respectively, while scoring for erythema was done as 0, 1, 2, 3, and 4 as none, well defined, moderate and formation of scar, respectively.

The primary irritation index was calculated by using the following formula:

Primary irritation index (PII) = (Score of erythema + Score of edema) / Total score

Based on the PII results, the cream was categorized into various categories such as non-irritating (PII < 0.5), slightly irritating (PII 0.5 to 2), moderately irritating (PII 2.1 to 5), and severely irritating (above 5) (Mutalik et al., 2006; Saraf and Kaur, 2010).

**Statistical analysis**

Data expressed as mean ± SD and compared using one-way analysis of variance (ANOVA) for swelling ratio, with Dunnett’s multiple comparisons test for percentage inhibition of inflammation. p < 0.05 to be considered significant. All the statistical analysis was carried out in GraphPad Prism trial version 7.

**RESULTS AND DISCUSSION**

**Preparation of CA loaded and NLCs loaded cream**

Three potential dosages for the topical CA cream 0.5% w/w, 1.0% w/w, and 1.5% w/w were compared. CA was formulated as NLCs and evaluated for various characterization parameters. The most potent dose of CA based on pharmacological evaluation was formulated as NLCs based cream to check whether it was able to produce a synergistic effect due to modification in the formulation. This paper highlights CA in the form of NLC based cream, as a topical anti-inflammatory agent for inflammatory conditions.

**Evaluation and characterization of CA Loaded NLCs**

**Particle size, PDI, and zeta potential of NLCs**

The particle size, PDI, and zeta potential of the NLCs were found to be in the range of 116.1 ± 1.20 to 214.4 ± 1.9 nm, 0.28 ± 0.001 to 0.435 ± 0.03, −2.28 ± 1.06 to −15.2 ± 2.3 mV, respectively. The composition of the NLCs formulation and the particle size, PDI and zeta potential for the same are shown in Table 1. Particle size is one of the important factors for the determination of actual effect of the active moiety. Generally, the
active moiety or the nanoparticles with smaller particle sizes easily helps to penetrate the skin membrane and show their activity. Also, it was observed or proved that the particle size of NLCs less than 500 nm is ideal for penetrating the layers of skin. The zeta potential is also another important factor that is responsible for the stability of the nanoparticles, and the higher the zeta potential value, the higher the stability of the lipidic nanoparticles as there are fewer chances of nanoparticle aggregation due to electric repulsion among like charges. The negatively charged particle can penetrate the skin to a greater extent (Correia et al., 2004; Gonzalez-Mira et al., 2010; Kohli and Alpar, 2004).

**Entrapment efficacy**

The encapsulation efficiency of the NLCs formulations were found to be in the range of 46.38 ± 2.45% to 66.23 ± 1.63%. In the case of solid lipid nanoparticles, almost similar drug entrapment efficiency reported by Fathi et al. (2013). The encapsulation efficiency of the prepared formulation is shown in Table 1. The less encapsulation of the CA in the NLCs may be due to less logP value of CA (logP 1.15), which indicates the slightly hydrophilic and hydrophobic nature, and thus it is found to be satisfactory encapsulation as NLCs proved that it is a good carrier for water-insoluble or poorly aqueous soluble moieties. Surfactants and other ingredients added in the formulation also has some ability to increase the encapsulation of the drug inside the NLCs carrier (Dikmen et al., 2015). The absorbance of blank formulation was also analyzed which showed that there was no interference of lipid observed during the UV analysis so we have proceeded with the same procedure for further analysis.

**Solid-state characterization of NLCs**

**FTIR**

The pure drug (CA), CA loaded lyophilized NLCs are shown in Figure 1 with comparisons. The pure drug, CA showed characteristic spectral peaks at 647.83 and 807.40, 1,111.04, 1,212.23 and 1,273.69, 1,445.56 and 1,606.59, 1,644.5 cm⁻¹, intense bands between 2,300 to 4,000 cm⁻¹ (2,339.47 and 2,386.44 cm⁻¹) represents the in-plane bending vibrations of carbonyl group, in-pane and out-of-plane C-C-C bending vibrations of aromatic ring, in-plane beding vibrations of aromatic and olefinic C-H bonds and C-C stretching vibrations, very strong carbonyl group stretching vibrations and intense vibrations of -OH stretching vibrations partly overloaded with weak C-H stretching vibrations of acyclic chain and benzene moiety. The CA loaded NLCs showed characteristics, spectral bands at 1,100.85, 1,280.58, 1,459.70, 1,520.94, 1,634.82, and 3,437.70 cm⁻¹. The FTIR spectrum of pure drug (CA) and CA loaded NLCs, and it can be concluded that there was less or negligible disappearance of some of the drug characteristics, which may be due to slight interactions between the drug and lipid moieties while it also showed the incorporation of drug inside the NLCs moiety (Catauro et al., 2020; Wang et al., 2020).

**DSC**

Thermal analysis by DSC indicated the sharp endothermic peak of CA at 222.4°C (Pinho et al., 2014). The presence of a sharp endothermic peak at 68.75°C and 39.83°C in drug-loaded NLCs indicates the presence of compritol and poloxamer-407 in their crystalline form, respectively (El-Badry et al., 2013; Xu Xuefan et al., 2011). The DSC thermogram of the pure drug (CA) and drug-loaded NLCs (DL-NLCs) is shown in Fig. 2. DSC results showed the absence of endothermic peak of the drug in drug-loaded NLCs, indicating the conversion of crystalline to amorphous form and homogeneous solubilization of drug in the lipid-solid matrix (Shah et al., 2016).

**Surface morphology**

The surface morphology was analyzed by AFM, which provides information about the 2D and 3D images of nanoparticle surface texture at different magnification. An important step in getting proper 2D or 3D images in preparation of samples slide with less aggregation of the particles in NLCs dispersion. AFM (a) and TEM (b) images of CA loaded NLCs is shown in Figure 3. Surface morphology of NLCs was analyzed by using AFM, which showed the presence of spherical structures with uniform size distribution in the dispersion with required stability and it was also confirmed by performing the TEM analysis. The TEM analysis showed the formation spherical shaped lipidic nanoparticles, and its size obtained with TEM analysis resembles the values obtained with zeta size.

**Evaluation and characterization of CA loaded cream and CA encapsulated NLCs loaded cream**

The NLCs encapsulated with CA was successfully incorporated into cream, which was evaluated and characterized for various parameters.

![Figure 1. FTIR spectrum of pure drug (CA) and DL-NLCs.](image)
Homogeneity and appearance

The appearance of cream was found to be creamy, smoothy, and light-yellowish, which may be due to the color of the drug. The prepared cream was consistent and homogeneous in nature (Aswal et al., 2013; Gupta et al., 2015).

pH

The formulation prepared for the skin application should have the proper pH according to the skin, such as pH 5.5 (human skin pH). The pH of the CA loaded cream, and CA encapsulated NLCs loaded cream was 5.38 ± 0.03 and 5.73 ± 0.05, respectively. The pH of the cream was found to be nearer to the skin pH. Hence, it can be concluded that the prepared cream can be compatible with the skin and non-irritating (Peana et al., 2009; Purushothamrao et al., 2010).

Viscosity

The viscosity of the CA loaded cream and CA encapsulated NLCs loaded cream was 11583.76 ± 21.21 and 9282.50 ± 31.82 cps, respectively. Based on the viscosity results, it was found that the NLCs loaded cream have required viscosity, which indicates the ease in application with less shear or pressure (Khan et al., 2014).

Spreadability

The proper application and its spreadability are some of the important parameters to assess any topical formulation. The majorly topical formulation should spread quickly with less or negligible shear or pressure on the skin. After placing the load, the increase in diameter of the circle drawn on petri dish was 3.18 ± 0.18 and 3.34 ± 0.10 cm (29.3 ± 0.3 and 31.9 ± 0.5 mm), respectively, while the spreadability factor of the CA loaded cream and CA encapsulated NLCs loaded cream was 0.64 ± 0.04 g.cm/s and 0.67 ± 0.02 g.cm/s, respectively. The spreadability studies results proved that cream has the required spreadability with ease in application on the skin (Kumar et al., 2011).

Centrifugation test

After centrifugation for 10 minutes at 5,000 rpm, none of the cream showed any sign of phase separation which test showed that formulated cream was stable on mechanical shaking and during transportation for a longer time.

Drug content and content uniformity

The drug content and content uniformity of the 0.5%, 1.0%, and 1.5% of the caffeic loaded cream and caffeic encapsulated NLCs loaded cream was 100 ± 1.75%, 99.20 ± 2.14%, 99.52 ± 0.87% and 93.47 ± 1.63%, respectively. Based on the drug content and content uniformity, the formulated cream showed uniform distribution of drug in the NLCs, and CA loaded cream.

In-vitro drug diffusion study

The CA loaded cream showed rapid release of the CA compared to the NLC loaded cream. The 0.5% CA-loaded cream showed 77.14 ± 10.82% of release when compared to 1.5% CA-loaded cream, which showed almost 100% release in 3.5 hours. Drug release profile of pure drug (CA) loaded cream and CA encapsulated NLCs loaded cream with different concentration in phosphate buffer (pH 5.5) are shown in Figure 4. In vitro drug release or diffusion, the study showed that drug loading significantly influenced drug release. NLCs also showed higher release in the cream loaded with more amount drug. As the amount of drug loading in NLCs cream was increased, the drug release was also found to be improved. However, the sustained release of CA in NLCs loaded cream compared to CA-loaded cream could be due to the presence of CA in the lipid matrix of NLCs, which diffused out slowly (Sathe et al., 2019).

Accelerated stability studies

The samples subjected for accelerated stability studies were removed at different time intervals and examined for various parameters such as homogeneity, appearance, viscosity, spreadability, pH, and drug content (Results are shown in Supplementary Table S1). Based on the results obtained from accelerated stability studies, it was concluded that prepared cream formulations were stable at accelerated stability conditions and for a longer time, thus can be considered as the stable formulation as there were significantly less or negligible changes in the cream properties when compared to initial cream samples (at 0th Day) (Maru Avish and Lahoti Swaroop, 2019).
Anti-inflammatory activity in wistar rats

Effect of CA loaded NLCs cream on carrageenan induced inflammation in wistar rats

Carrageenan solution induced inflammation on rat paws. The inflammation was remarkably attenuated by the local application of the CA test formulation T1 (0.5% w/w), T2 (1.0% w/w), and T3 (1.5% w/w). The one-way ANOVA of swelling ratio was plotted at $p < 0.05$, and calculation of column statistics of inflammation ratio showed that T1 dose had only 15.53 ± 0.32%, T2 had 22.82 ± 1.40%, and T3 had inflammation ratio of 20.86 ± 1.11 by the end of 120 minutes (2 hours). NLCs loaded cream showed a significant percentage of inflammation ratio 41.14 ± 7.77 as compared to the control group. To estimate the percentage inhibition of edema, a digital Vernier calliper was used to measure the edema in the right paw of the rats, and the measurement was taken in millimeters (mm). Multiple comparison test by two-way ANOVA for percentage inhibition of edema method was used for statistical analysis of the results. DC was used as the control group for the estimation of the results. The anti-inflammatory activity (% inhibition of paw volume and edema) of the pure drug cream and NLCs loaded cream is shown in Table 2.

Based on the anti-inflammatory study in wistar rats, it indicates T1 showed a good reduction in swelling and inflammation ratio and T2 showed a fair decrease in inflammation ratio as compared to the NC group. Based on the swelling ratio and percentage of inflammation ratio, it was identified that the T1 dose of 0.5% w/w is the most effective way to reduce the swelling condition. Therefore, the NLC of 0.5% w/w was prepared and tested in animals. In addition to this, the T1 group showed significant inhibition of edema at 0, 30, 60, and 120 minutes. In comparison, T2 and T3 showed significant edema inhibition towards the 120th minute. NLC loaded dose of 0.5% w/w of CA formulation showed a significant reduction in edema toward the 120th minutes. As per the skin visualization using histopathological study, it was found that the NC group did not show any changes in the skin structure, while the DC group showed major changes in the skin layers, which suggested successful development of inflammation. When different formulations were compared, it was found that there were no changes observed in skin layers for group T1 while significant differences were observed in group T2 and T3, which finalize that the 0.5% of CA can be considered as safe and incorporated into the NLCs for preparation of NLCs loaded cream. NLCs loaded cream formulation can be regarded as the alternative formulation for delivering the CA for a longer duration.

Histopathology analysis of the anti-inflammation study

The histopathology of paw skin of rats on the 7th day of the experiment is shown in Figure 5. DC groups showed ruptured epidermis with increased layers of cells consisting of 3–8 layers (epidermal hyperplasia), scab formation, and granulation in tissue with ruptured vessels. In addition this, the presence of more keratin was observed in stratum corneum (hyperkeratosis) with few inflammatory cells in dermis and congested blood vessels. NC groups showed intact epidermis and dermis layer, no scabs on the skin layer, and no granule formation in the dermis layer of the skin. The epidermis was thin with 1–2 cell layers and presence of small amount of keratin was observed. There was no inflammatory cells were observed in normal control group. SC group showed small scabs, small epidermis rupture, and minor granulation in the skin visualization using histopathological study, it was found that the NC group did not show any changes in the skin structure, while the DC group showed major changes in the skin layers, which suggested successful development of inflammation. When different formulations were compared, it was found that there were no changes observed in skin layers for group T1 while significant differences were observed in group T2 and T3, which finalize that the 0.5% of CA can be considered as safe and incorporated into the NLCs for preparation of NLCs loaded cream. NLCs loaded cream formulation can be regarded as the alternative formulation for delivering the CA for a longer duration.

Table 2. Anti-inflammatory activity (% inhibition of paw volume and edema) of the pure drug cream and NLCs loaded cream.

<table>
<thead>
<tr>
<th>Test details</th>
<th>% Inhibition paw volume</th>
<th>% Inhibition paw edema (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 minutes</td>
<td>60 minutes</td>
</tr>
<tr>
<td>NC</td>
<td>12.80 ± 0.00</td>
<td>-</td>
</tr>
<tr>
<td>DC</td>
<td>10.52 ± 0.72</td>
<td>24.92 ± 0.01</td>
</tr>
<tr>
<td>SC</td>
<td>10.52 ± 0.72</td>
<td>11.34 ± 0.38</td>
</tr>
<tr>
<td>T1 (CA 0.5% cream)</td>
<td>15.53 ± 0.32*</td>
<td>21.35 ± 0.04</td>
</tr>
<tr>
<td>T2 (CA 1% cream)</td>
<td>22.82 ± 1.40</td>
<td>16.43 ± 0.14</td>
</tr>
<tr>
<td>T3 (CA 1.5% cream)</td>
<td>20.86 ± 1.11</td>
<td>15.81 ± 0.16</td>
</tr>
<tr>
<td>NLCs (CA 0.5%)</td>
<td>41.14 ± 7.77*</td>
<td>19.44 ± 0.07</td>
</tr>
</tbody>
</table>

Values are mean ± SD ($n = 6$).

*Signifies that the data is statistically significant when compared to the % inhibition ration at 30 minutes; $p < 0.05$ when compared with NC was used as control group.

NC: Normal control; DC: Disease control; SC: Standard control; T1, T2, and T3 are and NLCs are formulations with 0.5% w/v, 0.1% w/v, 1.5% w/v, and 0.5% w/v CA, respectively; Results are analyzed by one-way ANOVA.
dermis layer of the skin. In addition to this, there was reduction in epidermal thickness and keratin in stratum corneum. T1 group showed no scabs in the skin layer, intact epidermis and dermis layer and normal tissue structure and blood vessels in the dermis layer. T2 showed ruptured epidermis and scab formation in the skin. Large granulation was observed in the dermis layer of the skin. T3 group showed small scabs and abrupt epidermis with ruptured blood vessels in dermis. Granulation were also observed in the tissue. NLCs group did not show any rupture of epidermis and dermis layer. No scabs or granulation were observed. As with application of 0.5% w/w, CA cream did not show presence of scabs with maintaining the structure of the epidermis, dermis layer, and also the blood vessels present. This concentration can be considered as safe and hence selected for incorporating into the NLCs formulation. NLCs loaded formulation can be considered as the alternative formulation for delivering the CA for longer duration.

**Primary in vivo skin irritation study**

Wistar rats were used for primary skin irritation studies of the NLCs loaded cream compared with positive control and control. Table 3 shows the values of the primary irritation index (PII). The scores obtained for erythema and edema showed that the formulated creams and control groups were significantly less ($p < 0.05$) compared to that of positive control. The PII value for the positive control group (PII = 2.34 ± 0.20) was found to be more when compared to the control group (0.00 ± 0.00), while the PII values for prepared formulation groups were found to be 0.00 ± 0.00 when compared to positive control. The images of the rat skin as control, positive control, and treated with different formulated creams are shown in Figure 6.

*Figure 5. The histopathology of paw-skin of rats on the 7th day of the experiment. *All the images were captured at a magnification of 10× and 40×
In vivo skin irritation study showed no signs of irritation, redness, and inflammation for up to seven days for the formulation and control groups compared to the positive control group. The scores obtained for erythema and edema showed that the formulated creams and control groups were significantly less ($p < 0.05$) than the positive control. Based on the observed results, it was shown that the prepared optimized cream formulation showed no or negligible signs of erythema, edema and irritation when compared to the positive control group (0.9% formalin as standard irritant) which showed moderate to a higher level of edema, irritation, and erythema. Based on Draize test score, the formulated creams can be considered nonirritant as their values are less than 0.5. Thus, it can be regarded as stable and safe for skin application.

Table 3. Primary skin irritation studies of formulated creams.

<table>
<thead>
<tr>
<th>Animal groups</th>
<th>Reaction grade observed</th>
<th>PIH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Erythema</td>
<td>Edema</td>
</tr>
<tr>
<td>I—Control</td>
<td>0.00 ± 0.00*</td>
<td>0.00 ± 0.00*</td>
</tr>
<tr>
<td>II—Disease Control (Positive Control)</td>
<td>1.99 ± 0.33</td>
<td>2.53 ± 0.21</td>
</tr>
<tr>
<td>III—Blank Cream Base</td>
<td>0.00 ± 0.00*</td>
<td>0.00 ± 0.00*</td>
</tr>
<tr>
<td>IV—CA Loaded Cream</td>
<td>0.00 ± 0.00*</td>
<td>0.00 ± 0.00*</td>
</tr>
<tr>
<td>V—Blank NLCs Loaded Cream</td>
<td>0.0 ± 0.00*</td>
<td>0.00 ± 0.00*</td>
</tr>
<tr>
<td>VI—CA encapsulated NLCs loaded cream</td>
<td>0.00 ± 0.00*</td>
<td>0.00 ± 0.00*</td>
</tr>
</tbody>
</table>

*Animals ($n = 6$) considered, and results showed as mean ± SD; Significant ($p = 0.05$) compared to positive control.

Figure 6. In vivo skin irritation studies.
CONCLUSION

The present study demonstrated topical anti-inflammatory efficacy of CA and successful formulation development of NLCs loaded cream. The cream containing 0.5% CA showed significant anti-inflammatory activity in the acute model. The NLC loaded cream exhibited a sustained release profile compared to drug-loaded cream and proved that NLC loaded cream showed an anti-inflammatory effect for a longer duration. Formulated creams were safe and stable on the skin based on the skin irritation study, which showed no signs of erythema, edema, and irritation than the positive control (0.9% formalin as a standard irritant), which showed no signs of erythema as a moderate sign of irritation, erythema and edema. 0.5% w/w CA was found to be safe for topical administration. Thus CA NLCs loaded cream can be used as a safe alternative to existing formulations.

ACKNOWLEDGMENT

The authors are grateful to BASF, Mumbai, India, for providing a gift sample of Kollicream® OD.

CREDIT AUTHORSHIP CONTRIBUTION

PPK and RR (equally contributed): Conceptualization, Methodology, Validation, Draft writing and Editing; SM: Methodology (in vivo), Validation, analysis, Resources; YN: Methodology, Resources, Draft writing, editing; RN: Methodology (in vitro), Result analysis, CHM: Methodology (in vitro/in vivo), Draft writing; VV: Methodology (analytical), AK: Methodology (histopathology); UYN: Conceptualization, Resources, supervising; Data analyzing; Draft writing, editing.

CONFLICT OF INTEREST

All authors declare that there is no conflict of interest in publishing the data generated.

ETHICAL APPROVALS

This work includes the animal study for which approval was obtained from Institutional Animal Ethics Committee (IAEC/KMC/65/2019).

FUNDING

There is no funding to report.

DATA AVAILABILITY

All data generated and analyzed are included within this research article.

PUBLISHER’S NOTE

This journal remains neutral with regard to jurisdictional claims in published institutional affiliation.

REFERENCES


How to cite this article:
# SUPPLEMENTARY MATERIAL

## Table S1. Accelerated stability studies.

<table>
<thead>
<tr>
<th>Stability conditions</th>
<th>Properties</th>
<th>0th day</th>
<th>15th day</th>
<th>30th day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CA loaded cream</td>
<td>NLCs loaded cream</td>
<td>CA loaded cream</td>
<td>NLCs loaded cream</td>
</tr>
<tr>
<td>Homogeneity</td>
<td>Homogeneous</td>
<td>Homogeneous</td>
<td>Homogeneous</td>
<td>Homogeneous</td>
</tr>
<tr>
<td>Appearance</td>
<td>Smooth, creamy and light-yellowish</td>
<td>Smooth, creamy and light-yellowish</td>
<td>Smooth, creamy and light-yellowish</td>
<td>Smooth, creamy and light-yellowish</td>
</tr>
<tr>
<td>Viscosity (cps)</td>
<td>11,583.76 ± 21.21</td>
<td>9,282.5 ± 31.82</td>
<td>11,513.5 ± 26.16</td>
<td>9,078 ± 63.64</td>
</tr>
<tr>
<td>pH</td>
<td>3.18 ± 0.18</td>
<td>3.34 ± 0.10</td>
<td>2.89 ± 0.05</td>
<td>3.22 ± 0.05</td>
</tr>
<tr>
<td>Drug content (%)</td>
<td>100 ± 1.75</td>
<td>93.47 ± 1.06</td>
<td>97.18 ± 6.38</td>
<td>92.99 ± 7.36</td>
</tr>
<tr>
<td>Homogeneity</td>
<td>Homogeneous</td>
<td>Homogeneous</td>
<td>Homogeneous</td>
<td>Homogeneous</td>
</tr>
<tr>
<td>Appearance</td>
<td>Smooth, creamy and light-yellowish</td>
<td>Smooth, creamy and light-yellowish</td>
<td>Smooth, creamy and light-yellowish</td>
<td>Smooth, creamy and light-yellowish</td>
</tr>
<tr>
<td>Viscosity (cps)</td>
<td>11,583.76 ± 21.21</td>
<td>9,282.5 ± 31.82</td>
<td>11,259.5 ± 89.80</td>
<td>9,159.5 ± 79.90</td>
</tr>
<tr>
<td>pH</td>
<td>3.18 ± 0.18</td>
<td>3.34 ± 0.10</td>
<td>3.04 ± 0.11</td>
<td>3.24 ± 0.04</td>
</tr>
<tr>
<td>Drug content (%)</td>
<td>100 ± 1.75</td>
<td>93.47 ± 1.06</td>
<td>99.49 ± 4.48</td>
<td>90.21 ± 6.96</td>
</tr>
</tbody>
</table>