Quercetin Loaded Nanostructured Lipid Carriers-based Gel for Rheumatoid Arthritis: Formulation, Characterization and \textit{in vivo} Evaluation

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Received September 21, 2017; accepted October 6, 2017

ABSTRACT

Present research work describes the development of potential topical treatment containing nanostructured lipid carriers (NLCs) for rheumatoid arthritis (RA). Quercetin (QCT) is a renowned flavonol useful as model drug for carriers. QCT loaded NLCs were prepared and evaluated for particle size distribution, polydispersity index, zeta potential analysis, \textit{in vitro} drug release study. \textit{Ex vivo} study was carried out to evaluate the effect of NLCs on cell proliferation (HIG-82 cell line) and inflammation (TNF-α induction in RAW264.7 cells). The QCT-NLCs showed mean particle size of 155.6 ± 1.8 nm and polydispersity index (PDI) was 0.236 ± 0.4, entrapment efficiency of 95.63 ± 0.14 % and zeta potential of -27 ± 1.2 mV. For the ease of application, NLCs were incorporated into the gel base and final formulation was evaluated for rheological study, texture profile, drug release and antiarthritic activity. QCT-NLC gel showed pseudo plastic flow behavior with excellent texture profile parameters. \textit{In vitro} drug release studies showed that, QCT-NLC gel has more prominent permeation profile as compared with QCT-loaded gel. \textit{In vivo} activity was carried out using Complete Freund’s adjuvant (CFA) induced arthritic model. Evaluation of the severity of rheumatoid arthritis was done by measurements of hind paw volume, arthritis score and haematological parameters such as rheumatoid factor (RF), C-reactive protein (CRP), red blood cells (RBCs), white blood cells (WBCs), erythrocyte sedimentation rate (ESR) and hemoglobin (Hb). Edema and erythema were not observed after administration of QCT-NLC- gel on the rat skin. In conclusion, the results of \textit{in vitro} and \textit{ex vivo} studies, QCT-NLC gel appears a viable formulation system for topical delivery of QCT in the treatment of RA.

KEYWORDS: Quercetin; Nanostructured lipid carriers; HIG-82; Rheumatoid arthritis.

Introduction

In the contemporary expertise, drug delivery systems are being developed using nanotechnology. Amongst all, nanocarriers based drug delivery system is rapidly emerging technology to enhance the therapeutic effect of the drug. It can overcome the difficulties with solubility, penetration, target specificity and bioavailability of the drugs. Therefore, with the above considerations the use of nanoparticulate drug delivery system like lipid nanoparticles can be an excellent approach to design a formulation system. NLCs are colloidal particles that exhibit a size range of 100 - 400 nm. In addition to the advantages of colloidal drug carrier systems like liposomes, polymeric nanoparticles, emulsions, NLCs avoid or minimize the drawbacks such as stability, target specificity etc. (Muller et al., 2002; Joshi and Muller, 2009). NLCs have advantage due to their solubility enhancement, well-established safety profiles, skin occlusive effect, variety of routes of administration, improved properties for drug loading, modulation of the delivery profile, stable drug incorporation throughout the storage period, low toxicity, biodegradability, drug protection and avoidance of organic solvents during manufacturing (Ali et al., 2012).

Rheumatoid arthritis is a chronic, inflammatory autoimmune disease that progressively destroys the synovial membrane, cartilage and bone. It constitutes a profound and uncertain clinical problem even though significant progress has been made in the management of the disease. Important factors in the pathogenesis of RA are TNF-α activity, abnormal antibody production, circulating autoantibodies i.e. ‘rheumatoid factor’ and abnormalities in synovial tissue. Amongst all, the cytokines like TNF-α and interleukins have significant role in the disease progression (Choy, 2012; McInnes and Schett, 2007).

Conventional treatment of RA with NSAIDs (non steroidal anti-inflammatory drugs) and steroids exhibit adverse effects such as stomach upset, nephrotoxicity, iron deficiency anemia, protein loss, toxicity and a low therapeutic index (Sivasudha et al., 2013). Furthermore these treatments don’t show any prevention of tissue damage, mobility or bone destruction. Disease-modifying
anti-rheumatic drugs (DMARDs), monoclonal antibodies (MAbs) may provide symptomatic relief and slowdown the progression of the disease, but they have disadvantages such as high cost, lack of specificity, immunosuppressive effect. As a result, the treatment of RA is a difficult challenge in rheumatology.

Quercetin (QCT) is a well-known flavonol present in commonly consumed foods including apples, citrus fruits, grapes, onion, garlic, tea, red wine, nuts, seeds and vegetables. QCT inhibits TNF-α and nitric oxide and can help in the management of oxidative stress related chronic diseases like arthritis, inflammation and diabetes (Kaul et al., 1985). The therapeutic value of QCT is limited by poor absorption from the GIT, low skin penetration, low solubility (7.7 µg/mL in water, 5.5 µg/mL in simulated gastric fluid), rapid excretion from the body, resulting in low absorption in vivo (Li et al., 2009; Khaled et al., 2003; Gugler et al., 1975). Limited bioavailability of QCT renders poor efficacy in its clinical applications.

In this study, we sought to prepare a novel formulation of QCT with NLCs for improved drug delivery and increase therapeutic efficacy. The QCT loaded NLCs were tested for its topical application against RA in vitro, ex vivo and in vivo in CFA-induced arthritis in rat models.

Materials and Methods

Reagents

QCT was obtained from Otto chemie (Mumbai, India), Glyceryl mono stearate (GMS) was obtained from Fine Chem Industries (Mumbai, India), oleic acid was obtained from Loba Chemie Pvt. Ltd. (Mumbai, India), tween 80 was obtained from Siaco Research Laboratories Pvt. Ltd. (Mumbai, India) and soya lecithin was obtained from Cargill Deutschland GmbH (Krefeld, Germany). All other chemicals and reagents used were of analytical grade and GRAS category.

Formulation of NLCs

In the present study, GMS (solid lipid) and oleic acid (liquid lipid) were selected with the help of lipid-screening tests. The rational was to choose those lipids that could effectively dissolve QCT, also suitable for the topical use. Tween 80 and soya lecithin were selected as a surfactant and stabilizer respectively. NLCs were prepared by hot high pressure homogenization technique (Li et al 2012). Briefly, the lipid phase containing GMS and oleic acid was heated up to 80°C in which QCT is dissolved, to obtain a clear homogenous lipid phase. Meanwhile, an aqueous surfactant solution with tween 80 and soya lecithin was prepared and heated at the same temperature. The hot surfactant solution was then dispersed drop by drop in the hot lipid phase under mechanical stirring (Remi Instruments Ltd, Mumbai, India) at 2000 rpm for 15 min. The resultant emulsion was homogenized at 80°C, using high pressure homogenizer (PANDA 2K, Niro Soavi, Italy) under a pressure of 600 bar and eight homogenization cycles. Finally, the homogenized hot o/w emulsion was cooled to 4±0.5°C, so that the recrystallization of the lipid can occur and QCT-NLCs can form (Souto et al, 2010). The QCT-NLCs were separated and collected by the centrifugation (Optima “MAX-XP” ultracentrifuge, Beckman Coulter, Nyon, Switzerland) at 50,000 rpm for 30 min at room temperature. The NLCs precipitates were collected and redispersed in the small amount of water.

Mannitol (5%) was used in the lyophilization process as a cryoprotectant. NLC suspension was frozen in an aqueous mannitol solution under −70°C using a refrigerator for 12 h and then lyophilized using lyophilizer (Vir-Tis Benchtop, SP Scientific, Warminster, PA). The lyophilization time was controlled in 72 h to get the NLCs powder which was collected and used for further experiments (Liu et al, 2010).

Characterization of NLCs

Particle size, PDI and zeta potential were determined by photon correlation spectroscopy (PCS) using a Malvern Zeta sizer (Nano ZS 90, Malvern Ltd., Malvern, UK). The mean particle size and PDI values were obtained at an angle of 90° using disposable polystyrene cells with 10 mm diameter, which were equilibrated for 120 s. For zeta potential, the dip cells were used for the measurements with the field strength of 20 V/cm and the average of the zeta potential was given from 30 runs. Prior to the measurements, all samples were diluted with double-distilled water to produce a suitable scattering intensity. All measurements were performed in triplicate at 25°C (n=3) (Liu et al, 2010, Madane and Mahajan, 2016).

Morphology and structure of the NLCs were studied using transmission electron microscopy (TEM) (Jeol/JEM 2100, USA). The TEM was equipped with digital imaging software to perform observations. About 1 mL of sample was dropped in the specimen place and covered with a 400 mesh grid. After 1 min, 1 mL of uranyl acetate was dropped on top of the grid, and this sample was allowed to dry for 30 min before observation under the electron microscope. This procedure was used to confirm the particle size of NLCs as measured using the particle size analyzer.

For determination of percentage drug entrapment efficiency (%EE), QCT-NLCs dispersion was centrifuged at 50,000 rpm for 30 min; 1.0 mL of the supernatant collected, diluted suitably with methanol and absorbance was measured spectrophotometrically at 258 nm using a UV– Visible spectrophotometer (UV 1700, Shimadzu, Kyoto, Japan) (Liu et al 2010). The % EE was calculated using the following equation:

\[
\%\ \text{EE} = \frac{\text{Amount of drug added} - \text{Amount of drug in supernatant}}{\text{Amount of drug added}} \times 100
\]

Differential Scanning Calorimetry

Thermal analysis was performed using a differential scanning calorimetry (DSC) (Mettler-Toledo, Greifensee, Switzerland). DSC thermograms were recorded for pure
QCT, solid lipid- GMS and QCT-NLCs. The samples, weighing 2 mg, were analyzed in sealed and pin-holed standard 40 µL aluminum pans, with a heating rate of 10°C/min from 30°C to 400°C and during the measurement; the sample cell was continuously purged with nitrogen at a flow rate of 40 mL/min.

X-ray Diffraction Studies

X-ray diffraction (XRD) patterns of QCT, solid lipid-GMS, physical mixture for NLCs and QCT-NLCs formulation were obtained using the X-ray diffractometer (Brucker Axs, D8 Advance, Karlsruhe, Germany) in which the Cu-Kα line was used as a source of radiation. Voltage of 40 kV and current 30 mA was applied. All samples were measured with 2θ and 20° with a scanning rate of 3°/min and a step size of 0.02°.

Ex vivo Cell Line Study

Synoviocyte proliferation: To study the effect of QCT-NLCs on proliferation of the cells, synoviocytes (HIG-82 cell lines, fibroblast cells, isolated from the intrarticular soft tissue from the knee joint of a rabbit) were used. The media used for culturing the cell line was Ham’s F12 medium 90% with 10% foetal bovine serum (FBS). Cell proliferation was determined by MTT [(3-(4,5- dimethylthiazolyl-2)-2, 5-di phenyl-tetrazolium bromide)] assay, which helps in evaluating cell metabolic activities.

On day one, 1,500–2,000 synoviocytes were plated per well on a 96-well plate, leaving the first column blank. The plate was placed back into the 37°C in CO2 incubator. The following day, QCT, QCT-NLCs and DCS (diclofenac sodium) were added at various concentrations of 20, 40, 60, 80 and 100 µM. The second column was kept untreated for control. The cells were exposed to cell culture media for up to 48 h. At the end of the exposure period, MTT was added (50 µg/well) and allowed to incubate for 4 h at 37°C. The medium was then aspirated and 200 µL of DMSO (dimethyl sulphoxide) was added. The plate was agitated for 30 min and the absorbance was measured at 562 nm. The graph was plotted as concentration of sample versus % cell viability (Burt et al, 2006). The results were expressed as mean ± SD (n=3).

Effect on production of TNF-α(tumor necrosis factor-α):

To study the effect of QCT-NLCs on production of TNF-α, RAW264.7 cells (macrophage cells isolated from the blood of the mouse) were used. The media used for culturing the cell line was DMEM (Dulbecco’s Modified Eagle’s Medium - high glucose) + 2mM Glutamine + 10% FBS. The cells were exposed to cell culture media for up to 48 h. At the end of the exposure period, MTT was added (50 µg/well) and allowed to incubate for 4 h at 37°C. The medium was then aspirated and 200 µL of DMSO (dimethyl sulphoxide) was added. The plate was agitated for 30 min and the absorbance was measured at 562 nm. The graph was plotted as concentration of sample versus % cell viability (Burt et al, 2006). The results were expressed as mean ± SD (n=3).

Preparation of NLC-gel formulation: On the basis of compatibility with NLC dispersion, feel and ease of spreadability, Carbopol 940 was selected as the gelling agent. About 1% (w/w) concentration of Carbopol 940 chosen for further studies which has shown the optimum viscosity. Carbopol 940 was firstly dispersed in purified water and subsequently, NLCs (freeze dried NLC powder) with the amount equivalent to the dose of drug, incorporated into the blank gel using a high speed stirrer at 2000 rpm for 2 h. pH of NLC-gel was adjusted by using triethanolamine. The formed gel was ultrasonicated for 15 min and left equilibrating for 24 h at room temperature.

Characterization of Gel

pH of the gel: Topical gel should be physiologically compatible and non-irritant. Incompliance with the pH may contribute to irritation. Hence, maintenance of the pH of the gel formulation is necessary. pH of the gel was determined by pH meter (µ pH system, 362, Systronics, Ltd., India) standardized using pH 4.0 and 7.0 standard buffers before use. Measurements were made in triplicate (n=3).

Drug content: For determination of drug content, about 1 g of the QCT-NLC gel equivalent to 10 mg of QCT, was weighed in a 100 mL volumetric flask and dissolved in 50 mL of methanol. The volumetric flask was kept for 2 h in an orbital shaker (CIS-24, Remi Instruments Ltd., India) to mix it properly. Then it was diluted appropriately and analyzed by UV-spectrophotometer at 258 nm. Experiments were performed in triplicate (n=3).

Rheological measurements: Brookfield Viscometer (DV-E Brookfield Engineering Labs Inc, Middleboro, MA, USA) was used to study the rheological behavior of gel. About 30 g sample was placed in a beaker and allowed to equilibrate for 5 min. Measurements were carried out by using spindle no.8 7 at 5, 10, 20, 30, 60 and 100 rpm. At each speed, the corresponding dial reading of torque and rpm was noted. Viscosity measurement of sample at each speed was carried out in triplicate (n=3).

Texture profile analysis: Texture profile analysis is a method to determine mechanical properties of the gel such as consistency, firmness, cohesiveness (attractive forces within the formulation) and work of adhesion (attraction between formulation and substrate), gumminess, deformation at hardness and springiness. The measurements were done by using a Brookfield CT3 Texture analyzer (Brookfield Engineering Labs Inc, Middleboro, USA) in TPA mode. A conical shape sample holder was filled evenly with the NLC-gel (Hongtao Li et al. 2007, Foegeding et al., 2011). The probe (TA3/100) was programmed to move down into the sample at a speed of 0.5 mm/s with a target value 20 mm and then go up back at the same speed to its original position. The force encountered by the probe to break away from the gel when starting to ascend (the point of maximum force) was measured. The TPA characteristics of the sample were evaluated from the resultant force–time curve. This
particular and suitable method uses a small quantity of sample and provides a large amount of data pertaining to the physical properties of the semisolid formulations in a desired form. Analysis was performed in triplicate (n=3).

**Stability study of gel:** Stability is mainly evaluated to make sure that the quality of the product will be retained throughout its shelf life. Poor storage stability is an important drawback of nanodispersion. To improve the storage stability, NLC dispersion is normally transformed into semisolid gel formulation. Stability study of gel was carried out in accordance to the ICH guidelines. Stability of QCT-NLC gel was assessed at 30 ± 2°C/ 65 ± 5% RH for a period of 6 months. Samples were withdrawn at predetermined intervals: 0, 60, 120, 180 days. Physicochemical parameters such as color, viscosity, phase separation, pH and drug content were evaluated. All the measurements were performed in triplicate (n=3).

**Ex vivo permeation studies:** Wistar rat skin was prepared appropriate for the ex vivo permeation study by cleaning the surface of the excised skin and clipping of the hairs along with removal of the subdermal fat and fascia. Further the skin was hydrated with phosphate buffer pH 5.8 for 1 h and mounted on Franz diffusion cell (cell volume 10 mL) with stratum corneum facing upwards. The receptor compartment was filled with phosphate buffer pH 5.8 and the assembly was maintained at 37°C ± 0.5 under constant magnetic stirring. A dose of QCT-NLC gel equivalent to 10 mg of QCT was applied to the membrane in the donor compartment and covered with aluminum foil to prevent from drying. About 0.5 µL samples were withdrawn through the sampling port of the diffusion cell at predetermined time intervals over a period of 24 h (0, 2, 4, 6, 8, 10, 12 and 24 h). The buffer was immediately replenished with 0.5 µL of fresh buffer (Bhalekar et al, 2015, Madne and Mahajan 2014). The permeability coefficient was calculated by using the following formula:

$$J_{SS} = \frac{dc}{dt} \cdot \frac{C_o \times A}{C_{i} \times A}$$

Where,
- $J_{SS}$ - permeability coefficient
- $C_o$ - initial concentration in the donor compartment
- $A$ - area of mucosal surface and
- $dc/dt$ - rate of permeability

**Antiarthritic Activity**

**Complete freund's adjuvant (CFA) model:** Wistar rats were divided in 4 groups. Each group contains six rats. Arthritis was induced by the 0.1 mL CFA intradermally. Group 1- treated with CFA, Group 2- treated with QCT-gel (10 mg/kg), Group 3- treated with QCT-NLC gel (10 mg/kg), Group 4- treated with DCS gel (10 mg/kg) (Patil et al, 2011) (Bhalekar et al, 2015)

**Evaluation of the Severity of Arthritis**

**Measurements of hind paw volume:** The arthritic lesions i.e. paw volumes of injected and non-injected paws were measured using a digital plethysmometer. The lesions were measured again on the 7th, 14th and 21st days after injection of the adjuvant. During the experimental period, the body weight was measured using a digital weighing balance every 3rd day after adjuvant injection.

**Arthritis scoring system:** The severity of arthritis was recorded by a blinded observer using the visual arthritis scoring system. The arthritis score ranged from 0 to 4, where 0 indicates the least but definite swelling and 4 represents the maximum swelling. This scoring system involves observations of all four paws and giving a separate score for each limb. Scores were assigned for evaluation of the pain associated with the arthritis.

**Measurement of haematological parameters:** Haematological parameters such as rheumatoid factor, C-reactive protein, RBCs, WBCs, erythrocyte sedimentation rate and hemoglobin were evaluated by routine laboratory methods using commercial kits according to the manufacturer's instructions (Patil et al, 2011 and Bhalekar et al, 2015).

**Skin Irritation Study**

Possibility of skin irritation with QCT gel and QCT-NLC gel was evaluated by carrying out skin irritation test on Wistar rats (Draize et al., 1944; Pople & Singh, 2006). These rats were acclimatized to the conditions for seven days before the commencement of the study. Hairs were depleted from the back side of rats with the help of depilatories, 4 h prior to the experiment and the area was marked on both sides. One side served as the control while the other side served as the test. The rats were divided into four groups each containing 3 rats. Gel (500mg/rat) was applied once a day for seven days, and skin irritation from the formulation was determined by observations of any skin sensitivity and reactions such as redness, edema, and skin rash. The skin irritation effect of the gel was graded as: A-no reaction; B-slight, patchy erythema; C-moderate but patchy erythema; D-moderate erythema, and E-severe erythema with or without edema (Williams AC et al,1991)

**Data analysis:** All experiments were done in triplicate (n=3) and the data were expressed as mean value ± standard deviation. Statistical data analyses were performed using Student's t-test and one-way ANOVA. A value of p <0.05 was considered statistically significant.

**Results and Discussion**

**Formulation of QCT-NLCs**

The QCT-NLCs were prepared using hot high-pressure homogenization technique. For the preparation of QCT-NLCs, GMS was selected as a solid lipid and oleic acid as a liquid lipid. Tween 80 and soya lecithin were selected as a surfactant and a stabilizer, respectively. The balance of emulsifiers is required at the oil–water interface for the stability of dispersions. Hot high-pressure homogenization method was simple and quick at laboratory scale as there is no use of organic solvents in the development of formulation. Therefore, for the preparation of QCT-NLCs, hot high-pressure homogenization technique was selected, which has shown highest drug loading capacity and entrapment efficiency.
Characterization of NLCs

The average particle size of optimized batch of NLCs was found to be 155.6 ± 1.8 nm and PDI is 0.236±0.4. The particles having average diameter up to 200 nm could be easily transported through transdermal route. In addition, PDI measures the width of particle size distribution. PDI lower than 0.5, indicates high homogeneity of the particles in the formulation, whereas high PDI values suggest a broad size distribution. The value of PDI near to zero indicates higher homogeneity between the particles (Madne and Mahajan, 2016). As per the size and PDI, small sized NLCs which can cover the skin surface more as compared to larger particle size were preferred as optimized batch for incorporation in to gel formulation.

The zeta potential value -27±1.2 mV indicates the better stability of the formulation. The use of non-ionic surfactant, Tween 80, might be responsible for the low zeta potential. In addition, oleic acid contains negatively charged free fatty acid that contributed to the negative zeta potential.

The morphology of QCT-NLCs was analyzed using TEM and result is shown in Fig 1. The particles were found to be distributed evenly and the particle size was uniform. Also, were spherical and with even surface and non-adherent to each other on a scale of 200 nm. The small particle size and uniform size distribution of QCT-NLCs was appropriate for the development of nanofomulation.

The percentage entrapment efficiency (% EE) of QCT-NLCs was found to be increased by increasing the amount of total lipid (Solid and liquid lipid) whereas, increasing only the liquid lipid concentration, %EE was decreased. This may be due to lipid precipitation, which occurs during particle production (Aggarwal et al., 2003). When NLC formulation was cooled, recrystallization of lipids occurs resulting into a drug-free core or a core with the reduced drug content. Therefore, increase in the lipid beyond certain extent leads to poor % EE. Additionally, a significant effect was observed with surfactant- Tween-80. The %EE was decreased by increasing the Tween 80 concentration. Drug entrapment efficiency in NLCs was found to be 95.63±0.14 % (w/w), which was selected for in vitro drug release.

Differential Scanning Calorimetry

DSC is a fundamental technique used to study the crystalline or amorphous state of drug in the formulation by determining the variation of temperature and energy at phase transition. Also DSC studies were performed to confirm the absence of drug-excipient interaction. DSC thermograms of QCT, GMS and QCT-NLCs were shown in Fig. 2 (A). DSC thermogram has shown a peak at 317.2°C, 59.6°C and 166.7°C for QCT (a), GMS (b) and QCT-NLCs (c) respectively. The area under the curve for the QCT-NLCs thermogram was less as compared to that of QCT alone. The possible mechanism is may be the melting of the lipid components and their interactions with QCT. The entire disappearance of QCT endothermal peak was observed. The probable phenomenon can be assumed as evidence of interactions between the components of the carrier system and the molecular inclusion of QCT in the melted lipid matrix i.e GMS and oleic acid. This outcome clearly suggests that there is an amorphization of QCT and/or formation of inclusion composite.

X-ray Diffraction Studies

XRD was used to study the nature of lipid, drug-loaded NLCs. Sharp high intensity reflections are generated by highly crystalline lipids whereas low intensity reflections were created by amorphous background of imperfect lattice of lipids. Lipids which are mixtures of mono-, di-, and triglycerides and containing fatty acids of different chain lengths like GMS usually forms crystals with many imperfections, offering space to accommodate more drug molecules. Fig 2 (B) shows the XRD diffractograms of QCT (a), GMS (b) and QCT-NLCs (c). Pure QCT reflections were sharp with high intensity, whereas lyophilized QCT- NLCs has shown low intensity diffractograms which clearly demonstrate the amorphous nature of NLCs.

Ex vivo Cell Line Study

Synoviocyte proliferation: MTT assay determines the cell viability, which is directly proportional to the degree of cell proliferation. Proliferation of HIG-82 cells was studied at various concentrations of QCT, QCT-NLCs and DCF (Diclofenac sodium). The cell proliferation inhibitory effect of QCT-NLC occurred at low (micromolar) concentration and with the IC_{50} of 42±0.2 μM (Fig. 3 A). QCT-NLCs showed significantly higher inhibition rates and markedly higher suppression efficiency than QCT solution. It may be possible because of improved dispersity and stability of QCT-NLCs than QCT. Also the use of lipid contents in the formulation could facilitate superior cellular uptake.

Effect of QCT-NLCs on the production TNF-α in RAW264.7 cells: TNF-α plays an important role in the cytokine network with respect to the pathogenesis of various inflammatory diseases including RA. It controls the proliferation of fibroblast, recruitment of leukocytes to articular tissue, stimulation of prostaglandin production, collagen synthesis by synovial cells and bone resorption. Inhibition of TNF-α production suppresses inflammation and cartilage destruction significantly and slow down the progression of arthritis.
Fig. 2A. DSC curves a) QCT b) GMS c) QCT-NLCs.

Fig 2B. XRD diffractograms a) QCT b) GMS c) QCT-NLCs.
With the evidence of the cell line study, LPS-induced TNF-α production in RAW264.7 cells was significantly suppressed by pretreatment with the QCT-NLCs (Fig 3 B). The anti-arthritic effects of QCT-NLCs complex could possibly be mediated by the inhibition of inflammatory cytokines like TNF-α. (Kaul et al, 1985). This happens may be due to interference of QCT with early signaling events in response to LPS or blocking of LPS receptor on macrophage membrane. So the inhibition of TNF-α synthesis can be achieved by multiple means such as: (1) inhibition of translation; (2) decrease of the mRNA half-life; and (3) inhibition of transcription (Ghosh et al 1999).

**Characterization of QCT-NLC Gel**

**pH of the gel:** pH of the QCT-NLC gel was found to be 5.81±0.16. This value of pH was found to be close with the pH of human skin and thus it can be considered that no skin irritation will occur after application of QCT-NLC gel. Hence, the formulation was acceptably complying with pH value required for topical application.

**Drug content:** Drug content was found to be 97.87 ± 0.34 %. Hence, uniformity of drug content was found satisfactory. The high percentage of drug content achieved could offer advantages in the drug delivery and therapeutic efficacy of QCT for rheumatoid arthritis.

**Rheological measurements:** Formulation characteristics, such as viscosity, rheology are the most significant factors in the development and ultimate behavior of semisolid formulations. Increasing the viscosity of the formulation increases its retention time at the site of application but also reduces its spreadability. Therefore, viscosity of these formulations plays the key role in their ultimate behavior, including their application qualities on the site. Viscosity of the NLC gel was found to be 72,340 ± 3.56 cP at 5 rpm. The viscosity was found decreased with the increase in stress. The flow index of
gel was found to be 0.426 ± 0.04 indicating the pseudoplastic flow behavior. Higher viscosity of the QCT-NLC gel may assist to increase in solid content, higher surface area and ease of application. Due to lipid content, permeation enhancer and small size, QCT-NLCs are supposed to penetrate easily in the stratum corneum and show high retention capacity thereby increasing the local effect of the drug in the joints rather than systemic effect. With the consideration of lipophilic nature of QCT, fundamental possessions of NLCs and gel nature of the formulation, it is believed to enhance the skin hydration and skin permeation by forming an occlusive layer on the skin and providing improved retention of QCT-NLCs in the skin.

**Texture profile analysis**: Formulations, which are intended for topical application, must show acceptable mechanical characteristics e.g., ease of application, stickiness, firmness or hardness etc. The mechanical properties of NLC-gel were studied by texture profile analysis (TPA). Adhesiveness, cohesiveness, gumminess was found to be 0.4±0.01 mJ, 0.73±0.02 and 13±0.31 mg respectively. Also the deformation at hardness and springiness was found to be 3.83±0.23 mm and 2.56 ± 0.18 mm.

**Stability study of gel**: The formulation did not show any significant change in the drug content, appearance, pH, viscosity and phase separation parameters. It indicates that this formulation was able to retain its physical consistency up to 6 months.

**Ex vivo Permeation Study**

**Ex vivo** permeation study shown that the drug from QCT-NLC gel diffuses with faster rate and linear permeation than QCT gel. About 61.32 ±0.71 % of the drug was diffused from QCT-NLC gel at the end of 24 h. Permeability coefficient was found to be 2.9 cm²/min. The faster diffusion rates could be due to the combined properties of solid lipid and liquid lipid with the surfactants in the formulation. Non-ionic surfactant, tween 80, may penetrate into the intercellular regions of stratum corneum, increase fluidity and eventually solubilize and extract lipid components (Nokhodchi et al., 2003). Also, they are able to emulsify sebum and enhance the thermodynamic coefficient of drugs allowing it to penetrate into the cells more effectively. Hence, the studies confirmed that QCT-NLCs could deteriorate the barrier function of stratum corneum and enhance the drug permeation.

**Antiarthritic Activity**

**Evaluation of the severity of arthritis**

**Measurements of hind paw volume**: Rheumatoid arthritis was evident in rats after 2 weeks of adjuvant administration. Significant increase in paw circumference, erythema, swelling, joint stiffness and hindrance in the movement was observed. Paw volume was recorded by using plethysmometer on 7th, 14th, 21st and 28th day after adjuvant injection. However, in rats that received QCT-NLC gel, a significant decrease was observed in paw circumference and swelling when compared to the arthritic control. Control group shows 71 mm of paw circumference, which was found to be decreased up to 62 mm with QCT, 53 mm with the QCT-NLCs and 49 mm with DCS treatment.

**Arthritis scoring system**: Arthritis scores were recorded on the 7th, 14th, 21st and 28th day after adjuvant injection. The CFA-induced arthritis control group has shown signs of arthritis development, as seen by the increase in the paw volumes. Results of evaluation done on 21st and 28th day expresses that the QCT-NLC gel treatment has considerably reduced an adjuvant-induced arthritic lesions such as paw circumference, erythema, swelling, joint stiffness and hindrance in the movement as compared with the CFA control group. Results of arthritic index, stiffness score and paw circumference of group 1 to 4 were shown in Table 1.

**Measurement of haematological parameters**: The CFA-induced haematological parameters, such as an increase in the WBC count, a decreased RBC count, a decreased hemoglobin (Hb) count and an increased erythrocyte sedimentation rate were also favorably altered by QCT-NLC gel treatment. The levels of C-reactive protein and RF were also found reduced by QCT-NLC gel. Results were shown in Table 2.

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Fig. 4. Texture profile analysis of QCT-NLC gel.
CFA induced arthritis is an extensively used arthritic model for development and evaluation of anti-arthritic and anti-inflammatory agents, since it is very similar to human rheumatoid arthritis with respect to pathological and serological changes and inflammatory mediators. CFA induction in rats activates T cells which then proliferate and secret additional cytokines, such as interleukins. B cells become activated through interactions with T cells and increases proliferation. These cytokines further stimulates macrophage. Cytokines also induces acute phase response proteins such as C-reactive proteins and increases ESR. Macrophages are rich sources of pro-inflammatory mediators such as nitric oxide, TNF-α and interleukins. They further release chondrocytes, osteoclast and synovial fibroblast. This results in cell proliferation, chronic swelling and pain in multiple joints via release of cytokines from inflammatory cells, ultimately causes bone and cartilage damage and immobility of the joints.

Increase in WBC and ESR level has been suggested to important characteristic diagnoses of arthritis, which is also observed in CFA–induced arthritic rats. QCT-NLCs treatment significantly decreased WBC and ESR confirming its beneficial role in arthritic treatment. (Sivasudha et al, 2013)

In conclusion, in consideration to the challenges in the management of RA and drawbacks of QCT administration, present research work emphasizes on the development of a novel formulation with sustained release through transdermal route. It helps in increasing the permeation of QCT to the site; thus increases therapeutic efficacy of QCT. QCT loaded NLCs were prepared and evaluated successfully for in vitro, ex vivo and in vivo parameters. The optimized QCT-NLCs showed inhibition of cell proliferation and TNF-α production effectively. In vivo activity of QCT-NLCs can be revealed by the mechanism that, the lipids from NLCs is supposed to get entrapped in the dermal layer of skin from which the drug is released and it is proposed to be acting on the fibroblasts, TNF-α existing in the dermis, which plays a crucial role in activation of the inflammation following bone and cartilage damage. Ultimately, it results in reduction in the severity of the disease. Hence, the study reveals that QCT-NLC gel confirmed significant result in improving the skin acceptability, which indicated its potential in improving patient acceptance and topical administration.

### Skin Irritation Study

The results of the skin irritation study showed that after the application of QCT-NLC and blank gel, for seven days there was no reaction (erythema or edema) found on the rat skin. Thus, QCT-NLC gel confirmed acceptance and topical administration.

### Conclusions

The authors declare that there are no conflicts of interest.
References

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Int J Pharm Sci Nanotech Vol 11; Issue 1 • January–February 2018