

Inhibitory Effect of Quercetin-Loaded Nanostructure Lipid Carrier on Compound 48/80-Induced Mast Cell Degranulation



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Abstract

Quercetin is naturally occurring flavanoids, it is abundant in nature and the human beings are taken up it through their daily meals. The different pharmacological activity of quercetin has been evaluated since a long time. The scientific exploration of quercetin revealed that it is effective as anti-oxidant, anti-inflammatory agent; it can reduce the elevated levels of cholesterol, triglycerides, low-density-lipoproteins etc. However, solubility and bioavailability has been an issue for the quercetin. In this experimental work we not only evaluate the antihistaminic activity of quercetin but also developed a nano structure lipid carrier system for quercetin and explore its effectiveness as an antihistaminic agent.

It was found that the quercetin loaded nano structured lipid carrier system was successfully fabricated with adequate drug loading and entrapment efficiency. The intracellular uptake of formulated delivery system by isolated mice peritoneal mast cells has also been studied. The antihistaminic activity of quercetin and quercetin loaded lipid carrier system was compared against the standard antihistaminic agents like prednisolone, cromolyn sodium and it was found that the quercetin loaded lipid carrier system have showed superior activity than the free quercetin and the standard antihistaminic agents.

Keywords: Quercetin; NLC; Lipid based formulation; Compound 48/80; Mast cell; Antihistaminic

Abbreviations: Nano structured Lipid Carrier; qNLC: quercetin Loaded Nano Structured Lipid Carrier

Introduction

Allergic diseases and asthma have been prevalent since a long ago and still, now the incidence of these types of disease condition increased in such a manner that it has appeared as a major health concern. As per world health organization, more than 1.8lakhs of mortality is caused by asthma annually [1]. Ages are no bar for the occurrence of this type of disease; it may happen in any age group. The diseases like asthma and allergy are initiated by the process of inflammation. CD4+T helper cell responses are important for the initiation and propagation of the disease condition [2]. Interleukin-4, 5, 9 and 13 are the cytokines which are secreted by TH2 cells and they are the most important mediators of the process of inflammation. Mast-cell degranulation, increased IgE level is the characteristic phenomenon of asthma and allergy related diseases [3].

The treatment of this kind of disease condition falls into different categories like mast cell stabilizers, corticosteroids, antihistaminic agents. They may act by blocking the mediators responsible for inflammation or by preventing the process which initiates the process of inflammation. The present chemotherapy for this purpose is efficient however long term side effects still be a concern. Skin fragility, immunosuppressant is associated with the long term use of oral corticosteroids. It was also observed that the new generation antihistaminic agents are showed sedative effects [4,5]. Hence, there is always a need for a new antihistaminic agent which could effectively treat the disease condition as well as tolerable in patients in long term.

Searching for a new antihistaminic agent as well as a new drug delivery system for this type of diseases we have developed

quercetin loaded nano structured lipid carrier system. Quercetin is a naturally occurring phenolic compound and it is flavonoid in class. Quercetin is abundant in nature and it is generally found in the food plants. The flavonoids commonly present as glycosides. Like, the aglycone quercetin linked to quercetin as 3-o-glycoside [6]. The beneficial pharmacological effects of quercetin always draw the attention of many scientists for several decades. According to Gupta et al, the quercetin has potent antioxidant activity [7]. Quercetin could scavenge the free radicals, chelate metal ions and it shows synergistic activity with other antioxidants [8-12].

Quercetin could be effective in the treatment of inflammation and oxidative stress as well [13]. It was found in a study that regular intake of quercetin could decrease the elevated levels of cholesterol, triglyceride, and low-density-lipoproteins [14]. Tzankova et al in his recent study have found that quercetin possessed hepatoprotective activity [15]. It was reported that more than 90% of quercetin was metabolized after an hour when it was administered intraperitoneally. When Justino et al. [16] was treated Sprague–Dawley rats with a single intraperitoneal injection of quercetin at a dose of 50mg/kg body weight, it was found that the plasma contains very low amount of free quercetin [16]. Therefore, the development of a drug delivery system with quercetin which could effectively increase the solubility and bioavailability of it, have always been a challenge. In these circumstances, the nano structured lipid carrier system (NLC) for quercetin was developed to evaluate its antihistaminic property which would open up a new beneficial pharmacological effect of the naturally occurring flavonol.

Material and Methods

Materials

Gattefose India Pvt. Ltd. supplied Comparitol® 888 ATO. Other substances like soy lecithin, compound 48/80, triolein, o- phthalaldehyde, sorbitol, Pluronic F68, and quercetin were purchased from Sigma- Aldrich. Fetal bovine serum, RPMI1640 and medium 199 were supplied from HiMedia, India. All the solvents used in the experiment were of analytical grade.

Instruments and Equipment

Sartorius balance, 700 MW sonicator (Vibra cell VCX750; Sonics, CT, USA), Vortex (CM101 cyclomixer; Remi, India), high-speed homogenizer (IkaT10 basic, Ultra-Turrax), magnetically stirred hot plate (Tarsons, Spinot digital model

MCO₂), lyophilizer (LaboGene-ApS, 6-B-DR-3450 Lyngø, Denmark), High performance liquid chromatography (HPLC) (Dionex ultimate3000; Thermo Fisher Scientific, MA, USA), UV spectrophotometer (Shimadzu UV-1800; Shimadzu, Tokyo, Japan), BOD incubator shaker (BOD Inc 1S, Kolkata, India), CO₂ incubator (MCO-15AC; Sanyo, Tokyo, Japan), Laminar Airflow (Stericlean; Deepak Meditech Pvt. Ltd, New Delhi, India), Cold Centrifuge (Rota 4R-V/FM; Plasto Crafts, Mumbai, India). Standard Borosil glass wares were used for experimental purposes. GraphPad Prism 5.0.1. was used for statistical analysis.

Ethics statements

BALB/c mice (20-25gm) of either sex or apparently same ages were used for the studies. Animals were housed in polypropylene cage. All of the experiments, where animals were used, were done according to the guidelines of Jadavpur University Animal Ethics Committee. Animals were fed with standard diet and water and they were exposed to normal day and night cycle.

Methods

Preparation of Cedrol-loaded NLCs: A combination of melt emulsification along with homogenization and ultrasonication method was employed to fabricate the quercetin loaded NLC (qNLC) [17]. Briefly, according to the drug and lipid ratio, the weighted amount of comparitol® 888 ATO (1.5mg) [(glycerol mono behenate 15%, glyceryl tribehenate 35% and glyceryl di behenate 50%)], triolein (0.31mg) and quercetin were melted together at about 85 °C. That melted mixture was stirred occasionally for 15mins to develop transparent and uniform oil phase. It was observed that there was no degradation of quercetin happened at that particular temperature condition.

The aqueous phase was prepared by dissolving Pluronic F68 and soya lecithin (0.31mg) at the same temperature condition and for the same period of time in a 60 ml double distilled water. Now, the aqueous phase was added drop-wise into the oil phase during homogenization at 20000rpm for 10mins. The formed emulsion was then sonicated for 10mins in an ultra sonicator at 85 °C. The formed NLC was cooled at 4 °C for overnight followed by membrane filtration through the 0.45µm membrane filter. After frozen at -80 °C NLC suspension was lyophilized to obtain a dry finished product.

Blank NLC was prepared by the same technique except adding the quercetin (Table 1).

Table 1: Composition of NLC.

Formulation	Solid Lipid (%w/w)	Liquid Lipid (%w/w)	Amphiphilic Emulsifier (%w/w)	Hydrophilic Emulsifier (%w/w)
qNLC	Comparitol® 888 ATO (69.2%)	Triolin (14.5%)	Soya lecithin (14.5%)	Pluronic F68 (10.0%)

Characterization

Particle size, zeta potential, and poly dispersity index:

The average particle size, zeta potential, and polydispersity index (PDI) of formulated NLCs were determined by the dynamic light scattering (DLS) technique by the Zetasizer Nano ZS90 (Malvern Instrument Ltd., UK) using the software Data transfer assistance (DTS). For these studies, 1 mg of the formulations were dispersed in 2ml of Milli Q water and then sonicated for half an hour before the analysis.

Quercetin loading and entrapment efficiency study: To carry out the drug loading and entrapment efficiency study, 5mg of qNLC was dispersed in 5 ml of 0.5% tween 80 in PBS buffer (pH 7.4). The dispersion was sonicated for 15mins. After that, it was centrifuged at 20000rpm for 15min. The supernatant was collected and the quantity of quercetin was determined by the liquid chromatographic method. For that purpose, C18 column (4.6 × 250 mm, 5μ) was used. Acetonitrile and phosphate buffer (pH 4.0 maintained by o- phosphoric acid) in a ratio of 50:50 was used as a mobile phase and the flow rate was 1ml/min. Quercetin was detected at a detecting wavelength of 292nm. Drug loading and the entrapment efficiency was calculated by the following equations.

$$\text{Drug loading} = (W_{\text{total}} - W_{\text{free}} / W_{\text{lipids}}) \times 100\%$$

$$\text{Entrapment efficiency} = (W_{\text{total}} - W_{\text{free}} / W_{\text{total}}) \times 100\%$$

Where W_{total} was the weight of drug added in the system, W_{free} was the analyzed weight of drug in the supernatant and W_{lipids} weight of lipids added to the system.

Field emission scanning electron microscopic study: The surface morphology of the formulated NLC was evaluated by field emission scanning electron microscopy. The lyophilized product was used for taking the SEM images. Briefly, lyophilized NLC was spread on the carbon tape then subjected to gold coating. After completion of the coating, particles were observed under FE-SEM (Model-JSM-6700F; JEOL, Tokyo, Japan).

In vitro cumulative drug release study: *In vitro* cumulative drug release study of formulated qNLC was carried out for 21 days. Briefly, 5mg of qNLC was suspended in 5 ml PBS buffer pH 7.4 and kept it in the dialysis bag (MW cutoff 12000 kDa). That dialysis bag was further kept into a 100 ml 0.5% v/v tween 80 with PBS buffer (pH 7.4). That system was kept in a shaker incubator. 1 ml sample aliquots were collected and 1ml of fresh release medium was incorporated for a predetermined time period (45 mins, 90mins, 3h, 6h, 12h, 24h and up to 504h). The collected samples were analyzed by the similar chromatographic condition as previously described.

Mast cell isolation and culture: Peritoneal mast cells were isolated from BALB/c mice [18]. Briefly, after sacrificing the mice by cervical dislocation, 5mL of heparinized (10 IU/mL) calcium- and magnesium-free Hank's solution (HBSS) was injected into

the mice peritoneal cavity. After 2mins, the peritoneal fluid was collected and centrifuged at 1,500rpm for 10minutes at 4 °C. The supernatant was discarded and the cells were resuspended in fresh HBSS containing 0.1% protease-free bovine serum albumin and then it was purified by continuous isotonic Percoll gradient (72%) for isolation of mast cell.

Mast cells thus purified were resuspended in fresh HBSS media containing calcium and magnesium. Toluidine blue and trypan blue exclusion staining were performed to evaluate the purity and the viability of mast cells, respectively.

After the tests for purification and viability, mast cells were transferred to RPMI-1640 medium containing 15% fetal bovine serum and 5μg/mL streptomycin and 50U penicillin. After every 3 days sub culturing was carried out, and the culture was maintained for 2 months.

Trypan blue exclusion assay of mast cell viability: Mast cell viability was assessed by trypan blue staining. For this, 0.2ml of mast cells were transferred aseptically into a 1.5ml eppendr of tube along with an equal amount of 0.4% (w/v) trypan blue solution prepared in 0.8% (w/v) sodium chloride and 0.06% (w/v) dibasic potassium phosphate and incubated it for 3min at room temperature. Cells were counted by using the hemocytometer under a light microscope. The count of viable and nonviable cells was recorded separately.

Mast cell sensitization by compound 48/80: HEPES-Tyrode's buffer containing mast cell suspension was incubated with 4μg/ml of compound 48/80 for the period of 10mins at 37 °C. Mast cells smears were prepared, fixed and stained with 0.1% (w/v) toluidine blue and kept it for 10mins. The morphology of the mast cells was observed under a light microscope [19].

Cellular uptake study: To carry out the cellular uptake study, coumarin-6 loaded NLC was prepared it was used instead of qNLC. For this study, sterile cover slips were placed in each well of the 24 well plate was 4×10^5 cells/well were seeded with coumarin-6 loaded NLC (0.5mg/mL) and incubated in a 5% CO₂ incubator at 37 °C for 20min. After the specific period of time, the cover slips were collected, washed for three times and then observed under confocal microscope (TCS SP8; Leica Microsystems, Wetzlar, Germany) using Leicasuitelas X software. The magnification of the objective was 63×.

Effects of anti-asthmatic drugs, quercetin, and qNLC on histamine release assay from mast cells: For the stabilization purpose, the isolated and purified mice peritoneal mast cells (2×10^5 cells/mL) were incubated at 37 °C in a 5% CO₂ incubator for 10mins. Standard anti-asthmatic drugs, quercetin, and qNLC at variable concentrations were added to the pre-incubated media containing the mast cells and further incubated at 37 °C. After the incubation period of 24h, the cells were washed and further incubated with 4μg/ml of compound 48/80 for the period of 20minutes. After that, the test tubes were kept in ice

water. The released histamines were separated from the mast cells by the process of centrifugation at 2,000rpm for 5 minutes at 4 °C. 0.05% (v/v) Triton X-100 was added to liberate the residual histamine. Then, 0.02% opt-methanol solution was added and histamine content in the supernatant and in the pellets of cells was determined spectrofluorimetrically (excitation 360nm, emission 450nm). For the estimation of the spontaneous release of histamine (spontaneous), exactly the same procedure without adding drugs and samples was followed. The following equation was used to calculate the release percentage of histamine

[20,21].

$$\% \text{ Histamine release} = \frac{\{(\text{Supernatant} - \text{Spontaneous}) / (\text{Supernatant} + \text{cell pellets}) - \text{Spontaneous}\} \times 100\%}$$

Statistical analysis: Experimental data were expressed as a mean ± standard deviation. One way analysis of variance was used to calculate the statistical significance. P <0.05 was considered as significant and P >0.05 was considered as not significant.

Results

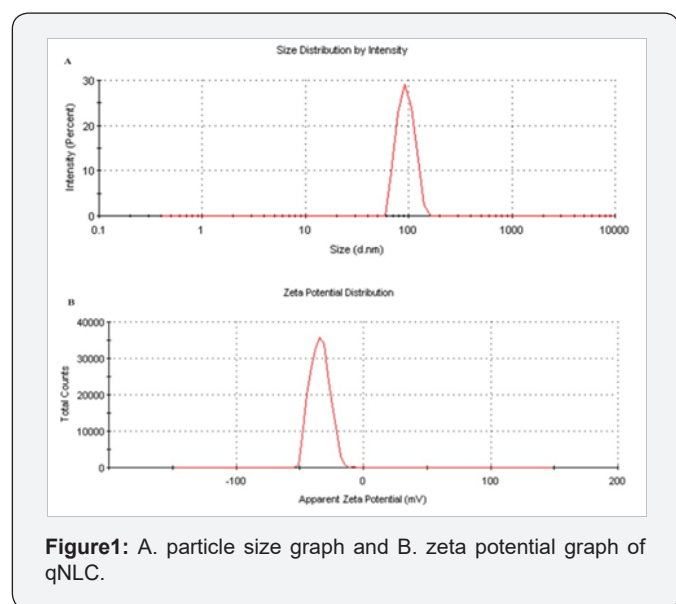
Average particle size, zeta potential, PDI

Table 2: Average particle size, zeta potential, PDI, drug loading, and entrapment efficiency of formulated NLCs.

Formulation	Drug: Lipid	% Yield (After Lyophilization) ^a	Avg. Particle Size (nm) ^a	Zeta potential (mV) ^a	PDI ^a	Drug Loading (%) ^a	Entrapment Efficiency (%) ^a
Blank-NLC	-	81.4±2.5	54.70±0.34	-20.3±0.03	0.213±0.05	-	-
qNLC	1:3	77.1±3.7	90.06±0.57	-29.2±0.019	0.257±0.03	19.3±0.72	98.7±0.92

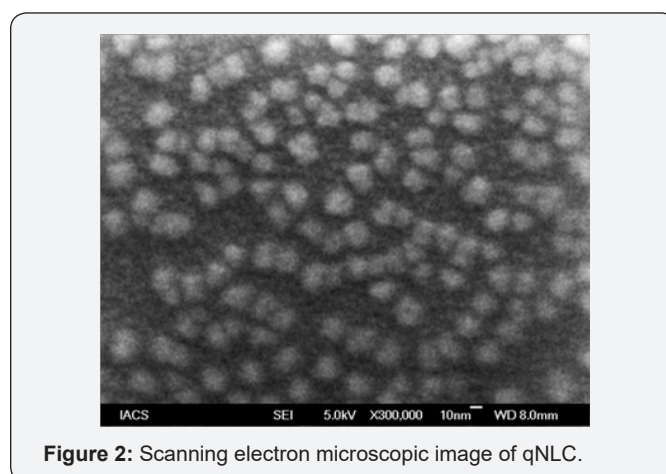
The average particle size, zeta potential, PDI values were calculated for blank-NLC and qNLC nanoparticles. According to the Table 2 the both types of formulated nanoparticles were in nano size range and their average particle sizes were less than 100nm. The percentage yields of the NLCs were more than adequate. The PDI values were found to be 0.213±0.05 and 0.257±0.03 for the Blank-NLC and qNLC, respectively. The NLCs have shown negative zeta potential values like -17.3±0.03 for Blank-NLC and -34.2±0.019 for qNLC.

Drug loading and entrapment efficiency



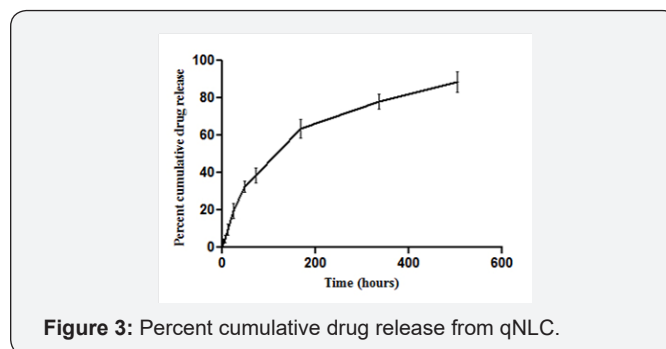
From the Table 2, it could be observed that the percentage drug loading for qNLC was 19.3±0.72% and the entrapment efficiency of the formulation was 98.7±0.92% (Figure 1).

Scanning electron microscopic study



The scanning electron microscopic study was carried out to evaluate the size and shape of the formulated NLC. It could be found from the Figure 2 that the formulated qNLC was in nano size range and particles were almost spherical in shape.

In vitro cumulative drug release study



The *in vitro* cumulative release study was carried out for 21 days. It was found that after 504h, $88.7 \pm 1.5\%$ of quercetin was released from the NLC core (Figure 3).

Mast cell uptake study

The confocal study was carried out to evaluate the

formulated NLC uptake by the isolated and purified mast cell. From the Figure 4, it was observed that within the period of 20 mins coumarin-6 loaded NLC was taken up by the mast cells. The green fluorescence appeared in the Figure 4B & 4C were the coumarin-6.

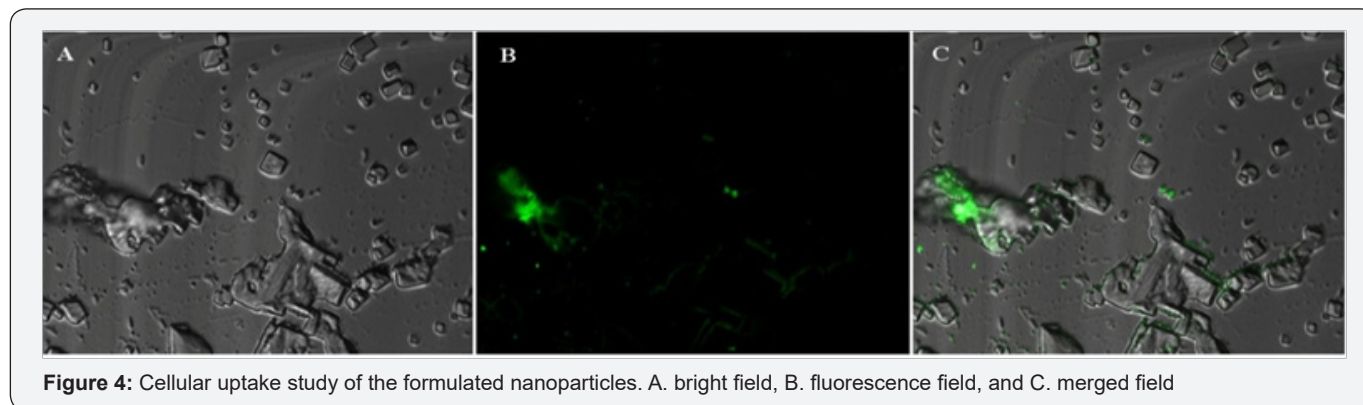


Figure 4: Cellular uptake study of the formulated nanoparticles. A. bright field, B. fluorescence field, and C. merged field

***In vitro* histamine release assay**

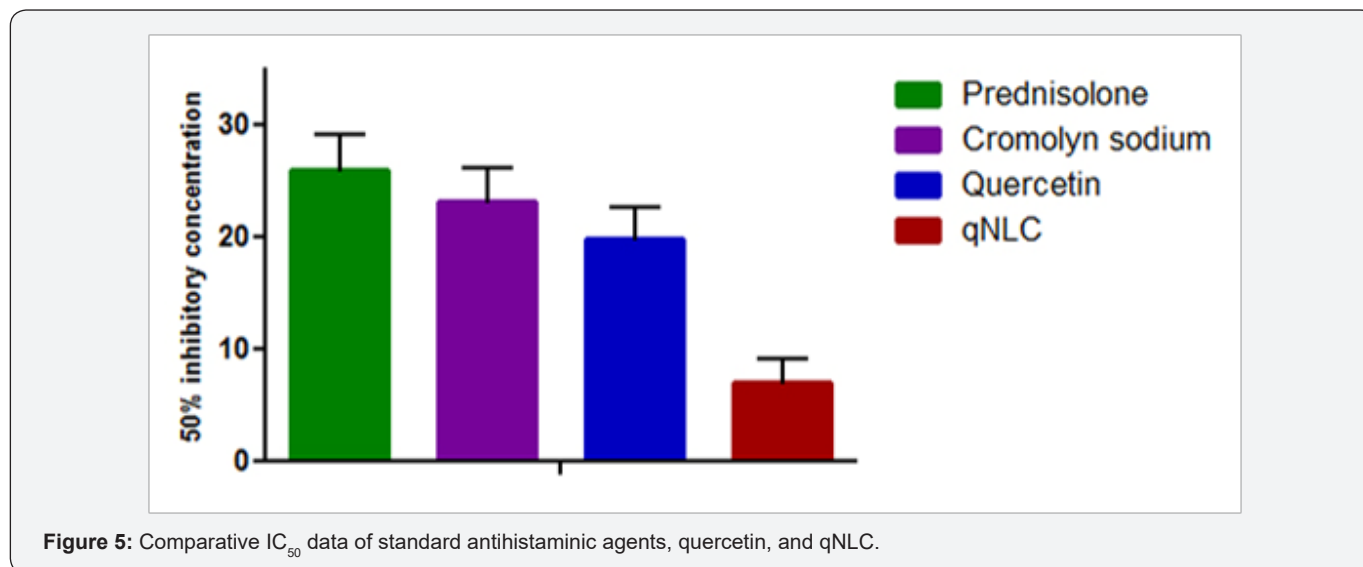


Figure 5: Comparative IC₅₀ data of standard antihistaminic agents, quercetin, and qNLC.

Table 3: Assay of histamine release in presence of standard antihistaminic agents, quercetin, and qNLC.

Drug/ Formulation	IC ₅₀ (mean± SD, n=3) (μM)
Prednisolone	25.86±3.21
Cromolyn sodium	23.15±3.04
Quercetin	19.84±2.76b
qNLC	7.06±2.14c

Table 3 represented the IC₅₀ values of Prednisolone, cromolyn sodium, quercetin and qNLC which indicated that

at the particular concentration of the drug or formulation the histamine release from the mast cells was 50% inhibited. It was observed from the Table 3 that the IC₅₀ of free quercetin was $19.84 \pm 2.76 \mu\text{M}$ which was less than the standard antihistaminic agents and the qNLC formulation of quercetin decreased the IC₅₀ value to $7.06 \pm 2.14 \mu\text{M}$ (Figure 5).

Discussion

Fabrication of quercetin loaded NLC was carried out by a combination of melt emulsification along with homogenization and ultrasonication method. It was observed from the character studies that the formed drug loaded particles were in nano size range. From Table 2 and Figure 1A it could be observed that the average particle size of qNLC was $90.06 \pm 0.57 \text{nm}$. The FESEM analysis was also confirmed the formation of nano size ranged

particles of NLC. From Table 2 it was also observed that the both the blank and qNLC possessed negative surface charge. Zeta potential is an important parameter which could depict the stability of the nanoparticles inside the formulation.

It was found from the Table 2 and Figure 1B that the zeta potential of qNLC was -29.2 ± 0.019 mV which indicated that the particles in the formulation would not agglomerate in suspension if it will remain suspended for an extended time period [22]. When the quercetin loading into the NLC was evaluated it was found that the formulation of qNLC exerted superior drug loading of $19.3 \pm 0.72\%$ with $98.7 \pm 0.92\%$ entrapment efficiency.

In vitro percentage cumulative drug release study was carried out for an extended period of time. It was an important parameter as it will indicate the amount of drug release for the predetermined period of time as well as the pattern of release. It was observed from Figure 3 that the qNLC formulation released the $88.7 \pm 1.5\%$ quercetin after 504h.

As the mast cell was our target so uptake of NLC by the mast cells was an essential parameter to evaluate and for that purpose confocal microscopy study was carried out. It was clearly observed from the Figure 4 that the coumarin-6 loaded NLC was well taken up by the isolated and purified mast cells within 20mins. Here the coumarin-6 was mimicking the quercetin which meant that quercetin will be taken up also when it will deliver through the NLC system.

Histamine released from the mast cells and inhibition of that phenomenon will be a property of an antihistaminic agent. For that purpose, 50% inhibitory concentrations of the free quercetin and qNLC formulation were determined and compared the data against the data generated from the standard antihistaminic drug like prednisolone and cromolyn sodium. From Table 3 and Figure 5 it was observed that the IC₅₀ values of prednisolone, cromolyn sodium, and quercetin were $25.86 \pm 3.21 \mu\text{M}$, $23.15 \pm 3.04 \mu\text{M}$, and $19.84 \pm 2.76 \mu\text{M}$, respectively and it was also found that there were no significant differences among them which could indirectly indicate the similar antihistaminic property of quercetin like the standard drugs. Moreover, when the quercetin was loaded inside the lipid core of the NLC it was found that the qNLC exerted superior inhibition of histamine release from the mast cells with an IC₅₀ value of $7.06 \pm 2.14 \mu\text{M}$. There was an extremely significant difference obtained among the IC₅₀ value of qNLC and the other drugs.

Conclusion

From this experimental work, it could be concluded that the qNLC was successfully fabricated. The formulated NLCs were in nano size range. The uptake study revealed the adequate homing of the drug of interest inside the mast cells and the qNLC was efficiently inhibited the histamine release from the mast cells and the quercetin loaded NLC delivered significantly higher efficacy than the standard antihistaminic agents.

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