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Reversion of Asthmatic Complications and Mast Cell Signalling Pathways in BALB/c Mice Model Using Quercetin Nanocrystals

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The tranquillizing effects of quercetin on allergic asthma are promising, but its poor water solubility and bioavailability is still a bottleneck. In this study, an ovalbumin (OVA) sensitized BALB/c mice asthma model was used to investigate the potential of quercetin nanocrystals (nQ) on relieving asthma aggravation. The water soluble nQ was prepared by the homogenization using the high energy sonication method. X-ray diffraction data showed the formation of nQ (10–30 nm) which was in agreement with transmission electron microscopy. The nQ was found to be more stable and soluble in PBS, and sera of BALB/c mice compared to bulk quercetin. Dose dependent experiments with nQ on OVA sensitized asthma mice exhibited significant anti-asthmatic potential of nQ at much lower dose (1 mg/kg body weight) compared to bulk quercetin. The treatment of nQ remarkably resulted in reduced OVA specific immunoglobulin E (IgE) production, anaphylaxis signs and type 1 skin test. The nQ also significantly modulated the expression of Th2 cytokines like IL-4 and IL-5, which are responsible for IgE class switching and suppressed the degranulation/secretion of different chemical mediators (PGD₂, mMCPT-1 Cys-L and TSLP) from activated mast cells. The levels of FcεR1, Syk, c-Yes, PI-3, p-PI-3, PLC-γ2, and p-PLC-γ2 were found to be reduced in the OVA sensitized BALB/c mice treated with nQ compared to those treated with OVA only. The results indicate that nQ alleviate pulmonary inflammation and airway hyporesponsiveness in allergic asthma at much lower dose compared to bulk quercetin and may be considered as a potential drug for the treatment of asthmatic patients.

KEYWORDS: Nanotized Quercetin, Asthma, Cytokines, Transcription Factors, Mast Cells.

INTRODUCTION

Asthma is a major cause of morbidity characterized by variable and recurring symptoms like airway hyporesponsiveness, tissue remodeling, bronchospasm and chronic airway inflammation with common features like coughing, congestion in chest, wheezing, and shortness of breath.^{1–3}

Recently, prevalence of asthma has increased substantially throughout the globe and approximately 250,000 asthma related deaths have been reported each year.⁴ Allergic asthma is the most prevalent form of asthma, which is typically triggered by allergens like pollen, mold, dust mites and foods.^{5,6} Asthmatic individuals need to pay additional attention as this population is at high risk from hazardous air particles (HAP) including fine particulate matter, tobacco smoke and other air born pollutants.⁷ Moreover, asthma is also triggered by several intrinsic

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factors like upper respiratory infection, physical exercise and weather changes.^{8,9} The different allergens and extrinsic factor elicit the immunological processes, which are characterized by enhanced levels of IgE, mast cells and activation of Th2 lymphocytes, setting off an allergic cascade that ultimately leads to airway hyper responsiveness (AHR) and chronic airway inflammation. Mast cells are dispersed in the mucosal tissue that play a central role in the pathophysiology of allergic asthma referred as immediate or type 1 hypersensitivity reactions. Studies have suggested that Th2 cytokines such as IL-4, IL-5 and IL-13 play pivotal role in the recruitment/activation of primary effector cells (mast cells, basophils, eosinophils) causing an allergic response.⁶⁻⁹ Furthermore, chemokines, including thymus and activation-regulated chemokine, play an important role in asthma in regulation of inflammation and IgE synthesis. Currently, the available treatment of AHR is done by decreasing the inflammation or relaxing airways, however these techniques are not good enough to fully control AHR. More than 80% of the world's population is using complementary and alternative medicines (CAMs) that are becoming an increasing component of US health care system and more than 70% of the population is using CAM at least once in lifetime that costs approximately \$34 billion.¹⁰ Among CAM therapies, dietary supplements and antioxidants are widely used to alter the immunogenic responses and have played a key role in the prevention of several diseases such as cancer, cardiovascular disease, Alzheimer's disease, allergic asthma.¹¹⁻¹³ Dietary antioxidants intervention may also reduce oxidative stress and prevent or minimize asthmatic symptoms.¹¹ Quercetin is a plant-derived bioflavonoid known to exhibit excellent anti-oxidant and anti-inflammatory properties.¹⁴ It has been suggested as a good candidate for the management of eosinophil-mediated diseases, such as allergic rhinitis and asthma.¹⁵ Quercetin relaxes airway smooth muscle through cAMP-mediated pathways and augments β -agonist relaxation via dual phosphodiesterase inhibition of PLC β and PDE4.¹⁶ Quercetin lowers the expression of pro-inflammatory cytokines and improves lung function in RV-infected mice.¹⁷ The acute effect of quercetin on experimental allergic asthma after single oral dose indicated that it is responsible for bronchodilation, both *in vivo* and *in vitro*.¹⁸ Anti-inflammatory effect of quercetin-loaded microemulsion (QU-ME) and quercetin suspension (QU-SP) has been studied on murine model of airways allergic inflammation.¹⁹ Potentiating the existing natural bioflavonoid quercetin using nanotechnology may pave the new direction for treating asthma.

In the last decade, Nanomedicine that promises for targeted therapy, drug delivery and personalized medicine has attracted tremendous research attention. Nanomedicine capitalizes on the enhancement of bioavailability of poorly water soluble drugs with small size that can easily penetrate and interact with cells.²⁰ A Nanomedicine consisting of Chitosan/carboxymethyl- β -cyclodextrin loaded with

unfractionated or low-molecular-weight heparin showed a potential effect in asthma treatment.²¹

The current study is focused on the preparation of water soluble quercetin nanocrystals that are poised to show enhanced bioavailability and therapeutic potential. We investigated well characterized nQ on OVA induced BALB/c mouse model of allergic asthma for various allergic parameters, like serum Immunoglobulin(Ig) concentration, histopathological changes of lung tissue, mediator release assay, mast cell signaling events, providing an effective strategy for treating allergic asthma.

MATERIALS AND METHODS

Materials

Quercetin, *o*-phenylenediamine (OPD), bovine serum albumin (BSA) and reagents for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) were purchased from Sigma Chemical Company, St. Louis, MO. The stabilizer Tween 80 (polysorbate 80) was procured from Uniqema, Belgium. Goat anti-mouse IgG1 and IgE-HRP linked antibodies were obtained from Southern Biotech, Birmingham, Alabama. All the other chemicals used were of the highest purity available from other commercial sources.

Preparation and Lyophilisation of Quercetin Nanocrystals

The preparation of nQ was carried out under high energy transfer using ultrasonicator (PCI Analytics probe sonicator, Mumbai, Maharashtra) as per described method by Sahoo et al.²² with minor modifications.²² Briefly, nQ (2% to 10% w/w) were prepared by mixing bulk quercetin in Milli-Q water (Millipore Corporation, Billerica, MA) having pH 6.8 and resistivity of 18 M Ω , with Tween 80 as a stabilizer (0.5 to 2.5 w/w). The solution thus prepared was subjected to high energy ultra-sonication to obtain quercetin nanocrystals. Thus obtained nQ was freeze dried at -80°C in a 20 ml flask using lyophilizer (Allied frost lyophilizer FD-5, New Delhi India) overnight. We have used nQ 2% (w/w) for all our experiment throughout the manuscript. It has been chosen on the basis of excellent antioxidant and reducing ability of nQ (2 wt%) compared to (5 wt%) and (10 wt%) as observed by Sahoo et al.²² These nanoparticles were characterized using dynamic light scattering (DLS), Transmission electron microscopy (TEM), X-ray diffractometer (XRD), Uv-Vis spectroscopy and Fourier transform infra-red (FTIR) spectroscopy for analysing the size, shape, structure and functionality.

Physical Characterization of Quercetin Nanocrystals

The FTIR spectra of bulk quercetin and nQ with KBr were recorded using Agilent Cary 630, machine in the scanning range of 500–4000 cm^{-1} with resolution of 1 cm^{-1} in triplicate. The UV spectra of bulk quercetin and nQ

(20 $\mu\text{g/ml}$) in methanol solvent were recorded on an Agilent Cary 5000 UV-Vis-NIR spectrophotometer. All spectra were recorded in the range from 200 to 600 nm in triplicate. The shape and size of nQ were seen by TEM measurements. In brief, a drop of diluted solution of the nQ (50 $\mu\text{g/ml}$) was placed in carbon coated copper TEM grid, negatively stained with 1% uranyl acetate (w/v) for 10 min and allowed to air-dry and the samples were imaged using Technai G2 S-Twin electron microscope operated at 200 kV accelerating voltage. The average hydrodynamic size, polydispersity index and zeta potential of nQ was analysed by dynamic light scattering (DLS) and phase analysis, light scattering, using a Zetasizer Nano-ZS equipped with 4.0 mW, 633 nm laser (Model ZEN3600, Malvern Instruments Ltd., Malvern, UK). Briefly, 1 mg/ml of bulk quercetin and nQ solution were prepared in double distilled water, followed by sonication for 30 s (PCI Analytics probe sonicator, Mumbai, Maharashtra, India). Zeta potentials of bulk and nQ were measured at 25 °C as per the above described protocol using the same instrument. All measurements were performed in triplicates. Further, the crystallographic structure of the nQ formulation was determined using X-ray diffractometer (Rigaku Miniflex 600, Tokyo, Japan). Measurements were performed at a voltage of 40 kV and 25 mA. The scanned angle was set from $2^\circ \leq 2\theta \leq 50^\circ$, and the scanning rate was 1° min^{-1} . All measurements were made in triplicate.

Solubility and Stability of nQ

Sedimentation kinetics of equivalent quantity of bulk quercetin and nQ suspended in PBS (3 mM) has been observed at different time points. The solutes were mixed properly at 0 hr and kept for observation at different time points to monitor aqueous solubility.²⁰ The stability of nQ with varying temperature and pH was also studied using high performance liquid chromatography (HPLC) according to the earlier described method.²³ For stability assay at a fixed concentration of 40 $\mu\text{g/ml}$ (at a total of 10 ml solution) were prepared in PBS (3 mM) at three pH (2.0, 7.4, 8.0) and incubated at 37 °C for a total of 12 h. At pre-determined time points, 100 μL aliquot of nQ (40 $\mu\text{g/ml}$) were taken out and diluted with 900 μL of methanol for HPLC analysis.

Animals

Female BALB/c mice, 6–8 weeks of age and free of murine-specific pathogens, were obtained from the animal breeding colony, CSIR-IITR, India. All experimental animals used in this study were maintained under a protocol approved by the Institutional Animal Ethics Committee of CSIR-IITR, India.

In-Vivo Pharmacokinetics of nQ

Animal studies were carried out to understand the pharmacokinetics of delivering bulk quercetin and nQ to female

BALB/c mice, according to the method described earlier.²⁰ BALB/c mice weighing 20–25 g were used. Mice were divided into two groups ($n = 10$) where group 1, received bulk quercetin dissolved in the Mili-Q with Tween 20 (1%, v/v) and group 2 received nQ dissolved in the Mili-Q. Each mouse of group 1 and group 2 was injected with bulk quercetin and nQ (30 mg/kg) respectively via a lateral tail vein, and the blood from these mice was collected from retro orbital plexus at 0, 2, 4, 6 and 12 hour after injection. Serum was isolated from the blood samples of treated mice and the concentration of delivering bulk and nQ were determined by HPLC.²⁴

Animal Treatment Protocol

The induction of allergic asthma in BALB/c mice was carried out via exposure of ovalbumin (OVA) according to the method described earlier with slight modifications.²⁵ Mice were immunized intraperitoneally (ip) with 20 μg of OVA (Sigma-Aldrich, St. Louis, MO) emulsified in 1 mg of aluminum hydroxide (DANPH Chemicals Co., Mumbai, India) on days 0 and 15. OVA sensitized mice were injected ip with 0.2, 1, 5 and 25 mg/kg/day of nQ in 200 μL of PBS each day for days 18–20 on consecutive days and designated as a OVA + nQ group. The group sensitized with OVA but not treated with nQ was designated as OVA group. The group treated with saline only was designated as a control group. All the groups of mice i.e., OVA, OVA + nQ and control were challenged intranasally with OVA (10 mg/ml of saline) each day for days 21–23 to ensure the therapeutic efficacy of nQ.²⁵ Blood was collected from retro orbital plexus of mice and tissue samples were taken 24 h after the last challenge to study the allergic parameters. The assay of specific IgE and IgG1 were carried out for selection of optimal dose of nQ (1 mg/kg). A graphical presentation of the animal treatment protocol is shown in Figure 3.

Total Serum IgE Assay

Total IgE (tIgE) was estimated with the Optia mouse IgE kit (BD Biosciences, Heidelberg, Germany) according to the manufacturer's instructions. Briefly, 96 well Nunc maxisorp microtiter plates (Nunc, Roskilde, Denmark) were coated with 100 μL of antimouse IgE antibody at 1:250 dilution in 0.1 M carbonate buffer (pH 9.5) and kept overnight at 4 °C. The plate was washed with PBS washing buffer having 0.05% Tween 20 and blocked with 3% Fetal Bovine Serum in PBS (Assay Diluent, BD Biosciences, Heidelberg, Germany). Serum samples from all the group of animals were added to the wells in triplicate. After 2 h at room temperature, the plate was washed and incubated with biotinylated antimouse IgE antibody and avidin horseradish peroxidase reagent for 1 h at room temperature. Colour was developed with orthophenylenediamine (OPD) and the reaction was stopped by adding 5 N H_2SO_4 after 20 min. The absorbance was read at 492 nm

using an ELISA plate reader (Biotek, Power Wave XS2). Total IgE levels were measured in triplicate and average value was calculated. Serum total IgE was quantitated by commercially available OptEIA kit (BD Bioscience, San Jose, CA).

OVA Specific IgE and IgG1 Assay

Specific IgE (sIgE) and sIgG1 levels against OVA were estimated by enzyme-linked immunosorbent assay (ELISA) using earlier described methods.²⁶ Briefly, the wells of microtiter plate (Nunc, Roskilde, Denmark) were coated with 1 μ g of OVA in coating buffer (0.05 M Carbonate-Bicarbonate, pH 9.6). After blocking with 3% bovine serum albumin (BSA), the plates were incubated with diluted sera (1:20 v/v for specific IgE and 1:500 for specific IgG1 respectively) of control, OVA + nQ and OVA groups and kept overnight at 4 °C. Further, the plates were washed and incubated with HRP-conjugated goat-anti-mouse IgE (1:1000 v/v) and HRP-conjugated goat-anti-mouse IgG1 (1:1000 v/v), respectively, for 3 h at 37 °C. Color was developed with orthophenylenediamine (OPD) and the reaction was stopped by adding 5 N H₂SO₄ after 20 min. The absorbance was read at 492 nm using an ELISA plate reader (Biotek, Power Wave XS2). Specific IgE and specific IgG1 levels were measured in triplicate and average value was calculated.

Measurement of Allergic Mediators in the Serum

Mediators of allergic asthma like Prostaglandin D₂ (PGD₂), cysteinyl leukotriene (CysL), mouse mast cell protease-1 (mMCPT-1) and mouse thymic stromal lymphopoietin (TSLP) levels were determined in the sera of control, OVA + nQ and OVA treated mice using commercially available EIA and ELISA kits (PGD₂ EIA kit, Cat No. 512031; CysL EIA kit, Cat No. 500390 Cayman chemicals, Ann Arbor, Michigan, USA; mMCPT-1 ELISA kit, Cat No. 555260 (BD Bioscience, San Jose, California) Ref. 88-7503-22 and TSLP ELISA kit, Ref. 88-7490-22, Ebiosciences, San Diego, CA, respectively) following the manufacturers' instructions.

Lungs Histopathology

To investigate histopathological changes in lung tissue, the mice were challenged and sacrificed by cervical dislocation and lungs were collected from control, OVA + nQ and OVA groups. Tissues were fixed in 10% formalin in saline for 24 h at room temperature, cut into 3–5- μ m thick sections and stained with Hematoxylin and Eosin. Stained sections were embedded in paraffin, and observed using a Nikon Eclipse TE2000-S microscope.

Immunohistochemistry (IHC) for Eosinophils Measurements

Detection of eosinophils in the lung tissue of control, OVA + nQ and OVA treated mice was performed using

IHC method. In brief, anti-mouse eosinophil major basic protein (EMBP (S-20): sc-18241, Santa Cruz Biotechnology, Santa Cruz, CA) was used as primary antibody and anti-mouse IgG HRP conjugate was used as secondary antibody. Further, staining with DAB (3,3' Diamino Benzidine Tetrahydrochloride) and counter staining with hematoxylin was done. The images were taken using Nikon Eclipse TE 2000-S microscope.

Real Time PCR for IL-4, IL-5 and Foxp-3 Expressions

The total RNA from the lung tissues of control, OVA + nQ and OVA groups was isolated using TRI-Reagent (Invitrogen Life Technologies, Carlsbad, CA) and cDNA were prepared using high capacity cDNA reverse transcriptase kit (Applied Biosystem, Foster city, CA). The qPCR was performed on cDNA samples using the SYBR Green system (Bio Rad, Richmond, CA). Primers used were IL-4: sense 5'-TCGGCATTGTGAACGAGGTC-3', antisense 5'-AAAAGCCCCGAAAGAGTCTC-3'; IL-5: sense 5'-TCA CCGAGCTCTGTTGACAA-3', antisense 5'-CCACACTT CTCTTTTGGCG-3'; and Foxp3: sense 5'-GCTCCCGG CCTGGTCTGCTC-3', antisense 5'-AGGTGGCGGGG TGGTTTCTGA-3' and GAPDH: sense 5'-TTCACCACC ATGGAGAAGGC-3, antisense 5'-GGCATGGACTG TGGTCATGA-3. Cycling conditions were 1 cycle at 50 °C for 2 min, 1 cycle at 95 °C for 10 min, and 40 cycles each corresponding to 15 s at 95 °C and 1 min at 60 °C. Analysis used the sequence detection software supplied with the instrument. The relative quantification value is expressed as $2 \pm \text{DCT}$, where DCT is the difference between the mean CT value of duplicates of the sample and of the GAPDH control.

Western Blot Analysis

The levels of Th1/Th2 transcription factors T-bet, GATA-3, c-maf, NfAT and SOCS-3 were studied in the lungs of control, OVA + nQ and OVA treated groups using western blot method.²⁷ Proteins from the lungs were isolated according to the method described earlier.²⁸ Goat anti-mouse T-bet; goat antimouse GATA-3 antibody (each from BD Bioscience, San Jose, USA) were used as primary antibodies. Goat anti mouse IgG, HRP conjugated was used as secondary antibody. The β -tubulin (Santa Cruz Biotechnology, Inc. Santa Cruz, CA) was used as internal control.

Lung Mast Cells Culture and β -Hexosaminidase Release Assay

The *ex vivo* relevance of nQ was carried out in the lung mast cell culture in the control, OVA + nQ and OVA groups. The lung mast cell culture was performed as per the previously described method.²⁹ Briefly, mice from the control, OVA + nQ and OVA groups were sacrificed and the lungs were collected. Lung mast cells were cultured

in Dulbecco's modified eagle medium (Sigma-Aldrich, St. Louis, MO) supplemented with 50 ng/ml IL-3 (Santa Cruz Biotechnology, Inc. Santa Cruz, CA) and 10 ng/ml stem cell factor (Sigma Life Science, USA). After 4 weeks, lung mast cells were stained with toluidine blue (Sigma-Aldrich, St. Louis, MO) to confirm its morphology. The β -hexosaminidase release in the lung mast cells were studied following the exposure of 10, 20, 30, 40 and 50 μ g OVA according to the previously described method.³⁰

Signaling Events in the Mast Cells

We further studied the role of nQ in the mast cell signaling pathway in control, OVA + nQ and OVA groups. For this study, we have carried out western blotting analysis of signalling molecules Fc ϵ R1, Syk, PKC, p-PKC, c-Yes, c-Fgr, Lyn, PI-3, p-PI-3, PLC- γ 2, and p-PLC- γ 2 (Santa Cruz Biotechnology, Inc. Santa Cruz, CA) of mast cells. The β -actin (Santa Cruz Biotechnology, Inc. Santa Cruz, CA) was used as internal control.

Statistical Analysis

Significant differences between mean values were assessed by means of ANOVA, followed by the Bonferroni's multiple comparison tests. *P* value less than 0.05 was considered to be significant.

RESULTS AND DISCUSSION

In this study, we have successfully demonstrated the enhanced bioavailability, solubility and counteractive efficiency of quercetin by synthesized quercetin

nanocrystals (nQ). Further, effect of nQ has been established by studying the pharmacokinetics, stability as well as anti-asthmatic property in the OVA induced allergic asthma mice. More importantly, mast cells, one of the significant pathological features of asthma, were found to be decreased by nQ leading to diminished asthma symptoms.

Figure 1(A) shows the FTIR spectrum of nQ and bulk quercetin. The IR bands at 676 cm^{-1} , 724 cm^{-1} , 792 cm^{-1} and 820 cm^{-1} are assigned to the C–H (s) (bending vibration) bonding of aromatic hydrocarbons in quercetin structure.³¹ The IR band at 1660 cm^{-1} attributed to carbonyl (–C=O) group, whereas band at 1601 cm^{-1} indicate the presence of C–O group. The multiplets between 1312–1005 cm^{-1} is due to C–O and multiplets in the range of 1559–1444 cm^{-1} is assigned to C=C group of aromatic ring.³² The FTIR spectra of nQ shows the presence of C=C and C=O moieties and other IR bands as observed in bulk quercetin. It can be seen clearly that there is no significant difference in the IR spectrum of nano and bulk quercetin indicating that native structure of quercetin remains intact in nano form. Therefore, it may be assumed that therapeutic pathways of nQ should not get affected compared to bulk on the basis of functionality. Further, the optical properties of bulk quercetin and nQ were studied using UV-Vis absorption spectroscopy (Fig. 1(B)). Bulk quercetin showed two absorption peaks at 256 nm and 372 nm corresponding to the benzoyl moiety and Cinnamoyl system respectively. However, nQ showed absorption peaks at 266 nm and 387 nm, which were slightly shifted compared to bulk quercetin. The shift and peak intensity for nQ could be attributed to the conformational

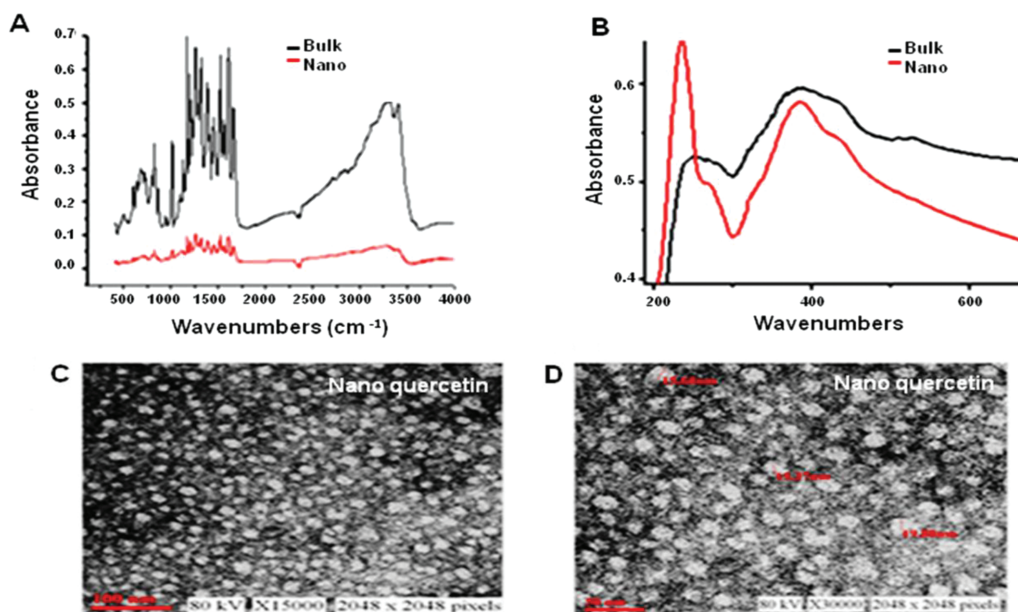


Figure 1. FTIR, Ultraviolet and Visible spectra and TEM of bulk and quercetin nanocrystals (nQ): (A) FTIR spectra of bulk quercetin (black), and quercetin nanocrystals (red). (B) Ultraviolet and Visible absorbance spectra of Bulk quercetin (black) and quercetin nanocrystals (red) at a fixed concentration of 1 mg/ml. (C), (D) Transmission electron micrograph (TEM) of quercetin nanocrystals at 100 nm and 50 nm scale.

changes in nQ compared to bulk quercetin. Flavanoids and polyphenolic compounds exhibit two distinctive bands, in a broad range of 240–400 nm where, Band I is considered to be associated with absorption due to B ring at 350–370 nm, and Band II is attributed to the benzoyl moiety with an absorption range of 240–280 nm.³³ It is shown that quercetin absorbs UV radiation with absorbance maxima in the UV-A ($\lambda_{\text{max}} = 365 \text{ nm}$, $\varepsilon = 28400 \text{ M}^{-1} \text{ cm}^{-1}$) and UV-C range ($\lambda_{\text{max}} = 256 \text{ nm}$, $\varepsilon = 28300 \text{ M}^{-1} \text{ cm}^{-1}$), thus results in photo protective mechanism of UV radiation, thereby preventing the formation of reactive oxygen species (ROS) and consequent DNA damage both *in vivo* as well as *in vitro*.^{33,34} The results of UV-Vis absorption spectra obtained in our experiments are in good agreement with reported works (Fig. 1(B)).^{33,34} TEM images showed the formation of nearly spherical monodispersed nQ with size of $16.95 \pm 1.3 \text{ nm}$ as shown in Figures 1(C)–(D). It has been reported that higher zeta potential helps the particles to repel each other, which ensure long term stability and avoid particle aggregation.³⁵ The zeta potential of the nQ ($-44.1 \pm 6.82 \text{ mV}$) was found to be higher compared to zeta potential of bulk quercetin ($-24.9 \pm 6.05 \text{ mV}$) corroborating the stability of nQ in water and is in good agreement with reported results (Data not shown).³⁶ The structure and crystalline analysis of nQ and bulk quercetin

was carried out using XRD that showed the well-defined diffraction patterns of nQ and native quercetin powder (Data not shown). These observations confirm the phase purity of nQ and ensure all the physical characteristics of bulk quercetin.

The pharmacokinetics of nQ were studied *in vivo* using well dispersed solution of nQ in aqueous media. Prior to *in vivo* studies, the stability of nQ in aqueous media was established using sedimentation kinetics. A known amount of bulk quercetin and nQ was dispersed in PBS (3 mM) and assayed with sedimentation kinetics at a time interval of 2, 4, 6 and 12 hours. It has been observed that sedimentation rate of nQ was significantly slow, indicating excellent solubility compared to bulk quercetin, which is poorly soluble in aqueous media (Fig. 2(A)). The suspending quality of quercetin increased by 50% in nanocrystal as it is clear from Figure 2(A) that at 6 hrs bulk quercetin settled down to 100% where as in case of nQ the settling rate was 50% only. Increased dispersibility has been reported to be linked with the enhanced bioavailability of the compound.³⁷ Similar to nQ other nanotized particles like curcumin have also been reported to exhibit increased dispersibility in aqueous medium.²⁰ In an attempt to study the biodegradation and instability properties of nQ, we incubated nQ in PBS (3 mM; pH 2, 7.4 and 8.0) and estimated

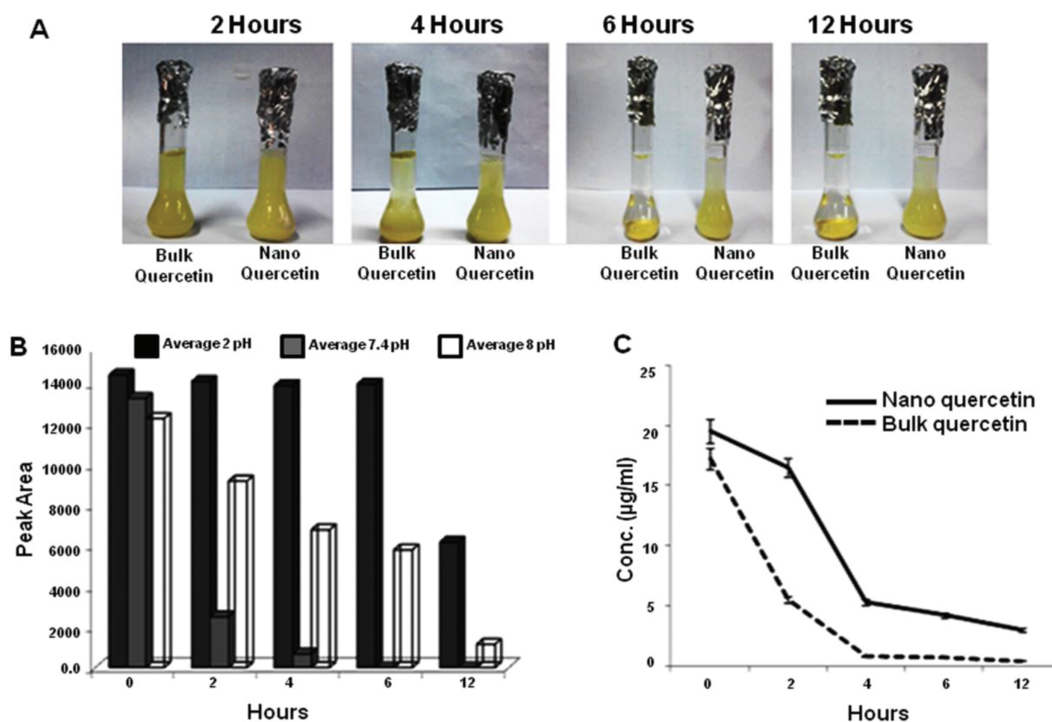


Figure 2. Solubility, stability and *in vivo* bioavailability of bulk and nanoparticulate quercetin: (A) Solubility study of quercetin (bulk and in formulation) in PBS. Bulk and nano Quercetin dissolved in PBS (3 mM) was insoluble in aqueous solution while equivalent quantity of nQ was fully soluble in aqueous solution. (B) Stability of nQ in PBS (3 mM; pH 2.0, 7.4 and 8.0) at 37 °C. (C) *In vivo* pharmacokinetics of bulk and nQ. The mice were divided into two groups ($n = 5$). Equivalent concentration of Bulk quercetin and nQ (30 mg/kg) was given to group 1 and group 2 mice respectively. Bulk quercetin and nQ were administered intravenously and blood was collected at different time intervals. Serum was separated and the concentration of quercetin was determined by HPLC analysis, as described in materials and methods.

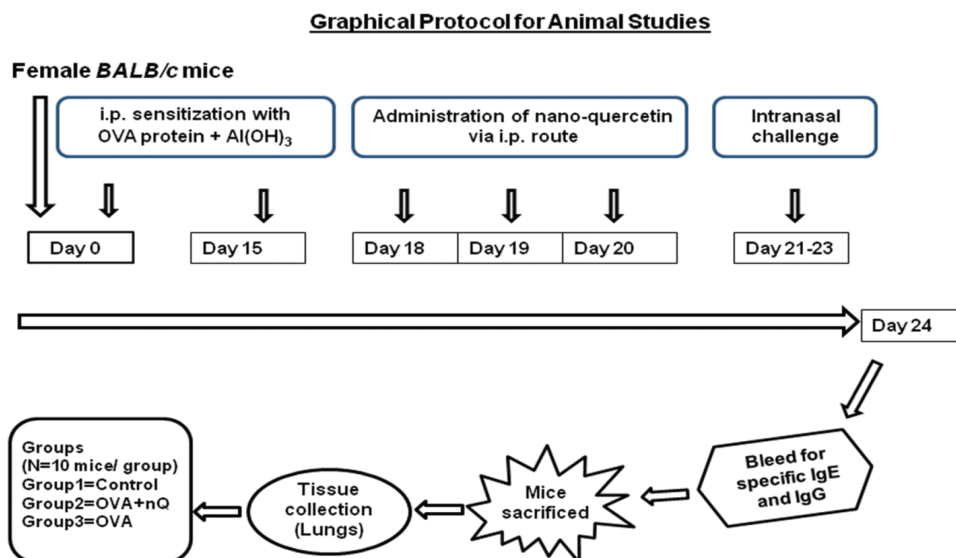


Figure 3. Graphical protocol for animal studies.

its concentration at different time points by HPLC. The nQ was found stable up to 12 h at pH 2.0 and 8.0 whereas at pH 7.4 nQ was stable up to 4 h only. The stability of nQ at different pH was in following order: 2.0 > 8.0 > 7.4 (Fig. 2(B)). Further, *in vivo* pharmacokinetics studies in BALB/c mice revealed the presence of nQ concentrations as 19.5, 16.5, 5.2, 4.2, and 2.9 $\mu\text{g/ml}$ in blood sera at 0, 2, 4, 6 and 12 h, respectively, following treatment of nQ (30 mg/kg via tail vein) which is much higher compared at all time points to bulk quercetin (Fig. 2(C) and Table I). The results clearly indicate that the bioavailability of nQ increased for a longer time compared to bulk quercetin. Among the bioavailability studies of polyphenolic compounds, quercetin received more attention due to its potential therapeutic effects but requires enhanced bioavailability and dispersibility.³⁸ Since bulk quercetin can be rapidly metabolized from plasma and other organs thus its effect gets restricted.³⁹ Therefore, the enhanced bioavailability and dispersibility for longer time in blood sera as shown in the present study indicates the superiority of nQ over bulk quercetin.

Enhanced level of allergen OVA specific IgE in mucosal tissues induces accumulation of mast cells with infiltration of eosinophils, basophils and macrophages, which is a fundamental feature of allergic asthma. Further, mechanism of class switching of IgE is a crucial event in the pathological progress of asthma that may lead to Th2 reactions.⁴⁰ Therefore, the levels of IgE and IgG1 have

to be evaluated for the pathogenesis of asthma. Animal models provide powerful tool in asthma research and to identify and evaluate novel therapeutic targets.⁴¹ OVA is widely used to induce an IgE response in mouse model of allergic asthma.⁴¹ Therefore, in present study OVA was used to sensitize and challenge BALB/c mice, as this strain has been reported to be one of the high IgE responder.⁴² OVA sensitized mice were injected ip. with 0.2, 1, 5 and 25 mg/kg dose to ascertain the optimum dose of nQ for suppression of IgE and IgG1 production. The levels of sIgE and sIgG1 were found lowest in the OVA group after 1 mg/kg dose of nQ. Further studies on various allergic parameters were therefore carried out using 1 mg/kg dose of nQ as an optimal dose. Earlier studies have shown to be effective dose of bulk quercetin in between 8–16 mg/kg for suppression of allergic asthma,²⁵ whereas, comparatively very less dose of nQ (1 mg/kg) is required to suppress the asthmatic symptoms effectively. The levels of tIgE, sIgE and sIgG1 are highly important in the allergic manifestations, which were observed to be significantly ($p < 0.05$) lower in the OVA + nQ group when compared to the OVA group as shown in Figures 4(A)–(C). The reduced levels of allergen specific serum Ig concentration in OVA + nQ group demonstrate the anti-allergic potential of the nQ.

Activated mast cells concomitantly release various type of degranulation products that include toxic granule proteins, lipid mediators (PGD2 and Cys-L), chemokines and other cytokines. These products together damage nerves, epithelial and mucosal cells, with the association of bronchoconstriction, vasodilatation and mucus hypersecretion that greatly exaggerate airway inflammation.^{43–46} PGD2, CysL, mMCPT-1, and TSLP are preformed and newly synthesized proinflammatory mediators in activated mast cells that are usually encountered during the allergic asthma.^{43–47} In this study, the mice of OVA group showed

Table I. Comparison of *in-vivo* bioavailability of bulk and quercetin nanocrystals in mice sera.

Time points (hours)	0	2	4	6	12
Nanotized quercetin ($\mu\text{g/ml}$)	19.5	16.5	5.2	4.2	2.9
Native quercetin ($\mu\text{g/ml}$)	17.2	5.4	0.8	0.7	0.4

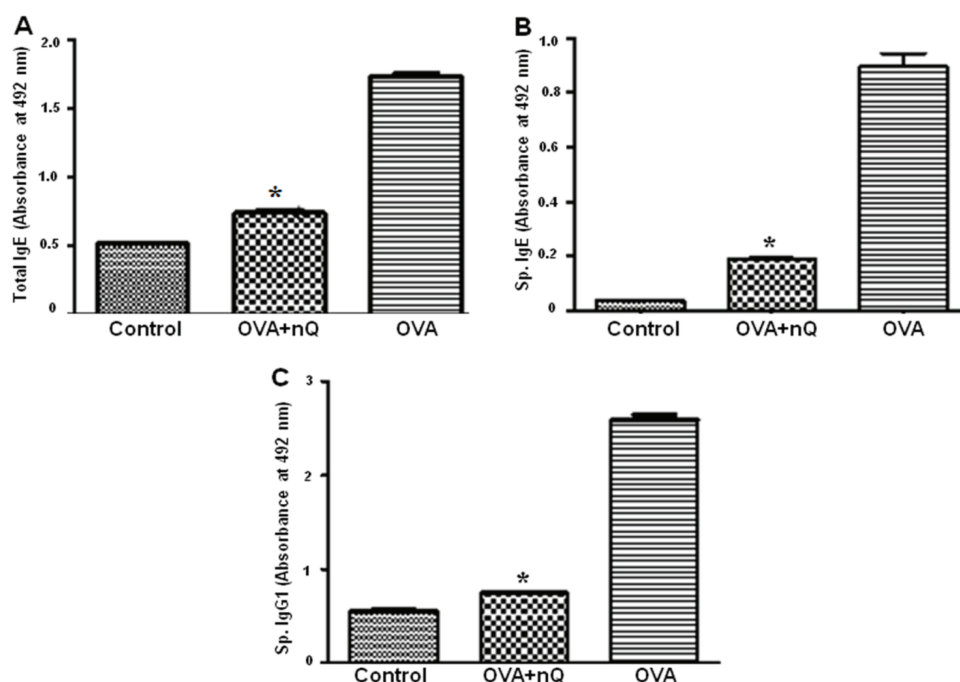


Figure 4. Immunoglobulin levels: (A) Level of total IgE in the serum of control, OVA+nQ and OVA; (B) Level of specific IgE in the serum of control, OVA + nQ and OVA; (C) Level of specific IgG1 in the serum of control, OVA + nQ and OVA treated groups. Following graphs are showing decreased levels of total IgE, specific IgE and IgG1 in the serum of OVA+nQ treated groups when compared with OVA group; The data from at least three independent experiments are expressed as the mean $\pm$ SEM. Significant difference * $p < 0.05$ when compared with OVA. Where ns = non-significant.

significantly increased levels of PGD₂, CysL, MCPT-1, and TSLP (Figs. 5(A)–(D)) while levels of these mediators returned close to control levels in the OVA + nQ group showing efficacy of nQ treatment. Accumulated mast cell followed by OVA exposure may lead to the release of above said mediators, which were efficiently reduced after nQ treatment along with other associated mediators. Earlier study has also shown that quercetin suppresses the release of allergic mediators in a peanut allergy model.⁴⁸

Lungs histopathology of OVA treated mice showed perivascular and peribronchial inflammatory cell infiltrate with mild narrowing of the bronchiolar lumen (Fig. 6(A)). Thickening of alveolar septa throughout the parenchyma was also observed in OVA treated mice compared to OVA + nQ groups. Lungs from control and OVA + nQ showed similar histology suggesting the potential action of nQ against inflammatory pathways (Fig. 6(A)). The higher concentration of mast cells including leukocyte infiltration indicated towards severe pathological changes.⁴² In the present study, the mast cell numbers were found to be increased in OVA sensitized mice in comparison to control group (Fig. 6(B)) pointing towards enhanced inflammatory pathways. The number of mast cells were found to be significantly reduced in OVA + nQ group mice suggesting that nQ is suppressed asthmatic disorders or other inflammatory responses. It is known that allergens cross-link to the IgE present on the surface of mast cells leading to degranulation and release of allergic mediators involved in

inflammatory process of allergic asthma.⁴² Based on the results of present study, the decrease in mast cell could be attributed to the downregulation of IgE by nQ. In addition, eosinophils are the other major effector cell type in IgE mediated immediate hypersensitivity, which produce cytokines, chemokines and lipid mediators possessing a high impact in aggravating airway inflammation.^{49,50} The OVA+nQ group showed significantly reduced eosinophil numbers when compared to OVA (Fig. 6(C)). Eosinophil numbers in OVA + nQ group were comparable to control level. Eosinophils, the key effector cells, has been shown to be source of numerous cytokines and chemokines such as IL-4, IL-5, IL-9 and IL-13 together with production of interferon- γ (IFN γ) and tumour-necrosis factor- α (TNF- α) supporting Th2-cell-mediated induction of increased mucus production.^{51–56} Several flavonoids including apigenin in OVA-induced asthma model have demonstrated the ability to inhibit OVA-induced increase in eosinophil counts.⁵⁷ Thus the reduction in the concentration of mast cells and eosinophils in the OVA + nQ group, compared to OVA treated animals, suggests that nQ markedly reduced the infiltration of inflammatory cells.

The inflammatory disease of the airways characterized by the presence of large numbers of CD4+ T cells that produce Th2 cytokines, which orchestrate the inflammation associated with asthma.^{58,59} In the present study, the mRNA expressions of IL-4, IL-5 and Foxp-3 were performed by Real time PCR in all the three groups. No

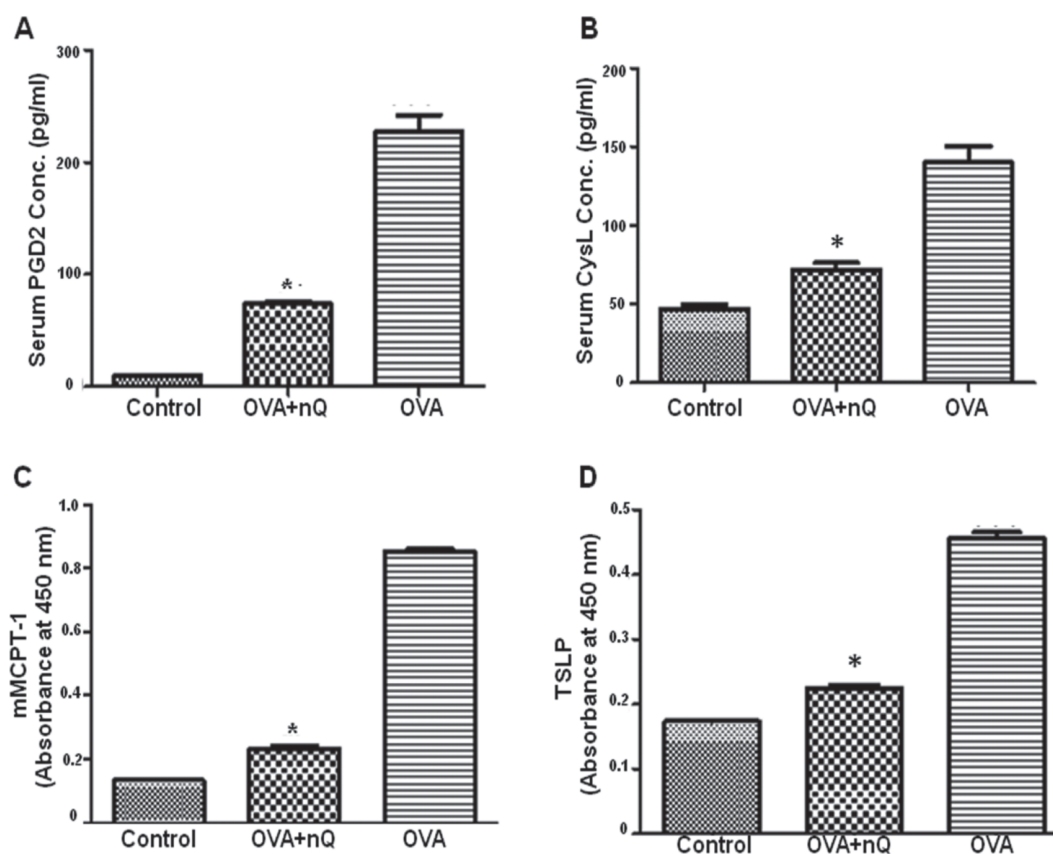


Figure 5. Serum concentration of PGD2, CysL, mMCPT-1 and TSLP: The mice of OVA + nQ group showed relatively lower levels of (A) Serum prostaglandin or PGD2 concentration, (B) Serum cysteine leukotriene (CysL) concentration, (C) Serum mouse mast cell protease-1 (mMCPT-1) and (D) Thymic stromal lymphopoietin (TSLP) when compared to OVA group. The data from at least three independent experiments are expressed as the mean $\pm$ SEM. Significant difference * $p < 0.05$ when compared with OVA. Where ns = non-significant.

significant differences were observed in the mRNA levels of IL-4 and IL-5 in OVA + nQ group and control, whereas, both of these cytokines were significantly enhanced in OVA group when compared to control (Figs. 7(A)–(B)). An up-regulated expression of Foxp3 was observed in the OVA + nQ group while it was reduced in the OVA group over controls (Fig. 7(C)). The over expression of Foxp3, family of transcription factors responsible for shutting down allergic response indicated that nQ efficiently alleviates the allergic asthma symptoms. In relation to this, earlier studies have shown that the isoquercitrin-treatment to mice showed the lowering of IL-5 levels.⁶⁰ The results of nQ treatment to OVA + nQ group showed decreased levels of IL-4, IL-5 (as observed in RT-PCR) and GATA3, which are in agreement with previous studies for bulk quercetin where levels of histamine, MMP-9, GATA-3 were also found to be significantly decreased, suggesting that the Th1 response played a dominant role in an OVA + nQ group of mice.⁶⁰

Furthermore, the Th2 transcription factors c-MAF, GATA-3, NFAT and SOCS-3 were quantified at protein levels. Significant reduced levels of c-MAF, GATA-3, NFAT and SOCS-3 were noticed in the OVA + nQ group

in comparison to OVA group (Fig. 8(A)). Figures 8(B)–(F) shows the densitometry analysis of transcription factors observed in western blot studies. The Th2 transcription factors c-MAF, GATA-3, NFAT and SOCS-3 have been reported to play a pivotal role in the fate of allergic reactions.⁶⁰ All these transcription factors play a crucial role in IL-4 and IL-5 synthesis. The down regulation of the above said transcription factors illustrate the potential of nQ to treat allergic asthma. At the same time the up-regulated expression of Th1 transcription factor T-bet was observed in the OVA + nQ that indicates the possibility of a shift toward Th1 from Th2 reactions.

In addition, β -hexosaminidase enzyme release following IgE activation is one of the significant marker of the mast cell degranulation.⁶¹ β -hexosaminidases are lysosomal enzymes involved in a variety of inflammatory diseases such as asthma and food allergy. In the present study, OVA treatment (10–50 μ g) enhanced the release of β -hexosaminidase in a dose dependent manner, whereas the release of β -hexosaminidase was found to be reduced in OVA + nQ treated group in comparison to OVA treated group (Fig. 9(A)). Further, mast cells isolated from lungs of OVA + nQ group showed suppressed the release of

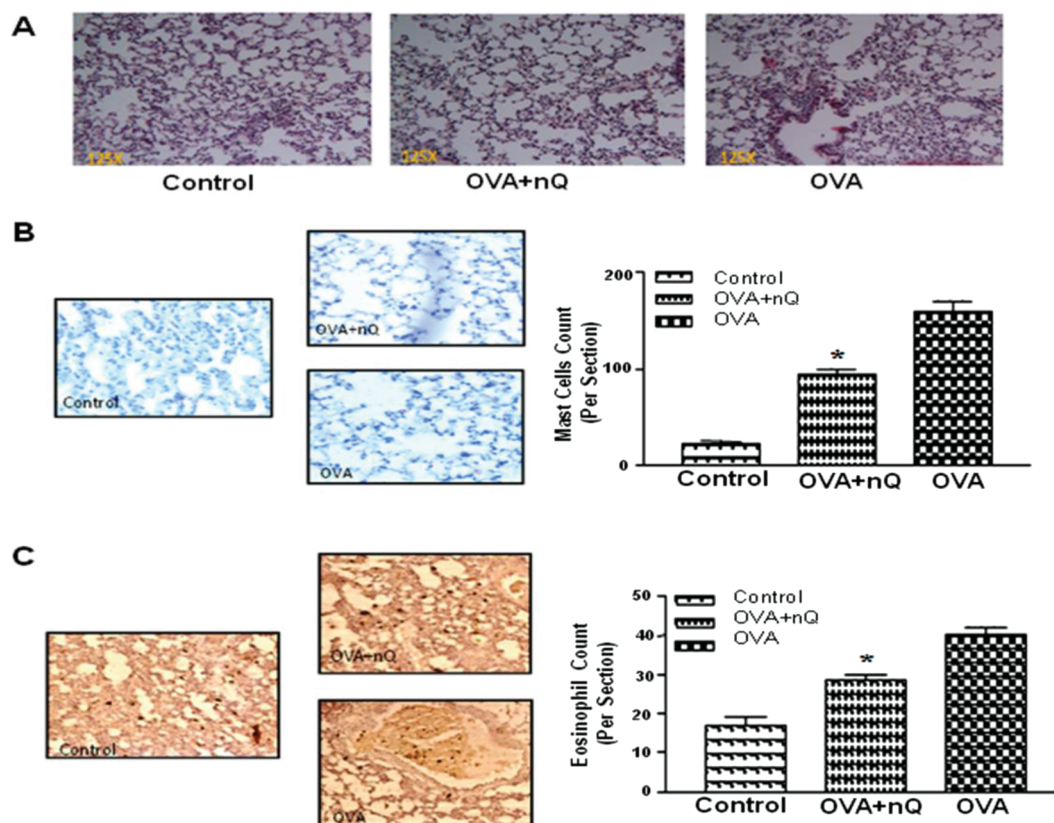


Figure 6. Histopathology, Mast cells and eosinophils counts: (A) Figure(A) shows the lung histopathology of control, OVA + nQ and OVA. The Lung histopathology of OVA mice showed perivascular and peribronchial inflammatory cell infiltrate with mild narrowing of the bronchiolar lumen as well as thickening of alveolar septa throughout the parenchyma. Mild bronchial epithelial hyperplasia along with moderate narrowing of bronchiolar lumen was observed in OVA + nQ. The control group showed normal histopathology. (B), (C) Figures (B) and (C) are showing images of mast cell staining with toluidine blue and eosinophils counts in lungs tissue. Decreased level of mast cell count along with eosinophils counts was observed in OVA + nQ group as compared to OVA as shown in graph. The data from at least three independent experiments are expressed as the mean $\pm$ SEM. Significant difference * $p < 0.05$ when compared with OVA. Where ns = non-significant.

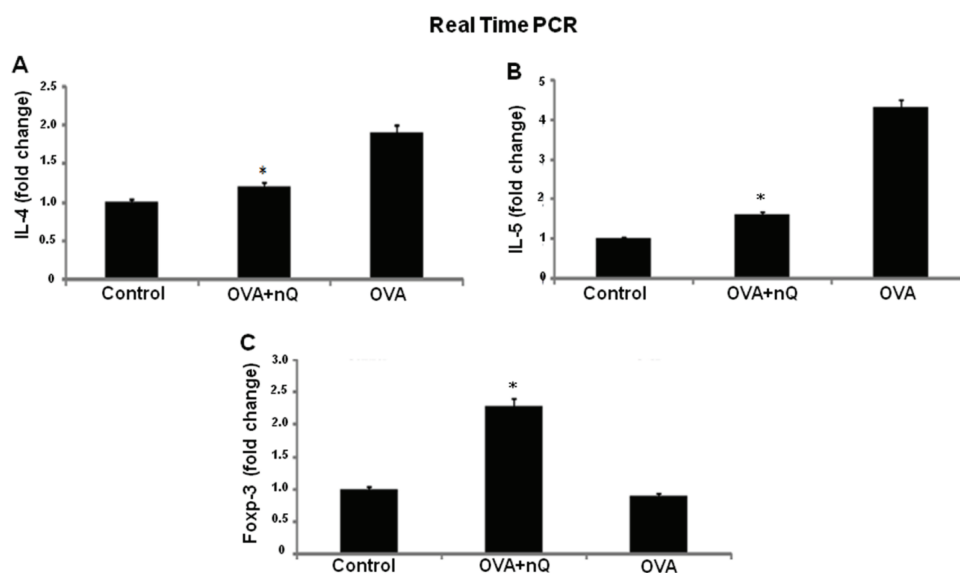


Figure 7. Real time PCR: (A), (B) RT PCR results are showing down regulated mRNA expression of Th2 cytokines (IL-4 and IL-5) and (C) Up-regulated expression of transcription factor Foxp3 in the lungs of OVA + nQ group as compared to OVA group. The data from at least three independent experiments are expressed as the mean $\pm$ SEM. Significant difference * $p < 0.05$ when compared with OVA. Where ns = non-significant.

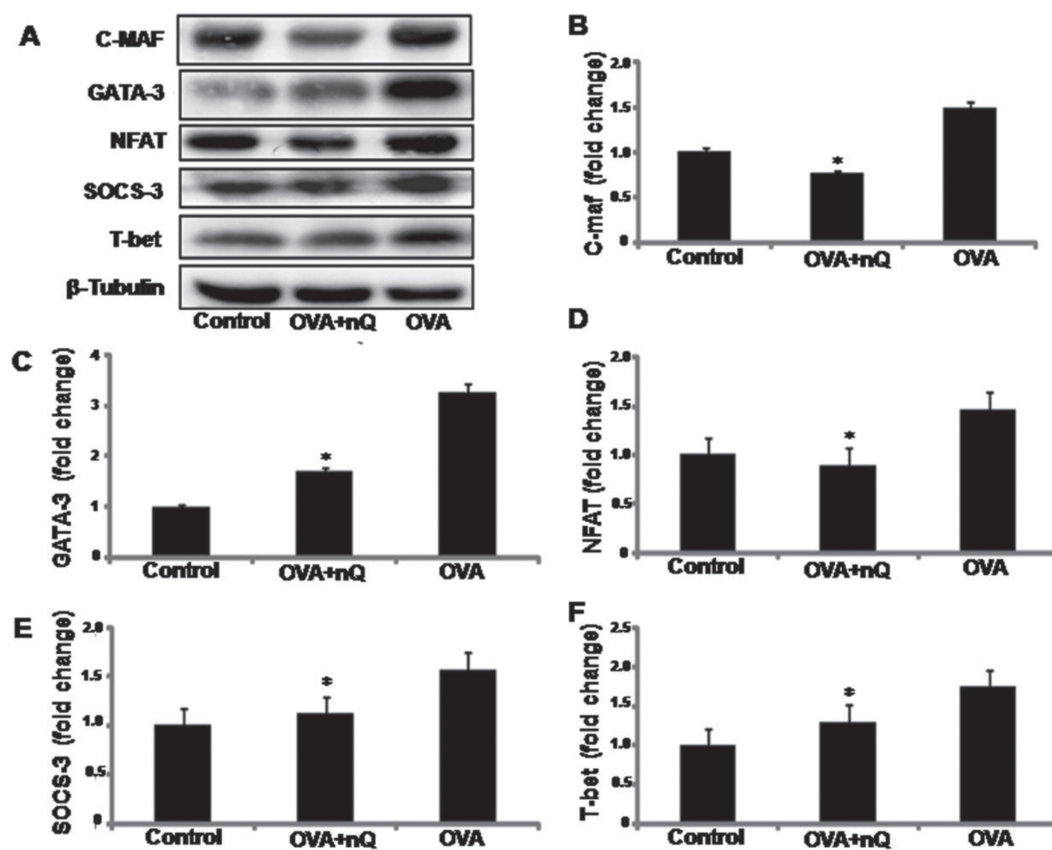


Figure 8. Western blots for transcription factors in mast cells: (A) Immunoblot results showed mixed expression of Th2 transcription factor (c-MAF, GATA-3, NFAT, SOCS-3 and T-bet), at the protein level in different groups. (B)–(F) Relative density (in fold terms) of each band measured by densitometry, showing in graph. The data from at least three independent experiments are expressed as the mean $\pm$ SEM. Significant difference * $p < 0.05$ when compared with OVA. Where ns = non-significant.

β -hexosaminidase, which suggest that even in the *ex vivo* condition nQ is equally effective.

Western blot analysis revealed significant increase in the levels of Fc ϵ R1, Syk, PKC, c-Yes, PI-3, phospho-PI-3, PLC- γ 2 and phospho-PLC- γ 2 proteins in the OVA treated group, while the levels of all these parameters returned close to control in OVA + nQ treated group when compared to control (Fig. 9(B)). There is no significant difference in PKC and PI-3 proteins but minor difference was visible for PI-3 in densitometry analysis (Data not shown). Further PKC was not supposed to change, as it is only activated in its phosphorylated form on interacting with its substrate (Fig. 9(B)). Following primary exposure of allergens in the sensitized animals, enhanced production of cytokines, especially Th2 directed class switching results in IgE release in the naïve B-cells.⁴⁰ IgE moves to mast cells/basophils and bind to the Fc ϵ R1 receptor. Following secondary exposure of same allergen, cross-linking of allergen and IgE molecules occurs in sensitized mast cells that ultimately activates the src family of protein tyrosine kinase (PTK) like Lyn and spleen tyrosine kinase (Syk).^{62,63}

Lyn is a one of the major Src family kinase involved in the regulation of degranulation of mast cells and

found to play role of positive and negative regulator during mast cell activation via the Fc ϵ RI.^{63–66} Several studies have demonstrated that Lyn has inhibitory effect on the mast cells degranulation as well as proliferation.^{65,68} Further, Lyn Kinase also inhibits the basophil proliferation, GATA-3 expression and induction of Th2 cell differentiation.⁶⁹ In the present study, enhanced level of Lyn was found in Ova + nQ treated group as compared to Ova treated group, suggesting the involvement of Lyn in the down regulation of mast cells activation and Th-2 type immune responses. We found that nQ preferably selects Lyn as a negative regulator for the suppression of mast cell degranulation. Further mechanism involved in the degranulation of mast cells include activation of multiple signaling pathways, including PI3K and phospholipase C (PLC) via Lyn and Syk phosphorylation. Moreover, the increased level of PI-3 and PLC leads to the enhanced PKC that ultimately increases Ca⁺⁺ ions, which results in mast cell degranulation and allergic reaction in the OVA induced murine model.^{70–72}

PKC, is a key enzyme of the T-cell receptor mediated signalling pathway, that regulates T-cell differentiation and T-cell mediated immune response.⁷³ Studies have revealed that PKC is involved in both Th2 as well as Th1 type

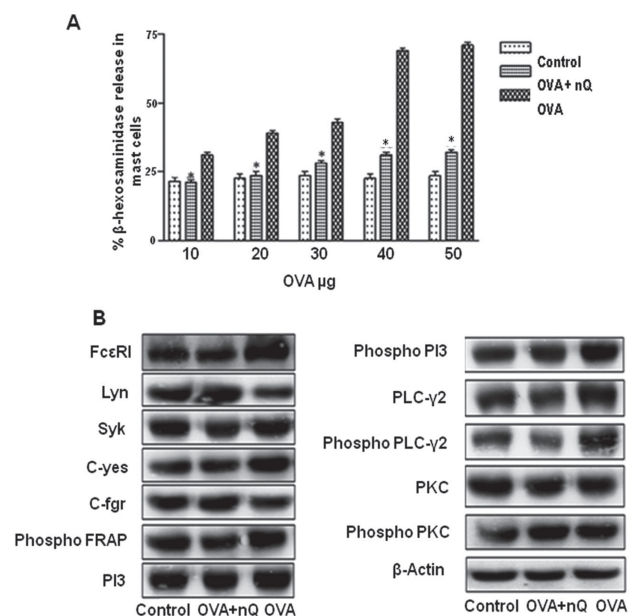


Figure 9. Western blots for signaling in mast cells: Immunoblotting of mast cell signaling molecules were carried out using their respective antibody as described in material methods. (A) β -hexosaminidase released by lung mast cell in OVA + nQ group as compared to OVA. (B) Immunoblot results showed mixed expression of signaling molecules (FcεR1, PKC, Ppkc, p-FRAP, c-Yes, c-fgr, Lyn, PI-3, p-PI-3, PLC-γ2, p-PLC-γ2 and Syk). The data from at least three independent experiments are expressed as the mean $\pm$ SEM. Significant difference $*p < 0.05$ when compared with OVA. Where ns = non-significant.

immune responses.⁷⁴ PKC is only activated in its phosphorylated form, thus, we have analyzed the activated form of PKC in our study. The level of pPKC was up regulated in OVA + nQ treated group that indicates its possible involvement in Th1 gene expression rather than Th2 genes because our entire data suggest that nQ is responsible for Th2 response suppression and Th1 immune response activation. It has been suggested that cells respond according to their micro environment.^{75,76} In present study, there is shifting of OVA induced Th2 response towards Th1 response after treatment of nQ, therefore, the micro-environment of the cells may have changing towards Th1 response. Higher expression of pPKC in OVA + nQ group may also be responsible for Th1 gene expression, which can be observed in the case of T-bet at mRNA level in nQ treated group.

Figure 10, schematically shows the probable mechanism related to the effect of nQ on mast cell degranulation like FcεR1, Syk, c-yes, PI-3, phospho-PI-3, PLC-γ2 and phospho-PLC-γ2 indicating intervention of nQ in the mast cell signaling pathways.

Several nanomedicine have been studied to cure asthma, including various antioxidants. But, nanotized quercetin may pose several advantages over other nanoparticles that supports normal respiratory health, cardiovascular health,

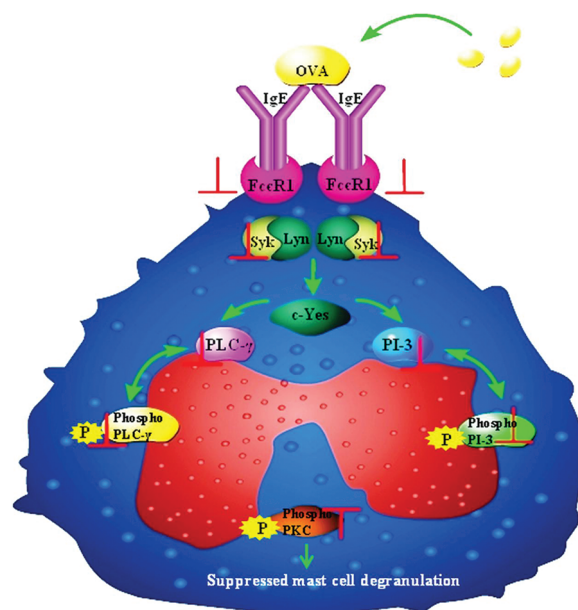


Figure 10. Schematic presentation of mast cell signaling.

promotes balanced blood pressure, reduce stress, improve upper respiratory conditions, provide nutritional support for overall health.^{16,77-82} Taken together, nQ has potential to suppress asthmatic conditions along with several other health ailments that makes it an effective therapeutic potion.

CONCLUSIONS

In conclusion, the prepared water soluble quercetin nanocrystals have shown potential as promising nanovehicle with *in vivo* and *ex vivo* stability, excellent bioavailability, and therapeutic efficacy in asthma murine model. The present study demonstrated that nQ strongly exerts suppressive effects on IgE production, inhibits the early- and late-phase allergic response, suppresses inflammation and provides improved control for allergic asthma. It has also been found to be effective in preventing anaphylaxis in murine model of allergic asthma. Furthermore, nQ inhibited the mast cell derived Th2 cytokines that include IL-4 and IL-5 and Th2 transcription factors like c-MAF, GATA-3, NFAT and SOCS-3 in OVA + nQ group. It strongly suppressed the allergic mediators like prostaglandin D₂, TSLP, mMCPT-1 and cysteinyl leukotrienes in OVA + nQ group. The nQ supports Th1 mediated immune response instead of Th2 via Lyn Kinase that inhibits the Basophil proliferation, GATA-3 expression and induction of Th2 cell differentiation. These observed results justifies that nQ is comparatively more effective than bulk quercetin. It is encouraging to suggest that nQ potentially reduced the symptoms of asthma by regulating IgE dependent mast cell degranulation, Th1/Th2 cytokine production, T-bet and GATA-3 gene expression in the OVA induced asthma model of BALB/c mice. In totality, the nQ has potential to substantially reduce the symptoms of

asthma and may play an important role in providing the most effective cure for asthma that is currently lacking. It may be considered a promising drug for the treatment of asthmatic patients, efficiently.

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