EVALUATION OF ANTI-ALLERGIC POTENTIAL OF QUERCETIN NANOCRYSTALS IN FOOD ALLERGY

A Thesis Submitted to
Babu Banarasi Das University
For the Degree of

Doctor of Philosophy
in
Biochemistry

By

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Dedicated to

My Grandparents

CERTIFICATE

This is to certify that the thesis entitled **"Evaluation of anti-allergic potential of quercetin nanocrystals in food allergy"** submitted by **Mrs. Kriti Gupta** for the award of Degree of Doctor of Philosophy in Biochemistry to Babu Banarasi Das University, Lucknow. It is a record of authentic work carried out by her under my supervision at Food Toxicology Division, Council of Scientific & Industrial Research-Indian Institute of Toxicology Research, Lucknow. To the best of my knowledge, the research embodied in this thesis is an original work done by candidate and has not been submitted elsewhere for the award of any degree or diploma.

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DECLARATION

I, hereby, declare that the work presented in this thesis, entitled **"Evaluation of anti-allergic potential of quercetin nanocrystals in food allergy"** in fulfillment of the requirements for the award of Degree of Doctor of Philosophy of Babu Banarasi Das University, Lucknow is an authentic record of my own research work carried out under the supervision of Dr. Premendra D. Dwivedi.

I also declare that the work embodied in the present thesis is my original work and has not been submitted by me for any other Degree or Diploma of any university or institution.

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DECLARATION OF PLAGIARISM CHECK

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Kriti Gupta

PREFACE

Allergy is an increasing health concern worldwide during the last few decades. A number of body's organ like skin, intestine, and lungs are found to be affected during allergic conditions. Various forms of allergy like food allergy and asthma are pathogenic immune response induced by pollen grains, dust mites, insect debris, fungal spores and foods. Food allergy is an imbalanced immune provocation induced by certain foods in sensitive individuals. It affects significant global population causing the morbidity and poor life style. Several foods including milk, egg, peanut, tree nuts, shellfish, fish, wheat, sesame seed and soy are frequently found to be associated with various allergic manifestations. These foods contain many culprit proteins also known as allergens which are responsible for more than 85% of food allergy. For instance, allergens like caseins in milk, vicillins in peanut, and ovomucoid in egg are proteinacious in nature and found to be resistant to denaturation by heat, or acid therefore, can remain intact even after processing, storage, cooking and digestion. The body's immune system responds to these allergens and produce antibodies. Together with mast cells, these antibodies cause a variety of clinical manifestations like hives itching of the mouth, lips, tongue, throat, eyes, and skin, difficulty swallowing, runny or congested nose, hoarse voice, wheezing, shortness of breath, nausea, vomiting, abdominal pain or stomach cramps, lightheadedness, and fainting.

In addition to food allergy prevalence of asthma is also on increase substantially throughout the globe. Asthma is a complex immunologic and inflammatory disease characterized by mucus hypersecretion, airway smooth muscle (ASM) remodelling with hypertrophy, hyperplasia, and cytokine secretion, and activation of inflammatory cells, including mast cells, T cells, eosinophils, and neutrophils. The symptoms of asthma include airway inflammation, airway wall remodelling, and bronchial hyperesponsiveness (BHR). Allergic asthma is the most prevalent form of asthma that is typically triggered by allergens like pollen, mold, dust mites and foods. A significant number of asthmatic patients also posses allergic sensitization to various foods.

Several therapeutic approaches are under scanner for the treatment of allergic diseases. In this regards agents of natural origin like polyphenols are considered as effective anti-allergy drug which is capable of influencing the multiple biological pathways and immune cell functions during the allergic immune response. The interaction of polyphenols with allergic proteins can modulate the process of allergic sensitization and suppress the activity of effector cells including mast cells, resulting in the alleviation of clinical symptoms. Among polyphenolic compounds, quercetin received more attention due to its potential therapeutic effects. Since quercetin can be rapidly metabolized from plasma and other organs, its effect gets restricted. However, considering the pharmacological activity and small side effects of quercetin, we hypothesize that quercetin nanocrystals (NQ) may be a potential natural drug for asthma and food allergy treatment. To accomplish this hypothesis, firstly we prepared and characterized water soluble quercetin nanocrystals that are poised to show enhanced bioavailability, aqueous solubility and therapeutic potential. The prepared quercetin nanoparticles were characterized by several techniques including dynamic light scattering (DLS), Transmission electron microscopy (TEM), X-ray diffractometer (XRD), Uv-Vis spectroscopy (UV) and Fourier transform infra-red (FTIR) spectroscopy for analysing the size, shape, structure and functionality. This study reveals that the prepared quercetin nanocrystals characterized with FTIR, UV, DLS, and TEM clearly showing 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. Afterthat, the solubility and stability of NQ with varying temperature and pH was studied in PBS using high performance liquid chromatography (HPLC). This study confirms that sedimentation rate of NQ was significantly slow, indicating excellent solubility compared to bulk quercetin, which was found to be poorly soluble in aqueous media**.** To study the *in vivo* pharmacokinetics of NQ a set of mouse designated as group 1 and group 2 was injected with bulk quercetin (BQ) 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. Similarly, it has been found that the presence of NQ

concentration in blood sera is much higher when compared at all time points to bulk quercetin. We also performed more specific extraction cum RP-HPLC procedure for the quantitative determination of NQ in serum as well as in different tissues of mice. Here we found that the HCl extraction method was 2-3 fold more efficient than DMSO: MeOH mix method. In addition, we also investigated well characterized NQ on OVA induced BALB/c mouse model of allergic asthma and food induced intestinal anaphylaxis using various allergic parameters, like serum Immunoglobulin(Ig) concentration, histopathological changes of lung and intestine tissue, mediator release assay, mast cell signaling. The finding of the study demonstrate that, a very small dose of NQ (2.5 mg/kg body weight) in case of food allergy and NQ (1 mg/kg) in case of asthma attenuates the allergic immune responses associated with the development of OVA induced food allergy with intestinal anaphylaxis and allergic asthma in a BALB/c murine model.

Possible application of research:

- Nanotized quercetin may provide numerous benefits, including improved efficacy, bioavailability, reduced toxicity, dose–response, targeting ability, personalization, and safety as compared to its bulk form.
- Treatment with quercetin nanocrystals would be also efficient to prevent other allergic manifestation like allergic rhinitis, atopic dermatitis, eosinophilic gastrointestinal disorders and other health concerns.
- Our finding also demonstrate that the method develop here would be implicated to estimate the other flavonoids in mice as well as in humans.

Suggestions/direction for future research:

Conclusively, a simple, highly sensitive and more specific extraction cum RP-HPLC procedure for the quantitative determination of NQ in serum as well as in different tissues of mice has been reported. This work deals with the additional steps of extraction of nanotized quercetin, which enhances the detection by two folds. Thus we feel that this work not only has the novelty, but has the utility. The

promising *in vivo* results gathered here for quercetin nanocrystals should refocus our efforts on other inflammatory diseases.

- The present HCL method also finds applicability in the extraction of other flavonoids.
- Clinicians and pharmaceuticals industry will also get advantage to this research work so that possible future advance treatment will be formulated to improve human health.
- Clinically effective and cost effective capsules may be developed using nanotized quercetin with anti-allergic potential.
- Consumption of nanotized quercetin as a supplement powder may provide relief from the allergic manifestations in the susceptible individuals.

List of Research papers / Publications:

1. **Gupta, K.,** Sharma, A., Gupta, R., Dixit, S., Singh, S.P., Das, M. and Dwivedi, P.D., 2018. Simple extraction cum RP-HPLC method for estimation of nanotized quercetin in serum and tissues of mice. *Pharmaceutical Chemistry Journal*, Vol. 52, Issue 2, pp.175-181.
2. **Gupta, K.,** Kumar, S., Gupta, R.K., Sharma, A., Verma, A.K., Stalin, K., Chaudhari, B.P., Das, M., Singh, S.P. and Dwivedi, P.D., 2016. Reversion of asthmatic complications and mast cell signalling pathways in BALB/c mice model using quercetin nanocrystals. *Journal of Biomedical Nanotechnology*, Vol. 12 Issue 4, pp.717-731.

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ABBREVIATIONS

µg	Micro gram
µl	Micro liter
µM	Micro molar
nm	Nanometer
mg	Miligram
ml	Mililitre
mM	Miliomolar
ANOVA	Analysis of variance
BSA	Bovine serum albumin
BQ	Bulk Quercetin
CBA	Cytometric Bead Array
CD4	Cluster of differentiation 4
CysL	Cystinyl leukotriene
DLS	Dynamic light scattering
DMEM	Dulbecco's modified eagle medium
EDTA	Ethylenediaminetetraacetic acid
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbent assay
FACS	Fluorescence activated cell sorting
FAHF	Food Allergy Herbal Formula-
FA	Food allergy
FIA	Food induced anaphylaxis
FTIR	Fourier transform infra-red
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HPLC	High Performance Liquid Chromatography
H_2O_2	Hydrogen peroxide
HRP	Horse radish peroxidase
IP	Intraperitoneally
IFN-γ	Interferon gamma
IL-4	Interleukin-4
Ig	Immunoglobulin
IgE	Immunoglobulin E

IITR	Indian Institute of Toxicology Research
LOD	Limit of detection
LOQ	Limit of quantification
Mol wt	Molecular weight
MCP-1	Monocyte chemotactic protein-1
MMCP-1	Mouse mast cell protease-1
MMP-9	Matrix metallopeptidase 9
NQ	Quercetin nanocrystals
PBS	Phosphate buffer saline
PLC	Phospholipase C
PGD2	Prostaglandin D2
PTK	Protein tyrosine kinase
PVDF	Polyvinylidene difluoride
OPD	Orthophenylenediamine
OVA	Ovalbumin
RIPA	Radioimmunoprecipitation assay
RAGE	Receptor for advanced glycation end products
RT	Room Temperature
RT-PCR	Real-time polymerase chain reaction
SDS	Sodium dodecyl sulphate
PKC	Protein kinase C
PA	Phosphoric acid
PAGE	Polyacrylamide gel electrophoresis
SEM	Standard error mean
Syk	Spleen tyrosine kinase
TEM	Transmission electron microscopy
TEMED	N N N’ N’ Tetra methyl ethyl diamine
Th1/Th2	T helper 1/ T helper 2
Tris	Tris-(hydroxymethyl) aminomethane
TSLP	Thymic stromal lymphopoietin
TNF-α	Tumor necrosis factor alpha
UV-VIS	Ultraviolet–visible spectroscopy
XRD	X-ray diffractometer

Chapter 1

Review of Literature

CHAPTER 1

REVIEW OF LITERATURE

1.1 Allergy & its Prevalence

Allergy is a serious, life threatening health concern developed due to imbalance in the immune system caused by environmental substances in susceptible individuals. It is an increasing health concern worldwide in both industrialized and non industrialized area (Amy et al., 2009). Allergic disorders are relevant and familiar health complaints with an increasing incidence. Among allergies, food allergy, rhinitis, asthma, dermatitis, eczema and arthritis appeared to be most problematic in western countries (Sicherer et al., 2011). About 30–40% of populations have been found to be affected by the different allergic diseases including allergic rhinitis, asthma, allergic conjunctivitis, eczema, and food allergy (Pawankar et al., 2011). Allergens are substances responsible for induction of allergy in the susceptible individuals. These substances belong to pollens, dust mites, molds, danders, foods, and medicines (Kumar et al., 2012). Allergies are characterized by adverse immunologic reaction to environmental factors as well as genetic factors (Ozdemir et al., 2009). The interaction of sensitizers such as indoor and outdoor allergens, air pollution, and various infections with immune system leads to various immune disorder (Kawai et al., 2007). Although the genetic factors are also involved in both adaptive as well as innate immune responses related to allergies.

Immunoglobulin E (IgE) is the most important immunoglobulin involved in the allergic reactions. Most of the allergic reactions are IgE mediated or type I hypersensitivity reactions. Due to its quick onset type I allergic reactions are also known as immediate type hypersensitivity. The mechanisms of allergic reactions involve the interaction between IgE and allergen that cause release of allergic mediators like histamine, prostaglandin D2 and leukotrienes following a cascade of molecular events (Janeway et al., 2001). The symptoms of allergy can be mild to severe including allergic rhinitis, allergic conjunctivitis, abdominal pain, vomiting, diarrhea, asthma, anaphylaxis and sometimes death. Several therapeutic approaches are under scanner for the treatment of allergic diseases (Kumar et al., 2012). According to World Allergy Organization (WAO), allergy is one of the leading causes of morbidity and mortality worldwide affecting 10-40% of world population (Pawankar et al, 2013). In Europian countries, more than 150 million individuals suffer from chronic allergic diseases and EAACI predicted that about half of the Europeans population may be affected by 2025. In UK, the rise in the prevalence of allergic diseases has continued affecting upto 20% of the population by one or more allergic diseases (Levy et al., 2004). National Health Interview survey shown that 5.6% or 4.1 million children reported food allergies in 2012 in U.S. Worldwide, food allergy affects upto 3-6% of children and pattern of food allergy depends upon various factors like dietary habbits, way of eating, ingestion of allergenic foods etc. In the UK, peanut is the most prevalent allergen with 1 in 40 breast fed infants followed by 1 in 20 with egg allergy (BSACI, 2011). Among food allergens, the most common food product is cow milk followed by peanuts and tree nuts that cause

severe allergic reactions like anaphylaxis in infants, children's and mixed population (Sicherer et al., 2006; Bock et al., 2007).

It is generally accepted that the prevalence of food anaphylaxis has been increasing in recent decades, particularly in western countries like US, UK, and Australia. About 1.5-50 per 100,000 individuals per year have experience severe allergic reactions like anaphylaxis (Yang et al., 2017). Earlier studies showed the rise in the prevalence of food induced anaphylaxis in past two decades to 2012 in UK. The rate of hospitalization of anaphylaxis patients increases upto 615 % alone in UK (Turner et al., 2015). Worldwide, up to 20% of individuals experience a fatal allergic reaction due to anaphylaxis. The occurrence of Anaphylaxis-type reactions is approximately 1 in 1000 of the general population. The incidence of anaphylaxis due to general anaesthesia occurs in 1 in 10,000–20,000 anaesthetics (Harper et al., 2018).

In addition to food allergy, asthma is considered as another severe allergic disorder with an increasing prevalence affecting 300 million individuals worldwide (Pawankar et al., 2006). The current prediction is that by 2025 upto 400 million of general population will be affected. Asthma is the leading cause of mortality each year among allergies (Pawankar et al., 2013). In 2012, the estimated prevalence of respiratory allergies was 10.6% or 7.8 million children in the past 12 months. In 2017, 5.4 million people, 1.1 million children (1 in 11) and 4.3 million adults (1 in 12) reported in the UK were reported to be diagnosed with asthma (Paul et al., 2012). Allergic rhinitis is another most common form of allergy characterized with sleep problems, including micro-arousals, leading to daytime fatigue and somnolence, and decreased cognitive functioning. In 2012, 7.5% or 17.6 million adults and 9.0% or

6.6 million children reported hay fever in the past 12 months. Approximately 30% adults and 40% children of total population were diagnosed with hay fever in 2013 (Pawankar et al., 2013). In past three decades, the rise in incidence of allergic eczema has continued in the industrialized world. Approximately, allergic eczema affects 15-30% in children and 2-10% in adults of the population (Pawankar et al., 2013). More than 45% of children have onset of atopic eczema during the first 6 months of life. During the first year 60% of these children are affected, and 85% are found to be affected before the age of five (Pawankar et al., 2013). The prevalence of allergy is worldwide, but still a permanent cure for allergic reactions is not available except strict avoidance of the offending allergens and symptomatic treatment of any adverse effects from accidental exposures (Otsu et al., 2012).

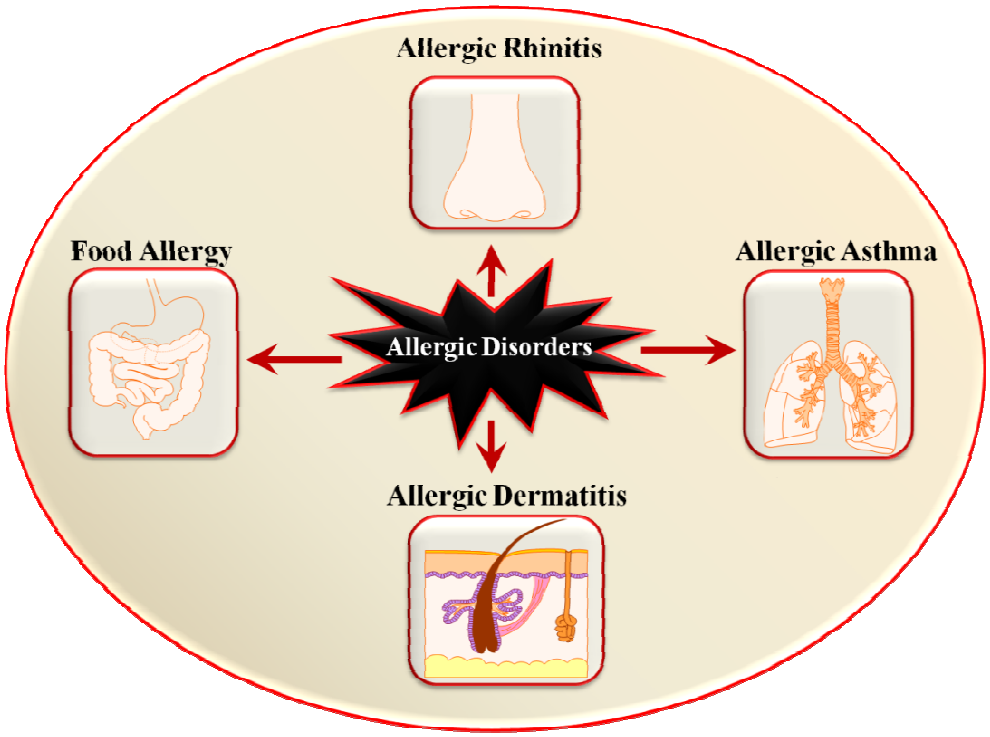

Figure 1.1: Different allergic disorder (Skin, Food, Respiratory, Nasal)

1.2 Types of allergy

Recent years have witnessed a marked increase in Japanese cedar pollen (JCP) allergic disease or pollinosis in Japan and it was estimated that approximately 23 million patients suffer from JCP from February to April, each year. JCP is a potent allergen that causes seasonal allergic rhinitis, asthma, rhinoconjunctivitis, and atopic dermatitis, in spring in Japan year after year (Okamoto et al., 2009; Maeda et al., 2008; Ozasa et al., 2002; Masuda et al., 2004). Among all IgE mediated allergies worldwide, cat allergy is one of the most prevalent allergies. The symptoms of cat allergy comprise asthma, conjunctivitis, hay fever, sneezing, chronic sore throat, watery eyes, and nasal congestion (allergyclinic.co.uk). More than five allergens from cat have been reported that are found to be responsible for inducing allergic reactions including two major allergens Fel d1 and Fel d4. The Fel d1 is a secretoglobin secreted by the cat's sebaceous glands and squamous epithelial cells (Ohman et al., 1974). The Fel d4 is an alipocalin found to be expressed in saliva, perianal, and lachrymal glands (Smith et al., 2004). The Fel d2, an albumin, Fel d3, a cystatin, and cat IgA have been reported as other allergens involved in cat allergy (Ohman et al., 1974; Ichikawa et al., 2001; Adédoyin et al., 2007). In addition panic effect of bee venom can be easily visualized by a majority of the population. Allergen Specific Immunotherapy (ASIT) with bee venom is associated with the risk of inducing systemic side-effects, redness and local swelling (Mellerup et al., 2000). Moreover, administration of T cell epitope peptides of allergen of interest to ASIT reduces the risk of ASIT-related systemic reactions. The use of peptides of bee venom improves safety and efficacy and may be an useful approach to combat allergenic manifestations induced by bee venom. The bee venom phospholipase A2

or PLA (Api m 1) is one of the key allergens in bee sting allergy (Carballido et al., 1993). Further, House dust mite allergy is the most awful form of allergy causing several health complications in the susceptible individuals. These are small microscopic organisms highly abundant in all homes, especially in warm and humid environments that presents optimal conditions for their growth; on the other hand, house dust mite can survive and reproduce easily even in dry climates, especially in bedding (Brooks et al., 2009). Proteins from house dust mite are some of the most potent elicitors of hay fever and asthma along with the symptoms like itchiness, sneezing, and watery and reddening of eye. About 1.2 billion people have been found to be affected by house dust mite (Basagana et al., 2004). Among 15 allergens of house dust mites, the most prominent and potent allergens are der p1 and der p2 (Dermatophagoides pteronyssinus). The der p1 is a cysteine protease enzyme that degrades polypeptides and affects most of the population. It has been reported that der p2 plays a vital role in immune modulation because it is recognized by the majority of house dust mite allergic patients and due to this fact, a new vaccine has been prepared that comprised of der p2 and a reduced IgE mediated side effect has also been observed during its implication (Pittner et al., 2004; Lynch et al., 1997).

Food allergy is an immune provocation induced by certain foods. It also defines as an allergic disorder that occurs when your immune system overreacts to a food and our body naturally defends against it. The symptoms of food allergy include, hives, itching of the mouth, lips, tongue, throat, eyes, and skin, or other areas of swelling of the lips, tongue, eyelids, or the whole face, difficulty swallowing, runny or congested nose, hoarse voice, wheezing, shortness of breath, nausea, vomiting, abdominal pain or stomach cramps, light headedness, and fainting

(Hoffman et al., 1983). The prevalence of food allergy has been increasing in recent decades, particularly in westernized countries among children's and mixed population. Food allergy affects nearly 5% of adults and 8% of children, and its prevalence is increasing continuously (Sicherer et al., 2014). The economic cost of children's food allergies is nearly $25 billion per year alone in United States. There are 170 foods that accounts for all food allergic reaction. Among them, the most common are big eight allergens– milk, egg, peanut, tree nuts, wheat, soy, fish and crustacean shellfish. These foods are responsible for 90% most of the serious food allergic reactions in the United States. In US population the estimated allergy prevalence of Peanut 0.6-1.3%, Tree nuts 0.4-0.6%, Fish 0.4%, Crustacean shellfish (crab, crayfish, lobster, shrimp), 1.2%, all seafood, 0.6% in children and 2.8% in adults. The food allergy and food anaphylaxis are on the rise in westernized countries like US, New Zealand, Australia and so on. In between 2008 to 2012, food anaphylaxis rates increased among children's aged 0-4 years from 1.2 to 2.4/105 alone in UK. However, the rate of food anaphylaxis is just four times from 2 to 8.2/105 population between 1998/1999 and 2011/2012 in Australia. The proportion of hospital admissions with food anaphylaxis cases are also increasing in United States between 2006 to 2009. These data suggest that there has been a actual increase in food allergy prevalence in kids and adults in the UK, United States and Australia in last two decades (Tang et al., 2017).

1.2.1 Food Allergy

1.2.2 Underlying Mechanisms of Food Allergy

Food allergy is defined as an abnormal immune response on exposure of allergens, particularly food. Symptoms of food allergic reactions include skin

rash, itching, hives, swelling of the lips, tongue or throat, shortness of breath, trouble breathing, wheezing, vomiting, stomach cramps, lightheadedness, fainting and diarrhea. Food allergies are characterized into non–IgE-mediated or mixed IgE- and IgE-mediated allergic reactions. The onset of IgE-mediated symptoms develop within minutes to 1-2 hours of ingesting the food, while non–IgE-mediated and mixed IgE- food allergies symptoms appears after several hours following food ingestion. Food allergy is not limited to IgE type sensitization, but also resulting from other mechanisms including eosinophil-driven disease or resulting from T-cell-mediated inflammation (Eigenmann et al., 2009). The underlying mechanism in food allergy includes-oral tolerance, sensitization to food allergens, and anaphylactic reactions to these food allergens (Johnston et al., 2014). Apart from this environmental and genetic factors also flock the immunopathogenesis of food allergy and its manifestations.

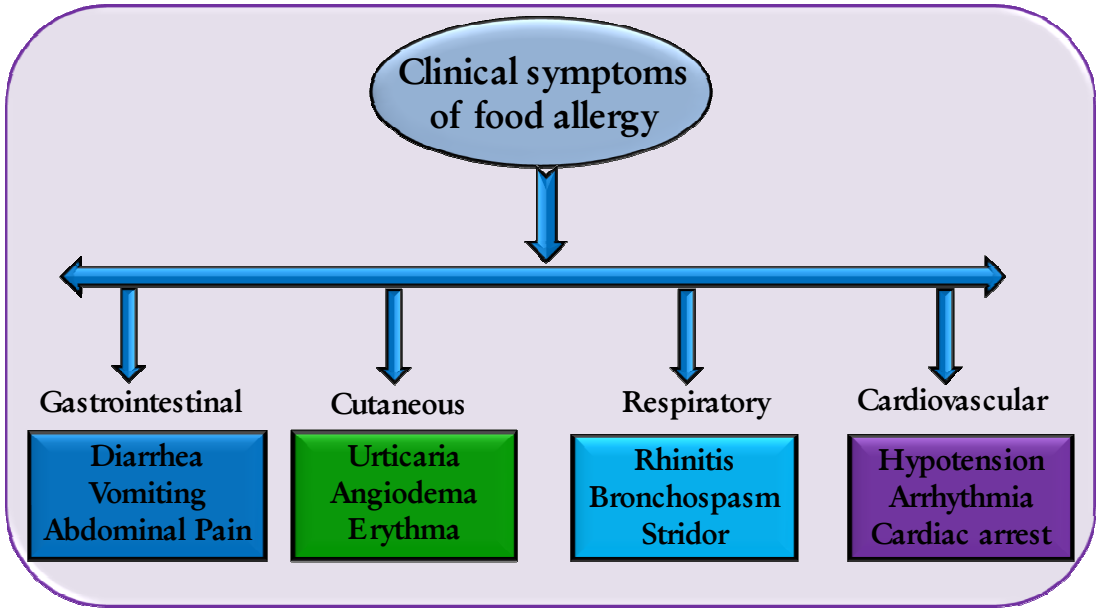

Figure 1.2: Clinical features of food allergy involving different organs

1.2.3 Oral Tolerance

Oral tolerance is the state of being immune unresponsiveness upon exposure to antigen derived from food proteins. Oral tolerance to food proteins through suppression of cellular or humoral responses plays a critical role in the prevention of food hypersentivity diseases Failure to oral tolerance results in induction of immune responsiveness to food allergens (Scott et al., 2015). The mechanism of tolerance is related to the gut immune system, the dose of antigen fed: anergy/deletion (high dose) or regulatory T-cell (Treg) induction (low dose), antigen specific tolerance and site of tolerance to oral antigens. The critical mechanism responsible for oral tolerance is the dose of antigen fed (Pabst et al., 2012). Low doses of antigen favors the induction of suppressor cells, called as regulatory T-cell (Tregs), which is characterized by the secretion of IL-10 and TGF-ß. While high doses of antigen favor the induction of anergy or deletion results in non responsiveness of allergen specific T cell. Dendritic cells (DCs) are professional antigen presenting cells that play a critical role in tolerance and immune unresponsive to self and harmless antigens. These cells are abundant in gut immune system located in the lamina propria (LP) of the intestinal villi, in the mesenteric lymph nodes (MLN), in lymphoid aggregates and Peyer's patches (PP) (Pabst et al., 2010; Rescigno et al., 2010a; Rescigno et al., 2010b). APC present in the LP and MLN are involved in the induction of oral tolerance, while PP has no role. $CD103^+$ and $CX3CR1^+$ are functionally two different major mucosal DC subsets. $CD103^+$ phagocytes express indoleamine 2,3-dioxygenase (IDO) (Matteoli et al., 2010), involved in the induction of T regulatory cell development via a TGF-β- and retinoic acid (RA)-dependent mechanism in the mouse (Coombes et al., 2007; Sun et al., 2007) as well as in the

human (Iliev et al., 2009b). $CX3CR1^{+}$ derive from monocytes induce the development of $Th17^{+}$ cells (Denning et al., 2007) presumably in response to microbial signals like Flagellin (Uematsu et al., 2008) or bacteria-released ATP (Atarashi et al., 2008). Schulz et al (2009) reported that CD103+ DCs supports the generation of T cell in both *in vitro* as well as *in vivo* immune responses in local lymph nodes LNs and serve as classical DC function. However CX3CR1+ is positioned directly in the directly in the mucosa and play an important role in initiating the first line of barrier against invading bacterial enteropathogens. Mutation in FoxP-3 locus, a variant of IPEX syndrome can (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) contribute to the development of food allergy as well as atopic dermatitis. Apart from concentration of antigens (low dose and high dose), expression of chemokines receptor CCR9 and integrin α4β7 on Tregs are crucial for tolerance, seeing as these molecules support gut homing (Johnston et al., 2014).

1.2.4 Sensitization

Defect in single epithelial layer which act as a barrier between gastrointestinal tract and mucosal–associated lymphoid tissue, results in sensitization (Herrick et al., 2000; Spergel et al., 1998). Sensitization is also defined as a cell mediated immune response in which unprimed or memory T cells interact with dendritic cells to become active lymphoblasts to eradicate antigens. Like tolerance, antigen presentation by DCs plays a special role in sensitization (Wang et al., 1996). The addressed possible routes for sensitization in food allergy include oral, cutaneous and respiratory. The mechanisms of sensitization in food allergy are well understood but the route of sensitization is incompletely followed. In previous

studies the oral route was described as the main route of sensitization but according to dual allergen hypothesis food allergen exposure via oral route results in the development of oral tolerance (Strid et al., 2005). However, exposure of food allergen through skin or the respiratory tract results in sensitization. It is also assumed that dysfunction in barrier of skin and respiratory tract leads to sensitization that results in food allergy (Hsieh et al., 2003).

1.2.5 Anaphylaxis

Anaphylaxis is severe life threatening allergic reaction that is rapid in onset and may cause death. Food induced anaphylaxis is the most common cause of anaphylaxis in which mast cells, basophils, macrophages and neutrophils have been shown to play key roles in anaphylactic shock responses (Finkelman et al., 2007). The symptoms of anaphylaxis occurs immediately after allergen exposure followed by late phase reaction of inflammatory cells. The symptoms of anaphylaxis appears within 5-30 minutes after intravenous exposure of allergen, while it will takes hours when ingested orally (Strait et al., 2002). The most common areas affected by anaphylaxis include skin (80–90%), respiratory (70%), gastrointestinal (30–45%), heart, vasculature (10–45%), and central nervous system (10–15%) (Sajjad et al., 2014). The symptoms of anaphylaxis typically include generalized hives, itchiness, flushing, or swelling (angioedema) of the afflicted tissues, runny nose and swelling of the conjunctiva, abdominal pain, diarrhea, vomiting, myocardial infarction, dysrhythmia, or cardiac arrest (Simons et al., 2009; Simons et al., 2010).The basis of anaphylaxis is the aggregation of high affinity receptor FcεR1 by an antigen recognized by IgE on activated mast cells, basophils, and eosinophils (Charles et al., 2001; Tsujimura et al., 2008). This results in a coordinated sequence of biochemical

and morphological events like exocytosis of secretory molecules, synthesis and secretion of newly formed mediators and cytokines. The four possible pathways of anaphylaxis are a "classic" pathway involving IgE, FcεRI, mast cells, and histamine; an "alternative" pathway mediated by IgG1, FcγRIII, macrophages, and PAF (Finkelman et al., 2007); an IgG basophil- PAF pathway (Tsujimura et al., 2008) and an IgG-neutrophil-PAF pathway via FcγRIV activation (Jonsson et al., 2011). In systemic anaphylaxis small dose of allergen results in a classical pathway while large dose activates alternative pathways (Strait et al., 2002)

Mast cells, basophils, macrophages, and neutrophils have been shown to contribute to anaphylactic shock responses in passive sensitization model from murine studies, while IgE, FcεRI, and mast cells play an essential role in allergic diarrhea in active sensitization model followed by platelet-activating factor (PAF), and 5- hydroxytryptamine (5-HT) (Vaz et al., 1977; Ishii et al., 1998). Strait et al 2002 showed that lacking FcεRI do not respond in a passive IgE-mediated systemic anaphylaxis model and have reduced responses in allergic diarrhea and food allergy. IgG also plays a key role in anaphylaxis and has several receptors including the high-affinity FcγRI and FcγRIV, and the low-affinity FcγRIIB and FcγRIII expressed on several cell types like mast cells, basophils, neutrophils, and macrophages. Strait et al (2002) showed that inhibition of low affinity FcγRII/III exterminates temperature drops associated with shock in IgG-, but not associated with IgE- mediated systemic anaphylaxis. Jhönsson et al (2011) reported that IgG antibodies, FcγRIIIA and FcγRIV, platelet-activating factor, neutrophils, and, to a lesser extent, basophils cells were involved in active systemic anaphylaxis. He also showed that not only IgG1, but also IgG2, antibodies could induce PSA in murine model. Arias et al (2009)

showed that combined blocking of PAF and histamine completely abolished the severity of peanut induced anaphylaxis in a mouse model and has supported the potential therapeutic approach to peanut and other food induced anaphylaxis.

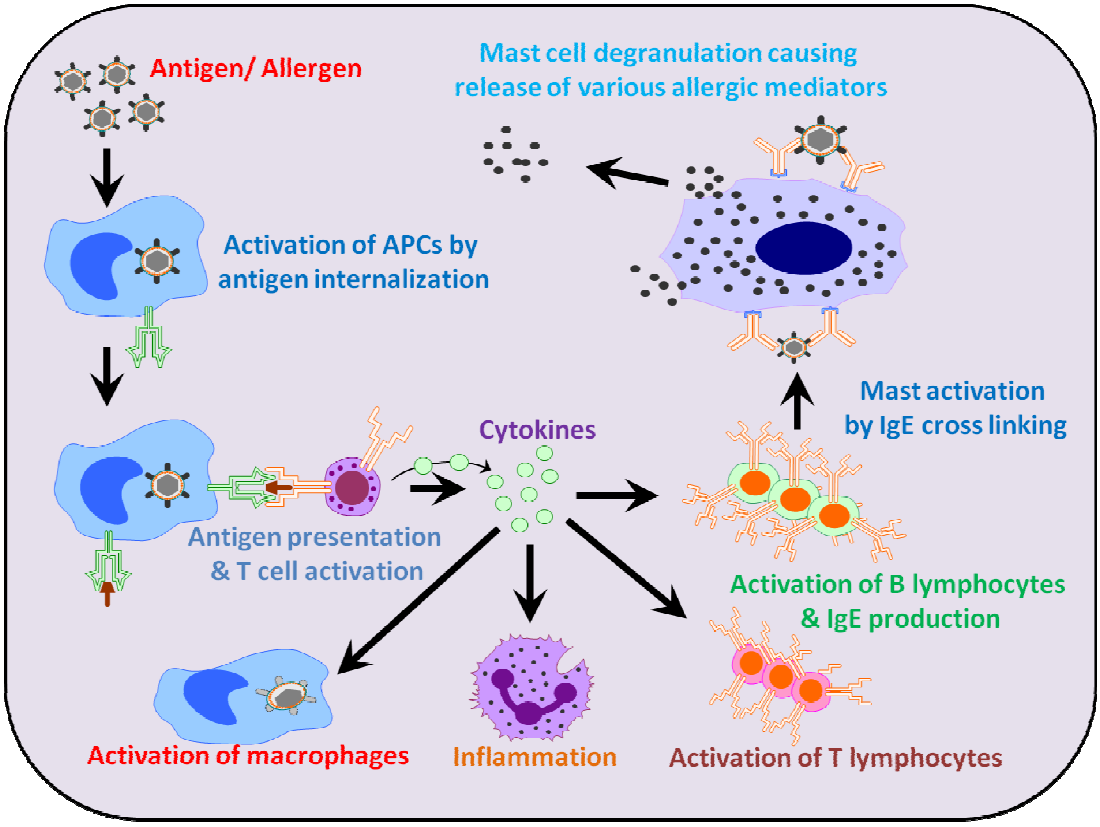

Figure 1.3: General mechanism of allergic Diseases

1.3 Asthma

Asthma is a set of various immunological and inflammatory responses in the lungs that is governed by different types of effector immune cells such as neutrophils, mast cell, eosinophil and lymphocytes. The phenotypic characteristics of asthma include epithelial damage with failure of healing and overproduction of growth factors and pro-inflammatory cytokines, mucous gland with associated mucus hyper-secretion, airway smooth muscle (ASM) remodelling with hypertrophy,

hyperplasia, bronchial hyper-responsiveness (BHR) along with cytokine secretion, and activation of inflammatory cells (Bradding et al., 2008). Once the airway passage becomes swollen and inflamed, it becomes narrower; thereby less air passes to the lung causing clinical symptoms like wheezing, coughing, chest tightness, and troubled breathing**.** In severe cases, the surrounding airways muscles tighten up and the above mentioned symptoms become more complicated than usual. Now a day, asthma is a most common chronic disorder affecting a significant number of childhoods as well as adults. The prevalence of asthma is increasing day by day over the past 2 decades. In the United States, 13.3 % of adults and 13.8 % of children have been found to suffering with asthma (CDC (Centers for Disease Control and Prevention., 2009; National Health Interview Survey Data: Lifetime Asthma Estimates., 2012). However, it affects up to 18% of the total population globally and accounts for 1 in every 250 deaths worldwide, resulting in 250,000 deaths throughout the year in different countries (Ober et al., 2011). The clinical association among three arms of allergic diseases i. e. asthma, allergic rhinitis, and atopic dermatitis, also called 'allergic triad', is well studied (Spergel et al., 2010)**.** Mostly, all of these allergic manifestations develop their own roots during childhood.

Numerous studies have demonstrated that allergic triads have a characteristic sequential progression during childhood. For instance, atopic dermatitis and food allergy typically may appear in infancy followed by asthma and/or allergic rhinitis in childhood and such type of sequential development is known as 'atopic march' (Rhodes et al., 2001; Rhodes et al., 2002; Gustafsson et al., 2000; Ohshima et al., 2002; Kulig et al., 1999; Lau et al., 2002). According to a study, about 30% of children having atopic dermatitis are found to develop asthmatic symptoms whereas

nearly 66% children develop allergic rhinitis symptoms (Illi et al., 2004). However, Most of the asthmatic patients (~80%) are reported with allergic rhinitis, while 19% to 38% of allergic rhinitis patients have coexisting asthma (Corren et al., 1997; Pawankar et al., 2006; Settipane et al., 1994). Several earlier studies carried out by our groups have demonstrated the correlation between allergic asthma and food allergy (Kumar et al., 2012). As mentioned, we have found that a significant number of asthmatic patients posses allergic sensitization to various foods. Among them, allergens from commonly consumed legumes such as pigeon pea, chickpea, red kidney bean, green gram, peanut and soya were potent allergy stimulus factors. Apart from asthma, patients with food allergy have also been found to have many other clinical complications involving skin and eyes (Kumar et al., 2012).

1.4 Flavanoids

For many years, phytochemicals like flavonoids, lactones, alkaloids, diterpenoids, glucosides and polysaccharides have become an essential part of human diet (Rice et al., 2001). Available evidence supports the role of these compounds in the management of pathophysiology of many diseases. Research has revealed that among phytochemicals like flavonoids has a surprisingly wide range of beneficial properties, including anti allergic, antibacterial, antiviral, antifungal, antidiabetic, anti-inflammatory, and antioxidant activity (Lakhanpal et al., 2007; Magrone et al., 2012; Joskova et al., 2011; Skrovankova et al., 2015; Gabor et al., 1986; Boesch et al., 2012). Flavonoids and flavones are considered as polyphenols that are present in different vegetable and fruits varieties like onions, broccoli, peppers, apple, berries, grapes and in some fruit juices and daily beverages like tea, wine and beer (Manach et al., 2004., Ko et al., 2015; Koh et al., 2009; Sun et al., 2007; Zielinski et al., 2014;

Skrovankova et al., 2015; Flamini et al., 2013; Tsanova et al., 2013; Jeszka et al., 2015; Martelo et al., 2014; Yoo et al., 2013).

Several research group have shown that among flavonoids, quercetin, a dietary phytochemical, become a promising agent in the prevention of various pathologies including cardiovascular, cerebrovascular diseases, a variety of cancer and Parkinson's as well as Alzheimer's diseases. Furthermore, it may also serve as the basis for new drugs in combination with others in the treatment of many diseases.

1.5 Quercetin, Its Structure and Main Sources

Quercetin extract, a natural effective agent obtained from seeds, stems, barks, roots and/or flowers, which become a prime choice in the treatment of human disease. Quercetin is a flavonoid with a broad set of pharmacological effects including antibacterial, antifungal, antiviral, antioxidative, antiinflammatory, and antiproliferative activities with different therapeutics range. It is found to be involved in UV filtration, symbiotic nitrogen fixation and floral pigmentation (Tsvetkova et al., 2006). In addition to the quercetin, its other derivatives like glycosides, ethers, sulfate and prenyl substituent's have demonstrated potential beneficial impact on human health.

Quercetin is multipurpose plant flavonoids widely strewn in red wine, grapefruit, onions, apples, berries, black tea, and, in lesser amounts, in leafy green vegetables and beans. It is also present in shrub, stem, seeds, fruits of different medicinal plants like Capparis spinosa, Pueraria tuberosa, Tephrosia pupurea, Amaranthus viridis Linn, Solanum Trilobatum, Emblicaofficinalis Ginkgo biloba, Hypercerium perforatum and in many others (Bhatt et al., 2009; Heijnen et al.,

2001). The major sources of active ingredient quercetin are green tea and onions and apart from that broccoli, asparagus, green pepper, tomatoes, and red leaf lettuce are also the alternative dietary sources in summer. It is aglycone form of glycoside flavonoid (e.g rutin, quercetin), which is a non sugar compound remaining after replacement of a glycosyl group from glycosyl by hydrogen bond (Manach et al., 1997). Quercetin flavonoid is a class of secondary metabolite characterized by more than one phenolic group fulfilling many functions. This is a hydrophobic natural compound exist in aglycone or sugarless form of other flavonoid glycosides, such as rutin and quercetin, highly abundant in citrus fruit, buckwheat and onions (Formica et al., 1995; Mukhtar et al., 1988; Comalada et al., 2005; Sri et al., 2009; Das et al., 1988; Gupta et al., 2016). However, the most common glycosylated structures of quercetin occur in nature is quercetin-3-O-β-glucoside, not the aglycone form (Ko et al., 2015). It is believed that the most *in vivo* bioactivity of quercetin is due to its conjugated derivatives like glucuronated, sulfated, and methylated conjugates (isorhamnetin and tamarixetin). Quercetin possesses five hydroxyl and one carbonyl at the carbon position of benzene ring and a double bond between 2 and 3 carbons. Quercetin showed two absorption peaks at 256 nm and 372 nm corresponding to the benzoyl moiety and Cinnamoyl system, respectively. 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. Quercetin absorbs UV radiation with absorbance maxima in the UV-A (max= 365 nm, = 28400 M−1 cm−1 and UV-C range (max= 256 nm, = 28300 M−1 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* (Gupta et al., 2016).

1.6 Bioavailibilty and pharmakokinetics of quercetin

Nowadays investigations are focused on the bioavailability and metabolism of quercetin derivatives like glycosides, as this type of quercetin predominates in diet. Although presence of quercetin is high in natural sources like fruits, vegetables etc, but their clinical application is restricted due to poor bioavailability and solubility. Among the bioavailability studies of polyphenolic compounds, quercetin received more attention due to its potential therapeutic effects but requires enhanced bioavailability and dispersibility. Since bulk quercetin can be rapidly metabolized from plasma and other organs, its effect gets restricted. The pharamcokinetics of quercetin in humans has been attempted by many researchers. Quercetin was found to have low plasma concentration, poor tissue absorption with rapid metabolism (Moon et al., 2008), thus limiting its effectiveness. In rats, quercetin did not undergo any significant phase I metabolism. In contrast, quercetin did undergo extensive phase II (conjugation) to produce metabolites that are more polar than the parent substance and hence are more rapidly excreted from the body. Four of the five hydroxyl groups of quercetin are glucuronidated by UDP-glucuronosyltransferase. The exception is the 5-hydroxyl group of the flavonoid ring that generally does not undergo glucuronidation. The major metabolites of orally absorbed quercetin are quercetin-3-glucuronide, 3'-methylquercetin-3-glucuronide, and quercetin-3'-sulfate. To improve bioavailability as well as therapeutic efficacy, new formulations have been developed by many researchers including complex forming with cyclodextrins and liposomes (Pralhad et al., 2004; Yuan et al., 2006). The new formulation may be

an improvement but is associated with risk of nephrotoxicity (Frijlink et al., 1991) On the other side, use of liposomal formulation is restricted because of stability problem during storage (Mu et al., 2006). Low solubility and the low stability of aqueous solution of quercetin is due to its hydrophobic nature. Researcher has used DMSO for improving the solubitlity but is not safe as higher dose of DMSO result in vasoconstrictor, neurological, cardiovascular and respiratory problems along with bradycardia and diffuse alveolar haemorrhage (Ader et al., 2000,). Rogerio et al (2010) tried two formulation of quercetin (QU-ME and QU-SP) and compared their effects in murine model of airways allergic inflammation. It was found that QU-ME at a dose of 10 mg/kg given orally exhibits pronounced anti-inflammatory properties as compare to QU-SP. However, Vicentini et al (2010) investigated the stability of quercetin and found a significant loss of quercetin content and antioxidant activity after 6 months of storage at 30°C/70% relative humidity and after 2 months at 40°C/70% relative humidity. Thus the above formulation required special storage conditions (at 4 ± 2°C) on longer term. Mulholland et al (2001) also synthesized water soluble quercetin (QC 12) and administered it in six cancer patients orally and intravenously (400 mg QC 12 i.e equivalent to 298 mg of quercetin). QC 12 can not be detected in plasma following oral administration, while it can be detected in plasma after i.v. administration. This study showed only 20%-25% bioavailability of quercetin released from QC12. Thus, there is a need for improvement of the formulation of quercetin and/or delivery system and many attempts have been made. Srinivas et al (2010) demonstrated the aqueous solubility of quercetin and its dihydrate at temperatures between 25°C and 140°C and they found that the aqueous

solubility of quercetin dihydrate was 1.5–2.5 times higher than that of anhydrous quercetin above 100 °C in subcritical water.

1.7 Nanotechnology

Over the last decades, nanoparticle engineering has been developed and reported with powerful pharmacological activity (Verma et al., 2009). Nanotechnology may be an alternative manner to solve the problems associated with various approaches described earlier. Nanotechnology is defined as the science and engineering carried out in the nanoscale that is 10^{-9} m. There are two approaches for synthesis and fabrication of drug nanoparticles form drug powder. Top down technique that includes the slicing or cutting of a bulk material into nano sized particle, while Bottom up refers to the buildup of a material from the bottom. Both techniques play important role in fabrication and processing of nanomaterials as well as enhanced solubility and efficacy of compound (Patel et al., 2011).

During 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 (Mohanty et al., 2010). A Nanomedicine consisting of Chitosan/carboxymethyl-cyclodextrin loaded with unfractioned or low-molecular-weight heparin showed a potential effect in asthma treatment (Oyarzun et al., 2012). Potentiating the existing natural bioflavonoid quercetin using nanotechnology may pave the new direction for treating asthma, cancer, diabetes and other diseases. Bioavailability and solubility of phytochemicals like flavonoids enhanced by various methods including

micronization, use of fatty solutions, use of penetration enhancer or cosolvents, surfactant dispersion method, salt formation, precipitation, etc., but still, these techniques have limited utility in solubility enhancement for poorly soluble drugs. Additional technologies involved for solubility improvement are liposomes formation, microemulsions, emulsions, formation of complexes (e.g., with $-cyclodextrin), and solubilization (Patel et al., 2011). Though these methods are successful, but have shown health risk in some instances along with loss of antioxidant activity of flavanoids. An alternative method to improve the solubility as well as bioavailability of a quercetin is the physical modifications that increase the surface area and wettability of quercetin by means of particle size reduction.

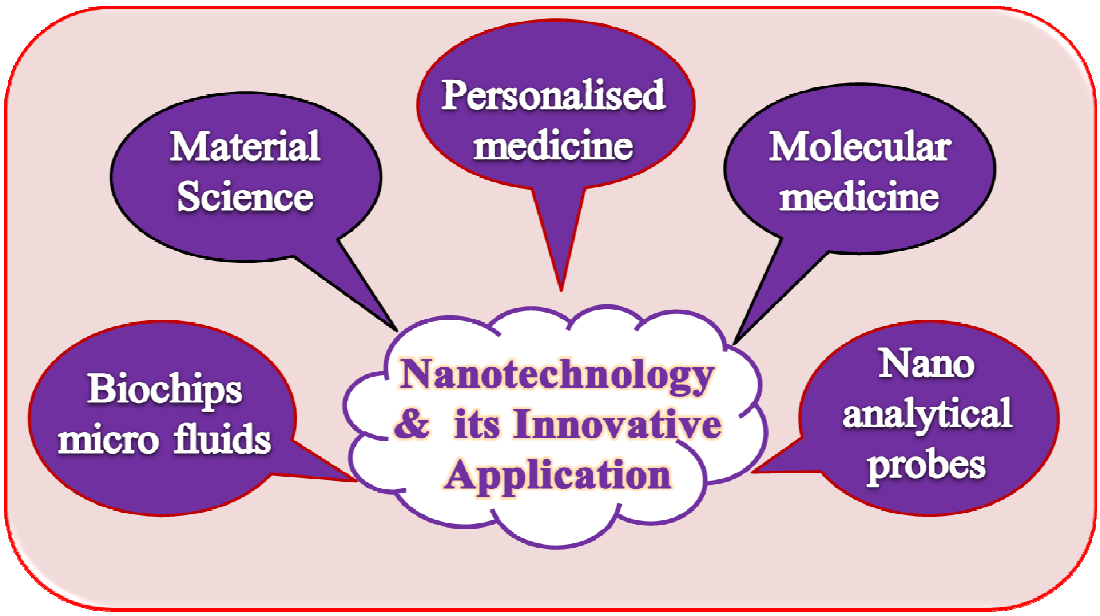

Figure 1.4: Different applications of Nanotechnology

1.8 Role of Quercetin in allergic diseases

Quercetin is a promising component, with various biological activities that can also prevent lifestyle related diseases (Jeszka et al., 2015). Quercetin extracts are

now largely used as nutritional supplements and curative ingredients for many illnesses such as diabetes related with obesity and circulatory dysfunction, including inflammation, as well as mood troubles (Jurikova et al., 2015). Quercetin exhibits similar anti-allergic potential as a Chinese herbal formula (Food Allergy Herbal Formula-FAHF) that has been related with blocking of anaphylaxis to peanuts in mouse models (Chirumbolo et al., 2011). Like other flavonoids, it has low retention and high excretion, but it exhibits a variety of pharmacological activities, including anti-inflammatory, anticancer, antioxidant, wound-healing, and anti-allergic (Formica et al., 1995; Mukhtar et al., 1988; Comalada et al., 2005; Sri et al., 2009; Das et al., 1988; Gupta et al., 2016). Ferry et al (1996) found that quercetin has very few side effects in phase I clinical trial with a recommended dose of 2.5 g for 70 kg individual. Lamson et al (2000) also found that oral administration of single dose of 4g of quercetin or 500 mg twice daily has no side effects after continuous administration for a month (Lamson et al., 2000). In this review, we have summarized the therapeutic role of quercetin and their different forms in allergic diseases.

1.8.1 Allergic rhinitis

Allergic rhinitis is the leading cause of chronic illness in many developed countries, including USA. In 2014, about 8% of the U.S. population has been diagnosed with allergic rhinitis. Allergic rhinitis has been substantially increasing worldwide and approximately 500 million people are affected globally and it is more common in children than adults. Allergic rhinitis is classified into four ways: causative antigens, predilection time, disease types, and severity of symptoms (Salo et al., 2011; CDC., 2016; Evans et al., 1993; Lamb et al., 2006).

Depending upon allergen exposure and symptoms throughout the year, allergic rhinitis is divided into two types i.e seasonal or perennial The seasonal allergens includes pollens, grasses & weeds while perennial allergens are derived from dust mites, molds, animal danders and other sources of occupational origin (’Rush University Medical Centre’’ Retrived 2008-03-05; Sampson et al., 1997). Allergic rhinitis is a complex IgE-mediated inflammatory disorder of nasal mucosa characterized by eosinophilic inflammation, hypersecretion of mucus, sneezing, lacrimation of eyes, nasal congestion, pruritis, and rhinorrhea. This may likely be due to the involvement of chemical mediators such as histamine and leukotrienes that are secreted by mast cells, basophils and eosinophils. In many cases the consequences results in the release of several cytokines and chemokines that are responsible for the allergic manifestations in nasal mucosa (Pawankar et al., 2011). Many people’s may experience the vasodilation, edema and activation of inflammatory cells in nasal mucosa due to antigenic stimulation on sensory nerves, leads to axonal reflex to produce neuropeptides, substance P (SP) and calcitonin gene-related peptides (CGRP). Cytokines and neurotransmitters leads to sensory nerve activation, plasma leakage and congestion of venous sinusoids. On the other hand, there is much evidence that nasal mucosa is innervated by sensory, sympathetic and parasympathetic nerves. After stimulation with aeroallergen, sensory nerves transmit signals generating sensations such as itching and motor reflexes, including sneezing (O’hanlon et al., 2007; Pfaar et al., 2009; Schaper et al., 2010; McDonald et al., 2013). Antigenic stimulation on sensory nerves also causes axonal reflex to produce neuropeptides, substance P (SP) and calcitonin gene-related peptides (CGRP) that are

responsible for vasodilation, edema and activation of inflammatory cells in nasal mucosa.

Direct and indirect intake of flavonoids has now become first choice for the treatment of several diseases including allergy, asthma, cancer, and diabetes (Lakhanpal et al., 2007; Magrone et al., 2012; Joskova et al., 2011; Skrovankova et al., 2015; Gabor et al., 1986; Boesch et al., 2012). Anti-inflammatory and anti allergic role of quercetin have been already proven by several studies. In earlier study by Truneh et al (1984) use of quercetin impaired mast cell degranulation and significantly inhibited antigen-stimulated histamine release. The same study also revealed the preeminence of quercetin over sodium cromoglycate which is used twice for the similar effect (Weng et al., 2012). Flavonoids like quercetin are known to inhibit the allergic disease specially, allergic rhinitis (Mlcek et al., 2016). In recent study by Kashiwabara et al (2016) found that oral administration of 25 mg/kg dose of quercetin for 5 and 7 days, inhibit sneezing and nasal rubbing movements, as well as turn down the level of substance P (SP), calcitonin gene-related peptide (CGRP) and nerve growth factor (NGF) contents in nasal lavage fluids induced by TDI (Toluene 2,4-diisocyanate) nasal challenge in rats.

1.8.2 Asthma

Asthma is a major devastating disease with a fewer therapeutic regimens. It is 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. (Manno et al., 2010; Gupta et al., 2016). It is responsible for approximately 25000

deaths each year (GINA., 2011). Asthma is strongly associated with allergens like pollen, mold, dust mites and foods (Richter et al., 2011; Rosenstreich et al., 2003). Furthermore, a big human population is at high risk of asthma because of hazardous air particles (HAP) including fine particulate matter, tobacco smoke and other air born pollutants (Leikauf et al., 2002). In addition, asthma is also triggered by several intrinsic factors like upper respiratory infection, physical exercise and weather changes (Chua et al., 2007; Tillie et al., 2005). It is a chronic inflammatory disease 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 a pivotal role in the recruitment/activation of primary effector cells (mast cells, basophils, eosinophils) causing an allergic response (Rosenstreich et al., 2003; Leikauf et al., 2002; Chua et al., 2007; Tillie et al., 2005). Furthermore, chemokines, including thymus and activation-regulated chemokine, play an important role in asthma in regulation of inflammation and IgE synthesis. These days, the treatment and prevention strategy for asthma has become the most important clinical issue. Currently, the available treatment of asthma includes β2-agonists, cysteinyl leukotrienes receptor 1 antagonists and corticosteroids. Although the effect of these drugs is beneficial but long term use may lead from mild to chronic side effects. Therefore, the treatment requires development of new drugs with long lasting clinical effects and subsequent reduction of the allergic inflammatory reactions with reduced side effects. 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 (Mainardi et al., 2009). Among CAM therapies, dietary supplement sand 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 (Blanc et al., 2006; Riccioni et al., 2007; Mukhtar et al., 1988). Dietary antioxidants intervention may also reduce oxidative stress and prevent or minimize asthmatic symptoms. Quercetin is a plant-derived bioflavanoid known to exhibit excellent anti-oxidant and anti-inflammatory properties (Das et al., 1988). It has been suggested as a good candidate for the management of eosinophil-mediated diseases, such as allergic rhinitis and asthma (Sakai et al., 2013). Quercetin relaxes airway smooth muscle through cAMP-mediated pathways and augments -agonist relaxation via dual phosphodiesterase inhibition of PLC and PDE4 (Townsend et al., 2013). Quercetin lowers the expression of pro-inflammatory cytokines and improves lung function in RV-infected mice (Ganesan et al., 2012). 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* (Joskova et al., 2011). Anti-inflammatory effect of quercetin loaded microemulsion (QU-ME) and quercetin suspension (QU-SP) has been studied on murine model of airways allergic inflammation (Rogerio et al., 2010).

Thus quercetin nanocrystals may hold great promise for developing new intervention strategies and could be used medicinally, either alone or as a

complement to other drugs used for the treatment of allergenic diseases such as food allergy and allergic asthma. Additionally, it could also be used as a nutraceutical.

1.8.3 Atopic dermatitis

Among different allergies worldwide, atopic dermatitis (AD) is one of the most prevalent forms of allergy (Werfel et al., 2014). It is a chronic inflammation characterized by release of vascular endothelial growth factor, mast cells and neuropeptides. Studies have suggested that the Th2 cytokines production such as IL-8 and TNF, inflammation related angiogenesis, mast cell mediators like histamine, PGD2 and LTs reported to be a key mediators of atopic dermatitis development. AD has also been characterized by enhanced level of IgE and eosinophils in blood sera of patients (Karuppagounder et al., 2016; Roesner et al., 2016). The prevalence of atopic dermatitis has been reported from several parts of the world. It affects nearly 10–20% of children and 1–3% of adults and its prevalence are increasing continuously (Werfel et al., 2014). The pathologies of AD involve disruption of skin barriers due to several factors including environmental allergies, malfunctioning of immune system, and genetic factors (Ring et al., 2012). The mechanism of action of atopic dermatitis includes both innate and adaptive immune system (Roesner et al., 2016). The exposure of allergens via skin results in the activation of antigen presenting cells (APCs) like macrophages, dendritic cells and lymphocytes causing activation and upregulation of proinflammatory genes. Further mechanism involved the activation of multiple signaling pathways including HMGB1 (High mobility group box 1 protein) and JAK-STAT pathways (Bao et al., 2013).

HMGB1 is a proinflammatory cytokines found to play a key role in the pathogenesis of several inflammatory and autoimmune diseases. Activated effector cells like macrophages secrete HMGB1 that further interacts with RAGE (receptor for advanced glycation end products), TLR2 and TLR4 considered as key event for activation of nuclear factor kappa B (NF-kB) along with ERK (extracellular signal-regulated kinase) 1/2 pathway in AD inflammation. The oral administration of quercetin (50 mg/kg) reduces the dermal changes including hyperkeratosis, parakeratosis, acanthosis, inhibit proliferative responses of mast cells and Th2 cytokines like IL-4 and IFNγ in house dust mite induced AD in NC/Nga transgenic mouse. Results from this study demonstrated a critical role of quercetin in HMGB1 /RAGE /NFκB signaling and activation of transcription factor Nrf2 (nuclear factor-erythroid-2-related factor) protein that plays potential role against oxidative damage (Karuppagounder et al., 2015). Dietary supplements and antioxidants are widely used to alter the immunogenic responses and have played a key role in mast cell blocking activities. Among antioxidant quercetin play a crucial role to inhibit the release of histamine, leukotrienes and PGD2 from human cultured mast cells in response to cross-linkage of high affinity surface IgE receptors (FcεRI). It has been reported that quercetin also inhibits histamine, IL-6, IL-8, TNF-a and tryptase release from human mast cells (Kempuraj et al., 2005; Min et al., 2007), as well as asthma development in an Ovalbumin induced murine model (Juríková et al., 2015; Chirumbolo et al., 2011; Park et al., 2009). In recent study by Weng et al. 2012 he found that the quercetin was found more effective than Cromolyn in inhibiting IL-8 and TNF release from LAD2 mast cells stimulated by substance P. Quercetin worked as more effective in reducing IL-6 release from human Cord blood-derived cultured mast

cells in a dose-dependent manner (Weng et al., 2012). It is found to be counteractive agent in inhibiting cytosolic calcium level increase and NF-kappa ß activation (Rogerio et al., 2007; Rogerio et al., 2010). Several studies advocated the preventive use of quercetin in allergic diseases including contact and atopic dermatitis. Recent study by Jung et al (2010) showed quercetin and tannic acid effectively suppressed the marked decrease in level of systemic serum IgE, suppression of angiogenesis and Th2-related cytokine expression, including thymic stromal Lymphopoietin (TSLP) and thymus and activation regulated chemokines (TARC), in an AD mouse model.

In addition to inhibiting HMGB1, quercetin also inhibits LPS induced type II LC3 production/aggregation and LPS induced HMGB1 translocation/release. Pretreatment with Quercetin at a dose of 50 and 100 mg/kg prevents endotoxin lethality and inhibits release of TNF-a and HMGB1 (Tang et al., 2009). Quercetin inhibits LPS-induced HMGB1 release via Mitogen-Activated Protein Kinase and NF-kB pathways in RAW 264.7 cells Endale et al., 2013. Many evidences support the use of quercetin in atopic dermatitis through several pathways. Quercetin has been shown to inhibit JAK2, TYK2, STAT3, and STAT4 activation and translocation induced via IL 12 that is essential for signaling in T cells in allergic encephalomyelitis (EAE) female SJL/J mice (Muthian et al., 2004). Similarly, in another study quercetin inhibited JAK/STAT pathway as well as iNOS and ICAM-1 expression in Cholangiocarcinoma (CCA) cells (Senggunprai et al., 2014). Quercetin was shown to be able to suppress phosphorylation of the JAK-STAT signaling pathway through the inhibition of TSLP-IL-7R/TSLPR complex (Arima et al., 2010).

1.8.4 Arthritis

Nowadays flavonoids are receiving huge attention for the management of arthritis and its complications, which have reached to epidemic levels globally (Jackson et al., 2006; Leyva et al., 2016). There are dozens of inflammatory diseases, the most common among that are arthritis characterized by inflammatory disorder of joints involving hands, wrists, feet and other joints (Feldmann et al., 2003; Andersson et al., 2008). It is a chronic inflammatory disease characterized as crystal-induced arthritis and rheumatoid arthritis (Jackson et al., 2006). The symptoms of arthritis can be mild to severe including bone erosion, cartilage degradation, joint stiffness, pain, swelling, redness, tenderness and warmth in the joints and many more complications. The mechanism of Crystal-induced arthritis or gout involves the recruitment of leucocytes triggered by the deposition of monosodium urate, calcium pyrophosphate, and basic calcium phosphate crystals in tissues resulting in the infiltration of neutrophils, mononuclear phagocytes and lymphocytes following cascade of molecular events. The alteration in serum urate levels is due to high purine rich diets, excessive alcohols intake, diuretic drugs, distress, surgery, leading to the release of microcrystals of monosodium urate or calcium pyrophosphate resulting in the release of inflammatory substances like prostaglandins, bradykinin, cytokines (in particular, interleukin (IL)-1b), and substance P. Furthermore, the other face of arthritis affecting most of the population is Rheumatoid arthritis. Rheumatoid arthritis is an autoimmune provocation in which both genetic and environmental factors play a crucial role resulting in the loss of immune tolerance. Basically it is induced by dysfunction of immune system and the recruitment of rheumatoid inflammatory cells such as neutrophils, lymphocytes, macrophages, fibroblasts and

synoviocytes in the joints, and other inflammatory sites characterized by synovial inflammation and hyperplasia, autoantibody production (rheumatoid factor and anti–citrullinated protein antibody [ACPA]), cartilage and bone destruction, matrix metalloproteinase (MMP) release, pannus formation with angiogenesis (Jackson et al., 2006).

Currently the available treatment of arthritis is done by non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin, diclofenac, sulfasalazine (SSZ), hydroxychloroquine (HCQ), methotrexate (MTX) and so on. But in many cases, treatment with these drugs is found to be risky and several side effects have also been reported that need to reduce for the betterment of the therapy. Side effects induce by NSAIDs include Nausea/vomiting, Dyspepsia, Gastric ulceration, bleeding, diarrhea and cardiovascular complications (Meraj et al., 2014). Considering the side effects of the above mentioned drugs, there is a need of a safe and efficacious approach to combat arthritis. Thus alternative therapies are necessary. The treatments of arthritis with antioxidant molecules like quercetin and curcumin could be one option (Jackson et al., 2006). Phytochemicals such as flavonoids have been extensively used in the treatment of arthritis because of its antioxidant and antinflammatory properties that are becoming increasingly accepted due to its powerful impact on human health. In this dissertation, a particular attention has been paid to the potential therapeutic effects of quercetin on crystal-induced arthritis and rheumatoid arthritis. Quercetin has been reported to have anti-inflammatory effects on rat adjuvant arthritis (Gardi et al., 2015). C. Gardi et al (2015) found that quercetin lowered levels of interleukin-1b, C-reactive protein, and monocyte chemotactic protein-1 and restored plasma antioxidant capacity. They also analyzed that quercetin inhibited the enzymatic

activity of pro-inflammatory 12/15-lipoxygenase in lung and liver and increased the expression of heme oxygenase-1 in joint and lung of arthritic rats. These findings suggest that, quercetin suppresses the 2-fold increase of NF-κB activity observed in lung, liver and joint after induction of arthritis. Quercetin may provide a safer alternative or adjunctive treatment for collagen induced rheumatoid arthritis by suppressing the production of nitric oxide, the accumulation of lipid peroxidation products. Meraj et al (2014) found that quercetin act as an antioxidant by up regulating the activity of antioxidant enzymes, down regulating COX2 and NF-κB p65 expression thus eliminating the accumulation and activation of polymorphonuclear cell.

Chapter 2

Preparation, Characterization and In Vivo Pharmacokinetics of Nanotized Quercetin

CHAPTER 2

PREPARATION, CHARACTERIZATION AND *IN VIVO* PHARMACOKINETICS OF NANOTIZED QUERCETIN

2.1 Introduction

Quercetin is a multipurpose plant flavonoids widely distributed in plants and is involved in UV filtration, symbiotic nitrogen fixation and floral pigmentation (Tsvetkova et al., 2006). It is aglycone form of glycoside flavonoid (e.g rutin, quercetin), a non sugar compound remaining after replacement of a glycosyl group from glycoside by hydrogen bond (Manach et al., 1997). It befalls the essential part of human diet by means of various sources such as apples, onions, grapefruit, tea, green vegetables, and beans. Research data demonstrate that 25 mg is the average daily dietary intake of quercetin by an individual in the United States (NTP Technical report 1992). It exhibits a variety of pharmacological activities, including anti-inflammatory, anticancer, antioxidant, wound-healing, and anti-allergic. Like many other flavonoids, it has low retention and high excretion. (Formica et al., 1995; Mukhtar et al., 1988; Comalada et al., 2005; Sri et al., 2009; Das et al., 1988; Gupta et al., 2016). Although quercetin has attracted tremendous attention but its poor water solubility and bioavailability has been a bottleneck in using it as anti-inflammatory and anti-allergic agent.

Bioavailability and solubility of phytochemicals like flavonoids can be enhanced by various methods including micronization, use of fatty solutions, use of penetration enhancer or cosolvents, surfactant dispersion method, salt formation, precipitation, etc. However, these techniques have limited utility in solubility enhancement for poorly soluble drugs. Additional technologies involved for solubility improvement are liposome formation, microemulsions, emulsions, formation of complexes (e.g., with ß-cyclodextrin), and solubilization (Patel et al., 2011). These methods are successful, but also associated with health risk in some instances along with loss of antioxidant activity of flavonoids. An alternative method to improve the solubility as well as bioavailability of a quercetin is the physical modifications that increase the surface area and wettability of quercetin by means of particle size reduction.

During the last decade, nanomedicine that could help in 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. A Nanomedicine consisting of Chitosan/carboxymethyl-cyclodextrin loaded with unfractioned or low-molecular-weight heparin showed a potential effect in asthma treatment (Mohanty et al., 2010; Oyarzun et al., 2012). Potentiating the existing natural bioflavonoid quercetin using nanotechnology may pave the new direction for treating asthma, cancer, diabetes and many other diseases associated with inflammatory response. Over the last decades, engineered nanoparticles have been developed with powerful pharmacological activity (Verma et al., 2009). Nanotechnology may be an alternative manner to solve the problems associated with

various approaches described earlier. Nanotechnology is defined as the science and engineering carried out in the nanoscale that is 10^{-9} m. There are two approaches for synthesis and fabrication of drug nanoparticles form drug powder i.e 'Top dowm and Bottom up'. Top down approach includes the slicing or cutting of a bulk material into nano sized particle, while Bottom up refers to the buildup of a material from the bottom. Both techniques play important roles in fabrication and processing of nanomaterials having enhanced solubility and efficacy of compound (Patel et al., 2011). Thus, Nanotized quercetin may provide numerous benefits, including improved efficacy, bioavailability, reduced toxicity, dose–response, targeted ability, personalization, and enhanced safety compared to its bulk form. Therefore, the present chapter focuses on the preparation and characterization of water soluble quercetin nanocrystals that are poised to show enhanced bioavailability and therapeutic potential.

2.2 Material and Methods

2.2.1 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). All the other chemicals used were of the highest purity available from other commercial sources.

2.2.2 Preparation and Lyophilisation of Quercetin Nanocrystals

The preparation of quercetin nanocrystals (nQ) was carried out under high energy transfer using ultrasonicator (PCI Analytics probe sonicator, Mumbai, Maharashtra) as per described method by Sahoo et al (2010) with minor modifications. Briefly, nQ (2% to 10% w/w) was prepared by mixing bulk quercetin in Milli-Q water (Millipore Corporation, Billerica, MA) having pH 6.8 and resistivity of 18 M with Tween 80 as a stabilizer (0.5 to 2.5 w/w). Prepared solution was subjected to high energy ultra-sonication to obtain quercetin nanocrystals. Thus obtained nQ was freeze dried at −80 ºC 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 study. 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 (2010).

2.2.3 Characterization of Quercetin Nanocrystals

These nanoparticles were characterized by several techniques including 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.

2.2.3.1 Fourier Transform Infrared and UV Spectroscopy

The Fourier Transform Infrared (FTIR) spectra of bulk querctein 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 µg/ml) in methanol solvent were recorded on an Agilent Cary 5000 UV-

VIS-NIR spectrophotometer. The UV-Spectra were recorded in the range from 200 to 600 nm in triplicate.

2.2.3.2 Transmission Electron Microscopy

The shape and size of nQ were seen by TEM (Transmission Electron Microscopy) measurements. In brief, a drop of diluted solution of the nQ (50 µg/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.

2.2.3.3 Dynamic Light Scattering

The average hydrodynamic size, polydispersity index and zeta potential of nQ were 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 seconds (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.

2.2.3.4 X-ray diffraction

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≤ 2≥ 50, and the scanning rate was 1 min^{-1}. All measurements were made in triplicate.

2.2.4 Solubility and Stability of nQ

Sedimentation kinetics of equivalent quantity of bulk quercetin and nQ suspended in PBS (3 mM) 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. The stability of nQ with varying temperature and pH was studied using high performance liquid chromatography (HPLC) according to the earlier described method (Mohanty et al., 2010). For stability assay at a fixed concentration of 40 µg/ml (at a total of 10 ml solution) was prepared in PBS (3 mM) at three pH (2.0, 7.4, 8.0) and incubated at 37 ºC for a total of 12 h. At predetermined time points, 100 µL aliquot of nQ (40 µg/ml) was taken out and diluted with 900 µL of methanol for HPLC analysis.

2.2.5 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 (Reference no.: IITR/IAEC/06/2016 - 48/17) of CSIR-IITR, India.

2.2.6 *In-Vivo* Pharmacokinetics of nQ

Animal study was carried out to understand the pharmacokinetics of delivering bulk quercetin as well as quercetin nanocrystals (nQ) to female BALB/c mice, according to the method described earlier (Mohanty et al., 2010). BALB/c mice weighing 20–25 g 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.

2.3 Results

2.3.1 FTIR and UV-VIS spectra of nQ

In Fig 2.1, FTIR spectra of nQ showed the presence of C=C and C=O moieties and other IR (Infra-red) bands as observed in bulk quercetin. It can be seen clearly that there is no significant difference between the IR spectrum of nano and bulk quercetin indicating that native structure of quercetin remains intact in nano form. 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 changes in nQ compared to bulk quercetin.

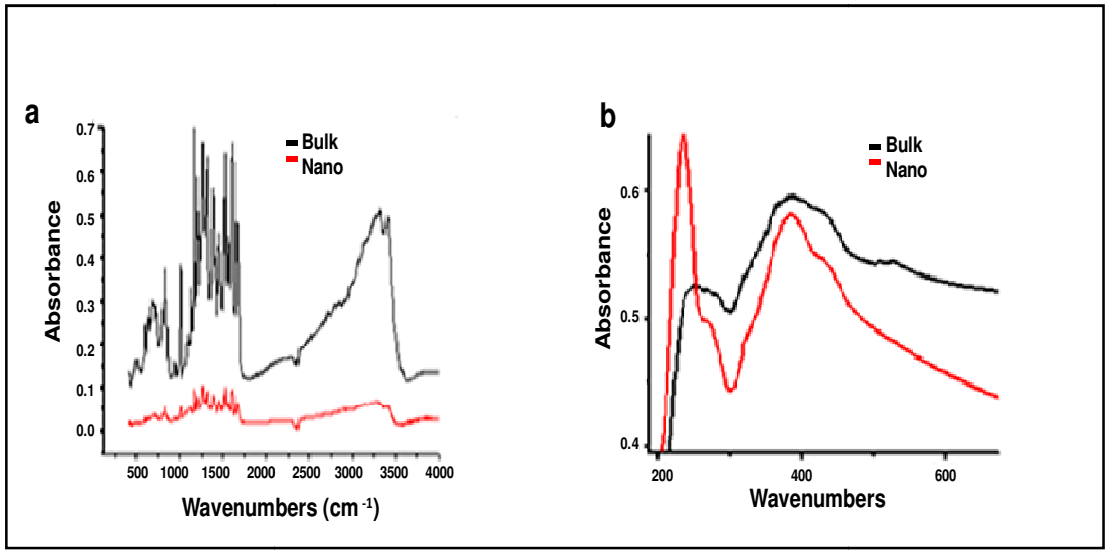

Figure 2.1: FTIR, Ultraviolet and Visible spectra of quercetin nanocrystals (nQ): (a) FTIR spectra of bulk quercetin (black), and quercetin nanocrystals (red). (b) Ultraviolet and Visible absorbance spectra of Bulk quercetin (black) and quercetin nanocrystals (red) at a fixed concentration of 1 mg/ml.

2.3.2 Transmission Electron Microscopy

TEM images showed the formation of nearly spherical monodispersed nQ with size of 16.95±1.3nm as shown in Fig 2.2 a and b.

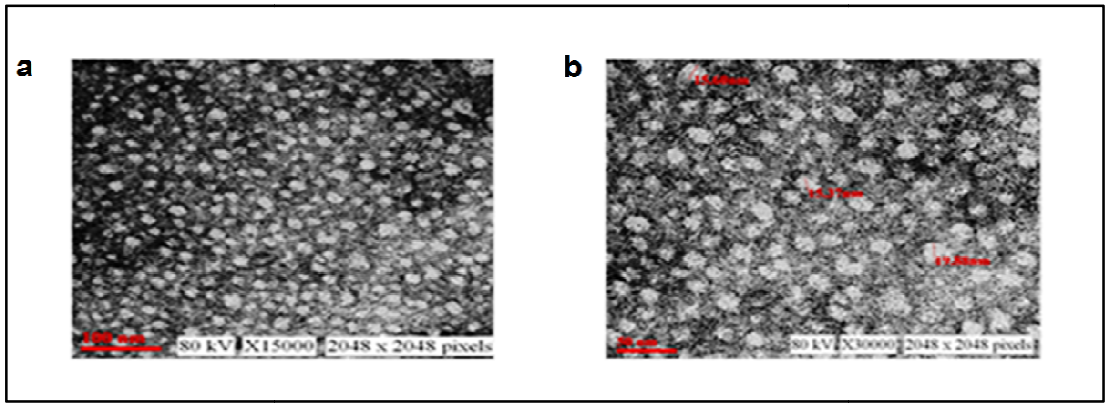

Figure 2.2: Transmission electron micrograph (TEM) of quercetin nanocrystals at (a) 100 nm and (b) 50 nm scale.

2.3.3 Dynamic Light Scattering

The zeta potential of bulk quercetin and nQ was analyzed using DLS technique. The zeta potential of the nQ (−44.1±6.82 mV) was found to be higher compared to zeta potential of bulk quercetin (−24.9±6.05 mV) corroborating the stability of nQ in water (Fig 2.3).

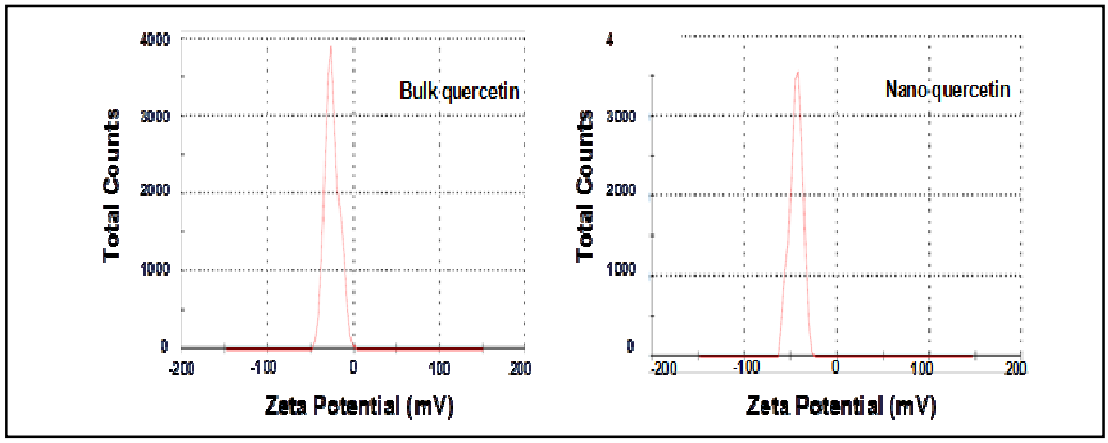

Figure 2.3: Zeta potential distribution of (a) Bulk quercetin and (b) Nanoparticulate quercetin measured by particle size analyzer.

2.3.4 X-ray diffraction

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 bulk quercetin powder.

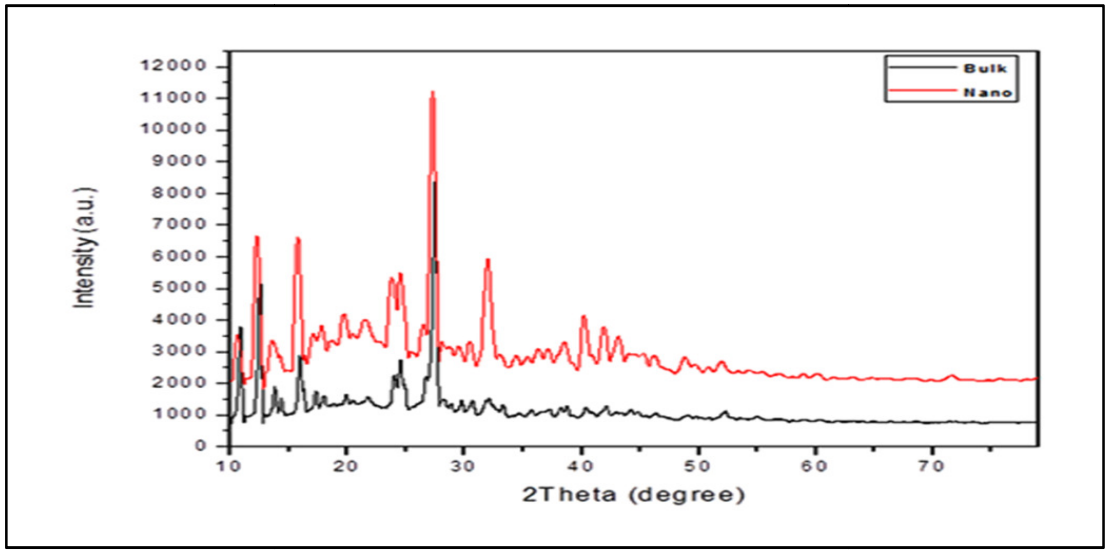

Figure 2.4: XRD of quercetin nanocrystals. All measurements were performed in triplicates.

2.3.5 Solubility, stability and *in vivo* pharmacokinetics of nQ

The pharmacokinetics of nQ was 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. 2.5(a)). The suspending quality of quercetin increased by 50% in nanocrystals as it is clear from Fig 2.5(a) that at 6 hrs bulk quercetin settled down to 100% where as in case of nQ the settling rate was 50% only.

The biodegradation and instability properties of nQ were determined at three different pH (3 mM; pH 2, 7.4 and 8.0) in PBS at various temperature using HPLC methods. 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. 2.5 (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 µg/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 when compared at all time points to bulk quercetin (Fig. 2.5(c) and Table I).

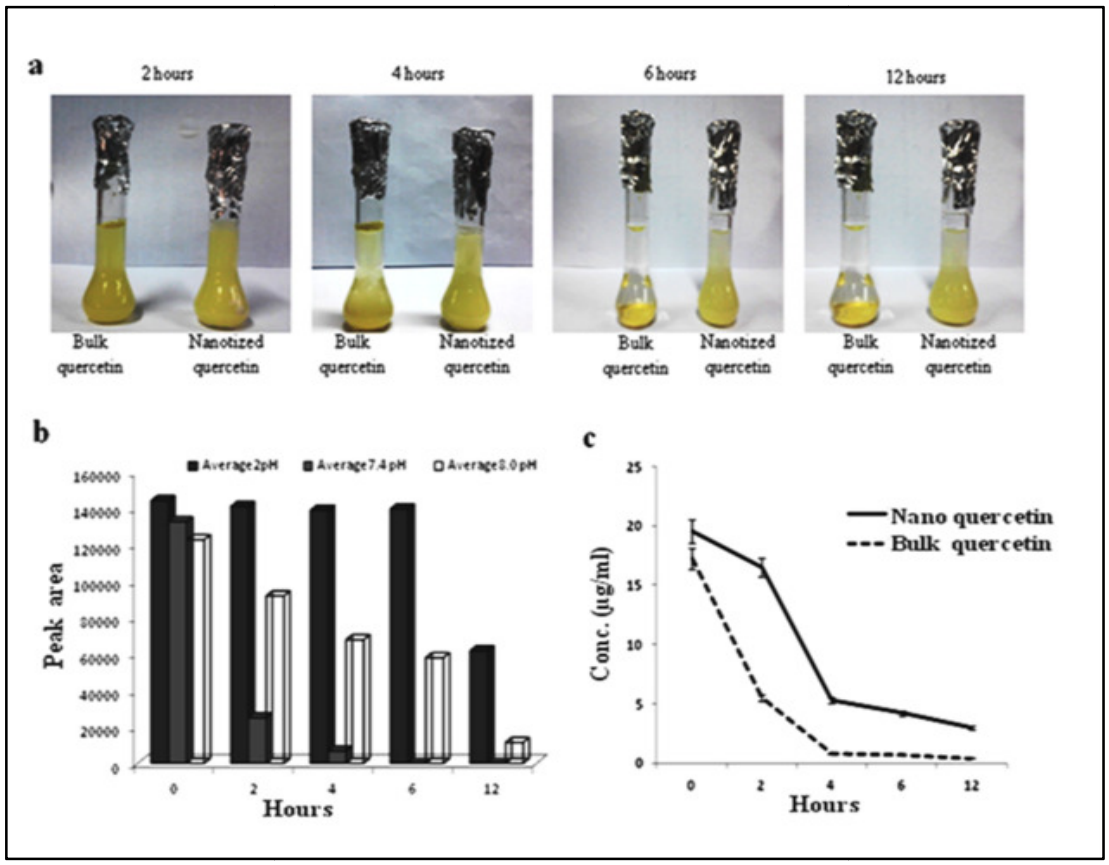

Figure 2.5: (a) Solubility study of quercetin (bulk and in formulation) in PBS. Bulk querectin (10 mg) dissolved in 10 ml PBS (0.01 M, pH 7.4) was insoluble in aqueous solution while equivalent quantity of nQ was fully soluble in aqueous solution. (b) Stability of querectin (bulk and nQ) in PBS (0.01M, pH 2.0,7.4 and 8.0) at 37 ºC. (c) In vivo bioavailability of bulk and nQ. The mice were divided in to two groups (n = 5). Equivalent concentration of Bulk quercetin and nQ (30 mg/kg) 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.

2.4 Discussion

In this study, we have successfully demonstrated the enhanced bioavailability, solubility and counteractive efficiency of quercetin by synthesized quercetin nanocrystals (nQ). 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 (Chourasiya et al., 2012). 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} are due to C–O and multiplets in the range of 1559–1444 cm^{-1} are assigned to C-C group of aromatic ring (Selvaraj et al., 2013). The FTIR spectra of nQ showed 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 and nano quercetin were studied using UV-VIS absorption spectroscopy. 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 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 (Fahlman et al., 2009). It is shown that quercetin absorbs UV radiation with absorbance maxima in the UV-A (max= 365 nm, ϵ = 28400 M^{-1} cm^{-1} and UV-C range (max= 256 nm, ϵ = 28300 M^{-1} cm^{-1}, resulting into 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* The results of UV-VIS absorption spectra obtained in our experiments are in good agreement with earlier reported works. (Fahlman et al., 2009, Ferreira et al., 2002). TEM images showed the formation of nearly spherical monodispersed nQ with size of 16.95±1.3 nm. It has been reported that higher zeta potential helps the particles to repel each other, which ensure long term stability and avoid particle aggregation (Usman et al., 2012). The zeta potential of the nQ (–44.1±6.82 mV) was found to be higher as compared to zeta potential of bulk quercetin (–24.9±6.05 mV) corroborating the stability of nQ in water and is in agreement with reported results (Kakran et al., 2012). 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**.** These observations confirm the phase purity of nQ and ensure all the physical characteristics of bulk quercetin.

The pharmacokinetics of nQ was 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 was found to be poorly soluble in aqueous media**.** Increased dispersibility has

been reported to be linked with the enhanced bioavailability of the compound (Saffoon et al., 2011). Similar to nQ other nanotized particles like curcumin have also been reported to exhibit increased dispersibility in aqueous medium. 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 its concentration at different time points by HPLC**.** The stability of nQ at different pH was in following order: 2.0 > 8.0 > 7.4. Like other flavanoids quercetin gets degraded at neutral and alkaline pH while it remains stable at acidic conditions. (Gupta et al., 2016)

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 (Manach et al., 1997). Since bulk quercetin can be rapidly metabolized from plasma and other organs thus its effect gets restricted (De Boer et al., 2005). 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.

Chapter 3

A simple extraction cum RP-HPLC method for estimation of pharmacokinetic parameters of nanotized quercetin in mice.

CHAPTER 3

A SIMPLE EXTRACTION CUM RP-HPLC METHOD FOR ESTIMATION OF PHARMACOKINETIC PARAMETERS OF NANOTIZED QUERCETIN IN MICE

3.1 Introduction

Among the bioavailability studies of polyphenolic compounds, quercetin received more attention due to its potential therapeutic effects but still required enhanced bioavailability and dispersibility. Since bulk quercetin can be rapidly metabolized from plasma and other organs, its effect vanishes quickly. The pharmacokinetics of quercetin in humans has been attempted by many researchers and it was found that the quercetin has a low plasma concentration, poor tissue absorption with rapid metabolism (Moon et al., 2008). Therefore, in order to have an effective treatment with quercetin, its low bioavailability needs to be improved. To improve bioavailability as well as therapeutic efficacy, new formulations have been developed by many researchers including complex forming with cyclodextrins and liposomes (Pralhad et al., 2004; Yuan et al., 2006). The new formulation may be an improvement but it was associated with risk of nephrotoxicity (Frijlink et al., 1991) On the other side, use of liposomal formulation has been restricted due to its poor stability during storage as a very short life span of liposomes encapsulated quercetin was observed (Mu et al., 2006). Low solubility as well as stability of quercetin in aqueous solution is due to its hydrophobic nature. To minimize this, DMSO has been

used for improving the solubility of quercetin but is not safe as higher dose of DMSO had resulted in vasoconstriction, neurological, cardiovascular and respiratory problems associated with bradycardia and diffused alveolar haemorrhage (Ader et al., 2000). Rogerio et al (2010) tried two formulation of quercetin-loaded microemulsion (QU-ME) and quercetin suspension (QU-SP) and compared their effect in murine model of airways allergic inflammation. They were found that QU-ME at a dose of 10 mg/kg given orally, exhibits pronounced anti-inflammatory properties as compare to QU-SP. However, Vicentini et al (2010) investigated the stability of quercetin (ME + Q) and they found a significant loss of quercetin content and antioxidant activity after 6 months of storage at 30°C/70% relative humidity (RH) and after 2 months at 40°C/70% RH. Thus the above formulation required particular storage conditions (at 4 ± 2°C) on longer term. Mulholland et al (2001) also synthesized water soluble quercetin (QC 12) and administered it into six cancer patients orally and intravenously (400 mg QC 12 i.e equivalent to 298 mg of quercetin). QC 12 could not be detected in plasma following oral administration while it was detectable in plasma after intravenous (i.v) administration, suggesting that only 20%-25% bioavailability of quercetin released from QC12. Thus there was an urgent need for improvement of the formulation of quercetin and/or delivery system and many attempts have been made. Quercetin has a tendency to bind with the plasma proteins, making the extraction quite complicated (Manach et al., 1997). But whether the available methods would also be applicable for nQ was not clear due to probability of higher binding capacity with protein. So, before proceeding for the *in vivo* experiments, the protein extraction by two different methods was compared. Wang et al (1993) demonstrated that a methanol-DMSO (4:4 v/v) mixture has been

used to extract protein bound drugs from plasma efficiently. Manach et al (1997) has been also extracted quercetin from plasma but resulted in insufficient recovery because of co-precipitation of the drug with proteins. Sri et al (2009) reported the extraction of bulk quercetin in human plasma by DMSO: MeOH mix (Sri V et al., 2009). Jones et al (1998) also used DMSO: MeOH (4:1 v/v) mixture for the precipitation–extraction of quercetin in plasma. The reported HPLC methods are tedious due to long colorimetric process and are applicable for bulk quercetin only (Sri et al., 2009). Therefore it can be stated that till date there is no method developed for detection of *in vivo* bioactivity of nQ. Thus the present work tries to develop a simple extraction cum reverse phase HPLC method to estimate nQ in various tissues and serum of mice treated with nQ. This study will also help in understanding the *in vivo* bioavailability and distribution of nQ and try to understand the benefits of increased bioavailability of quercetin that could not be apparent right now to scientific medical fraternity.

3.2 Materials and Methods

3.2.1 Reagents

Quercetin was purchased from Sigma Chem. Co. (Poole, Dorset, UK). The stabilizer Tween 80 (polysorbate 80) was procured from Uniqema, (Belgium). Ammonium acetate, disodium ethylenediaminetetraacetic acid (EDTA) and dimethyl sulphoxide (DMSO) all Analar grades were obtained from BDH (Poole, Dorset, UK), while methanol (HPLC grade) from Fisher (Loughborough, Leics., UK). The nano particulate quercetin was prepared and characterized as described in Gupta et al (2016).

3.2.2 Stability of nQ

The stability of nQ with varying temperature and pH was studied using high performance liquid chromatography (HPLC) according to the earlier described method (Mohanty et al., 2010). For stability assay at a fixed concentration of 40 µg/ml (for a total of 10 ml solution) nQ was prepared in PBS (3mM) at three pH (2.0, 7.4, 8.0) and incubated at 37, 4, -20, -80 ºC for a total of 96 h. At predetermined time points, 100 µL aliquot of nQ (40 µg/ml) was taken out and diluted with 900 µL of methanol for HPLC analysis.

3.2.3 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 (Reference no.: IITR/IAEC/06/2016 - 48/17) of CSIR-IITR, India.

3.2.4 Comparison of protein extraction of two methods

Before carrying out any further experiment the protein extraction using DMSO: methanol method and HCl method were carried out and the results of these two procedures were compared. Two sera samples of 100 µl from control mice were taken and protein was precipitated separately. In the first set, 200 µl DMSO: MeOH (1:4) was added. The mixture was vortexed for 2 min, centrifuged at 3000 rpm and supernatant collected. In the second set 100 µl 2N HCl was added and the samples were boiled at 90^0 C for 30 min. The resultant pellet was centrifuged at 12000 rpm

for 15 minutes. The supernatant liquid from the first set and the precipitated pellets from second set of protein were processed further for protein estimation.

3.2.5 *In-vivo* pharmacokinetics of nQ

Animal experimental study was carried out to analyze the pharmacokinetic aspects of delivering nQ according to the method described earlier (Mohanty et al., 2010). A set of 5 mice weighing approximately 20 g was injected with nQ (30 mg/kg body weight) via a lateral tail vein (i.v), and the blood from each mouse was collected from retro orbital plexus at 0, 2, 4, 6 and 12 hours after injection. Different tissues (lungs, kidney, liver, and brain) were collected after 4 hr from each of the mouse. All the samples were then further processed for HPLC determination of nQ.

3.2.6 Preparation of sample

Different tissues (100-200 mg) were homogenized with 500 µl of RIPA buffer. The samples were centrifuged at 16000 g for 30 min and supernatant were processed further for isolation of nQ. The serum samples were taken as such for isolation of nQ without any treatment of RIPA buffer.

3.2.7 Isolation of nQ

Extraction of nanotized quercetin from sera and tissues was optimized by 2 different methods. The first method was based on the extraction of nQ from sera and tissues by DMSO: MeOH mix (Sri et al., 2009). In the second method 100 µl of sera and 100-200 mg of tissue was precipitated with 0.1 ml of 2N HCl and heated at 90 ^{0}C for 30 minutes. Finally the samples were centrifuged at 12000 rpm for 12 min and supernatants collected.

3.2.8 Cleanup

The supernatants from both sets were passed through Oasis SPE cartridges for further clean up according to the method of Ishii et al (Ishii et al., 2003). Keeping in view of lower bioavailability of nQ in different tissues, the supernatants of each tissue of five mice were pooled for clean up. However the sera samples were processed individually from each mouse. The cartridges were conditioned with 1 ml of 0.5 M phosphoric acid (PA) and the supernatants from both sets were loaded. The cartridges were then washed with 1 ml of 5% MeOH in 0.5M PA followed by 1 ml of 50% MeOH in 0.5 M PA. The sample was eluted with 3 ml MeOH and evaporated to dryness at 40 $^{\circ}$C in vacuum. The residue was finally dissolved in 100 µl MeOH and 20µl was injected in HPLC for further analysis.

3.2.9 Chromatographic conditions

Chromatographic analysis was carried out with a Waters LC module (Waters Associates, Vienna, Austria) equipped with a dual pump (Model 515), Rheodyne injector with a 20 µL loop and a tunable absorbance detector (Model 2489). The UV detector was set at 389 nm and isocratic mobile phase (MeOH: 0.2% PA) of flow 1 ml/ min was used (Liu et al., 2011). The chromatograms were recorded and processed by Waters Empower III software version.

3.2.10 Linearity

The linearity of the assay was confirmed by running duplicate sets of standard in the range of 0.1-20 µg/ml and the calibration graph was obtained by plotting the peak area versus concentration.

3.2.11 Limit of detection (LOD) and Limit of quantification (LOQ)

In order to determine the LOD and LOQ, concentrations of nQ in the lower part of the linear range of the calibration curve were used. The LOD and the LOQ were calculated using the following equations: LOD = 3.3 (σ/S) and LOQ = 10 (σ/S), respectively, where σ is the standard deviation of the response and S is the slope of the corresponding calibration curve.

3.2.12 Precision

Repeatability values were determined by using 3 replicates of nQ at two different concentrations of 5 and 10 μg/ml. The intraday precision was evaluated by using triplicates at these two concentrations of the nQ on the same day. Interday precision was evaluated by using standard nQ at 2 concentrations on 3 different days.

3.2.13 Accuracy

Known quantity (1, 3, 6 or 9 μg) of nQ, as a solution was added to the sera and processed by HCl method and injected into HPLC to determine the accuracy of the analytical method. Accuracy was expressed in terms of the percent recovery.

3.2.14 Statistical analysis

All data were expressed as the mean +/- standard error of the mean (SEM). Differences between all groups were compared by one-way analysis of variance (ANOVA). A probability value of $P < 0.05$ was considered statistically significant.

3.3 Results

3.3.1 Stability of nQ

The nQ was found stable up to 96 hours at pH 2.0 at all the four temperatures (37, 4, -20 and -80 ºC). However, at pH 7.4 and pH 8.4 nQ was completely degraded at 6 hrs. The pattern of degradation of nQ followed similar trend at both pH 7.4 and 8.4 (Fig. 3.1).

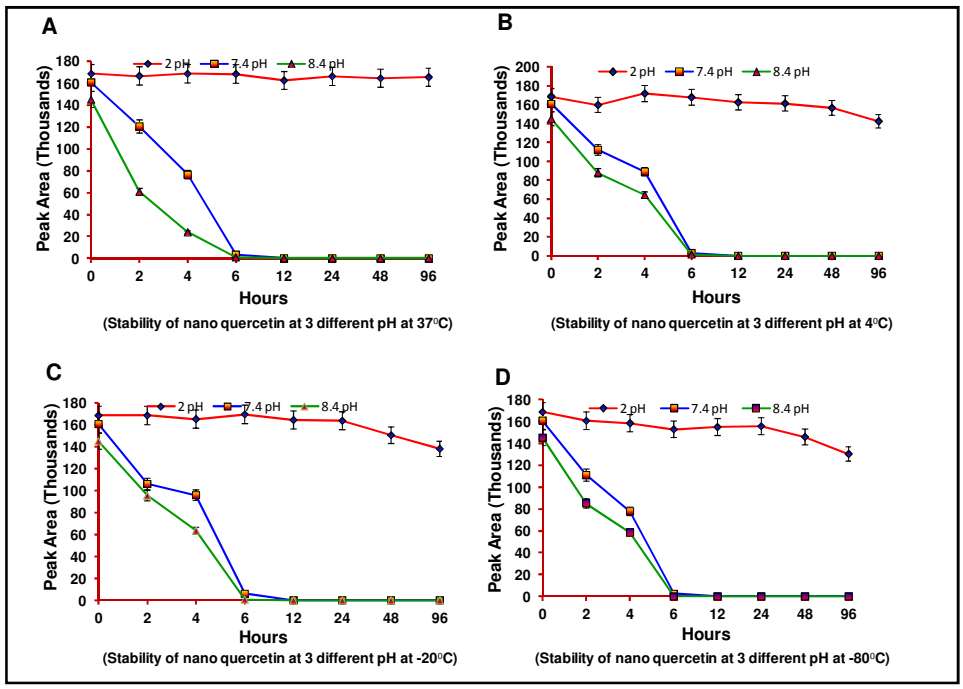

Figure 3.1: Stability of nQ at three different pH and temperature (4º, 34º, -20º, -80º C). The data from at least three independent experiments are expressed as the Mean percent of recovery (± SEM) (n = 3).

3.3.2 Validation of the method

The linearity of nQ was checked between the ranges of 0.1-20µg/ml. The calibration graph exhibited good linear relationships with excellent value of the regression equation (0.9990) and small intercepts (Table 3.1). The LOD and LOQ values of nQ were found to be 0.19 µg/ml and 0.58 µg/ ml respectively. The inter-day and intra-day precision was excellent with % RSD value varied from 0.9-1.2 and 1.2-1.3 respectively (Table 3.2).

Table 3.1: Statistical parameter of calibration graph of nQ

Equation Y=b+ mx*	Linearity range (μgmL^{-1})	Regression Coefficient	LOD (μgmL^{1})	LOQ (μgmL^{-1})
$-1.78x10^{3}+3.23 x10^{4}$	0.1-20.0	0.9990	0.19	0.58

Table 3.2: Precision for the estimation of nQ from serum

Inter-day			Intra-day % recovery		
Concentration (µg/ml)	**Average Peak area**	**% RSD**	**Concentration (µg/ml)**	**Average Peak area**	**% RSD**
5	154284	0.9	5	153888	1.2
10	323307	1.2	10	312567	1.3

3.3.3 Comparison of protein extraction of two methods

Protein Bovine serum albumin (BSA) was extracted by the reported DMSO: MeOH mix method and compared to 2N HCl method (Table 3.3). The results showed BSA precipitation is approximately 2 folds higher in case of 2N HCl.

Table 3.3: Comparison of protein precipitation by DMSO and HCl method

S.No.	Precipitated by	Amount of protein present in the pellet (mg/ml)
1	DMSO:MeOH	17.8±1.2
2	2N HCL	36.4±1.4

Values are expressed as means ± S.E.M. (n = 3).

3.3.4 Accuracy

A recovery experiment was performed by spiking 1, 3, 6 and 9 µg/ml of nQ in serum samples in triplicates (Table 3.4). The percentage recovery of nQ was found to be in the range of 81- 98 %, that is an advantage of our method over Sri et al (2009) reported 82-85% recovery of bulk quercetin from human plasma (Sri et al., 2009).

Table 3.4: Percentage recovery of nQ extracted from serum

S.No	Amount added(µg/ml)	Amount recovered (µg/ml)	% recovery
1.	1	0.8	81.0±0.2
2.	3	2.45	81.7±0.3
3.	6	5.30	88.3±0.2
4.	9	8.81	97.9±0.1

3.3.5 Comparison of extraction of nQ from serum and tissues by two methods

The extraction of nQ from tissues by HCl method was 2-3 fold higher than DMSO: MeOH mix. The comparison of extraction of nQ from different tissues by the two extraction methods is illustrated in Table 3.5. Similar results were obtained when serum samples were collected at different time intervals and extracted by these two methods and showed 2-3 fold higher concentrations of nQ in HCl extracted samples (Table 3.6). The HPLC profiles of extraction of nQ from serum samples with 2NHCl and DMSO: MeOH mixture is given in Fig. 3.2. The quantity of nQ extracted by HCl method was found to be more than DMSO: MeOH mix method (Fig. 3.2).

Table 3.5: Comparison of extraction of nQ in different tissues after 4 h following administration by tail vein.

Tissue	Extracted by	
	2N HCl (µg/g)	DMSO:MeOH mix (µg/g)
Lungs	12.7 ± 0.1	5.7 ± 0.3
Kidney	9.1 ± 0.2	4.1 ± 0.1
Liver	5.7 ± 0.2	2.6 ± 0.1
Brain	1.1 ± 0.3	0.4 ± 0.01

Values are expressed as means ± S.E.M. (n = 3).

Table 6. Comparison of extraction of nQ in serum at different time points following administration by tail vein.

nQ extraction from serum at different time points	Extracted by 2N HCl (µg/ml)	DMSO:MeOH mix (µg/ml)
0.5 hr	19.8 ± 0.1	9.8 ± 0.3
2 hr	16.3 ± 0.06	5.2 ± 0.1
6 hr	5.8 ± 0.2	2.7 ± 0.1
12 hr	1.5 ± 0.2	0.5 ± 0.2

Values are expressed as means ± S.E.M. (n = 3).

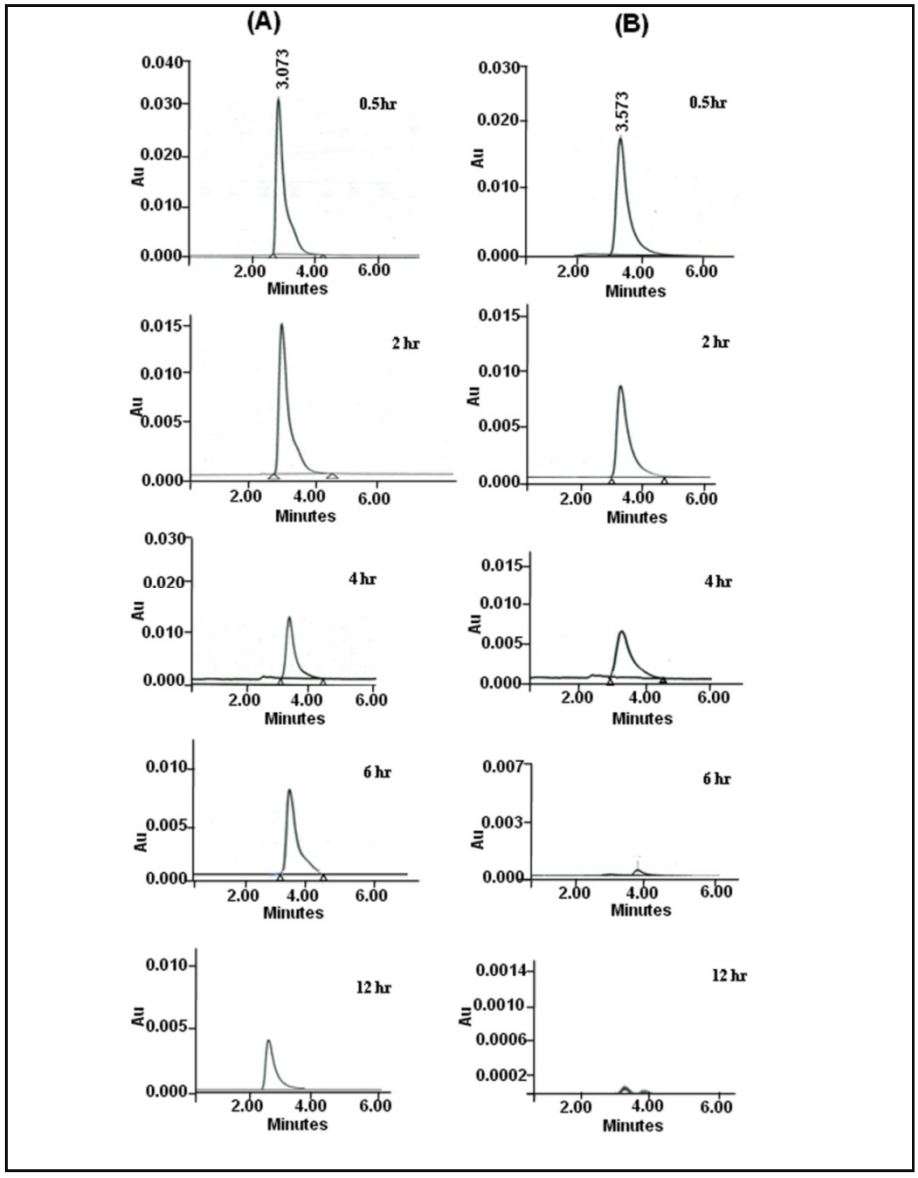

Figure 3.2: HPLC profile of extraction of nQ from sera at different time intervals with 2N HCl (A) and DMSO:MeOH mix (B). Values are expressed as means ± S.E.M. (n = 3).

Furthermore, no nQ was detected after 4 hrs when extracted by DMSO: MeOH mix method, whereas nQ was present up to 12 hrs when precipitated by 2N HCl method which is great advantage of this method.

The result of extraction of nQ from different tissues by both the methods also showed that nQ extracted by HCl method is superior to DMSO: MeOH mix method (Fig. 3.3 (a-b)).

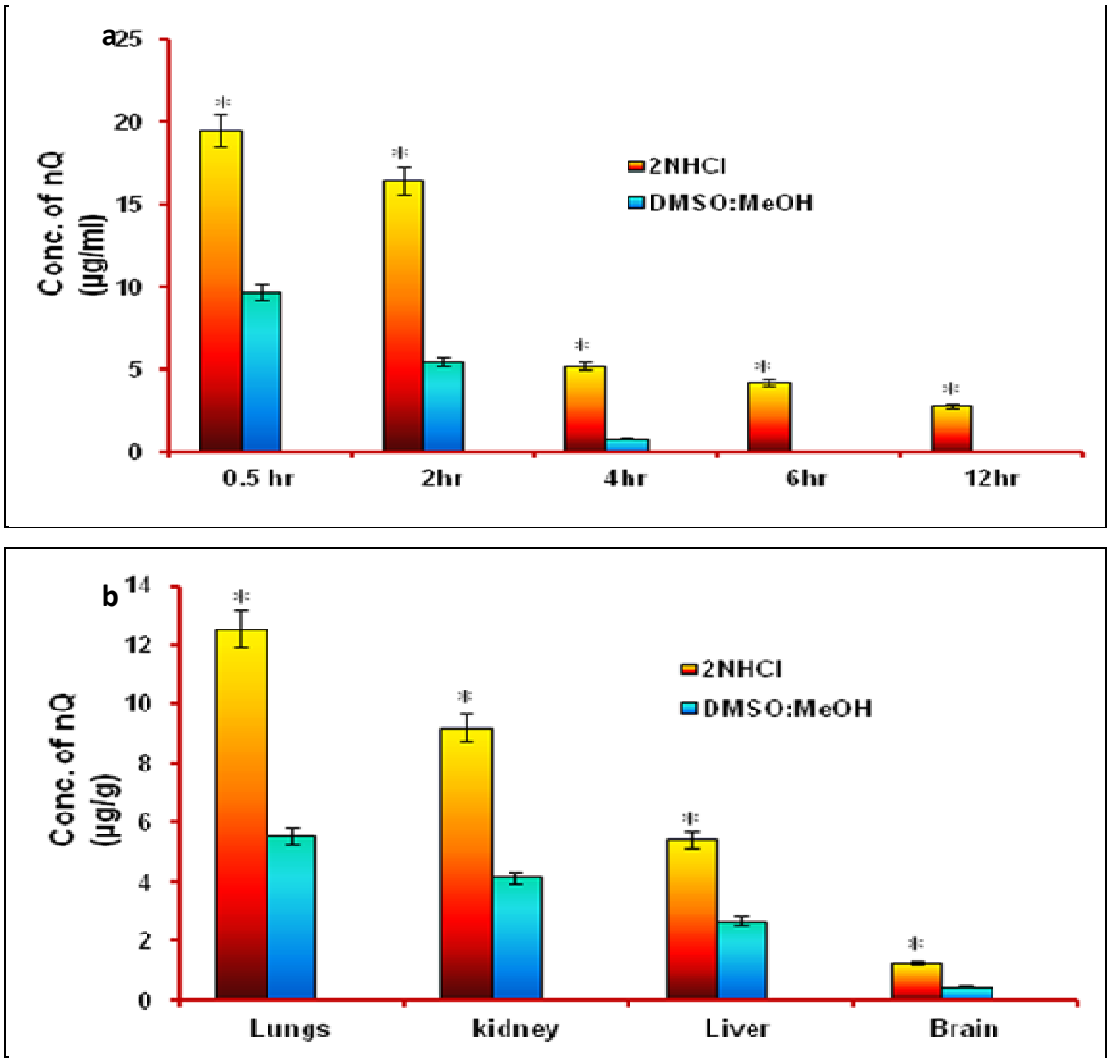

Figure 3.3: (a) Comparison of extraction of nQ from serum when given by intravenous (i.v) route. The data from at least three independent experiments are expressed as the Mean percent of recovery (± SEM) (n = 3). (b) Comparison of extraction of nQ from tissues when given by intravenous (i.v) route. The data from at least three independent experiments are expressed as the Mean percent of recovery (± SEM) (n = 3).

3.4 Discussion

During the development of new therapeutic products, an essential parameter such as stability as well bioavailability must be investigated as an integral part of the process. In an attempt to study the biodegradation and instability properties of nQ, we incubated nQ in PBS solution (3mM) at three different pH concentration of 2.0, 7.4 and 8.4 with varying temperature of 37, 4, -20, -80 ºC and estimated its concentration at different time points by HPLC. It is well known fact that at higher pH, the flavonoids get degraded and the degradation kinetics is more rapid at pH around 7. The degradation and instability properties of flavonoids like quercetin depend on their pH, temperature as well as chemical structure. Polyphenolic compounds like flavonoids exhibit more hydroxyl groups corroborrated its lower stability. Quercetin is found to be more susceptible with change in temperature as well as pH due to its 4 to 5 hydroxyl groups. When assessing the stability of quercetin nanocrystals from acidic to alkaline pH, it was found that it is more unstable at neutral and alkaline pH (7.4 and 8.4) but it was found to be stable under acidic condition with varying temperatures. These results are in support with our previous study where the zeta potential of the nQ (−44.1±6.82 mV) was found to be higher as compared to zeta potential of bulk quercetin (−24.9±6.05 mV) corroborating the stability of nQ in water that is in good agreement with reported results (Gupta et al., 2016). The higher zeta potential helps the particles to repel each other, that ensures long term stability and avoid particle aggregation.

Sri et al (2009) showed linearity of bulk quercetin in the range of 0.2-30 µg/ml with correlation coefficient value 0.997. The % RSD of inter-day and intra-

day precision reported by Sri et al (2009) ranged between 0.9-1.6 and 0.1-2.2 respectively that further validates our method.

It was reported that though quercetin is soluble in methanol, but may not result in proper extraction due to its binding capacity of quercetin with proteins. DMSO: MeOH mixture has been used for complete extraction of quercetin (Jones et al., 1998). It was assumed that the mixture can precipitate protein, but perhaps could not break the covalent bond of quercetin with sera and tissue samples, therefore free quercetin may not be available for estimation. Serum and tissue samples treated with 2N HCL was sufficient to break the covalent bond as well as precipitate protein efficiently and hence total quercetin becomes available for estimation. The higher recovery of nQ in the present case may be due to treatment of 2N HCl.

In several studies, bulk quercetin extraction from plasma and tissues has been carried out using DMSO: MeOH mixture, but, could have resulted in insufficient recovery because of co-precipitation of the drug with proteins. Also, there are no reported methods for extraction of quercetin nanocrystals from biological samples. Considering the same issues, we have tried to develop an effective method to extract the quercetin using 2N HCL instead of DMSO:MeOH mixture. The present method was found to be more efficient with better recovery of bound quercetin in comparision to DMSO: MeOH mixture.

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not break the covalent bond of quercetin with sera and tissue samples, therefore free quercetin may not be available for estimation. Serum and tissue samples treated with 2N HCL was sufficient to break the covalent bond as well as precipitate protein efficiently and hence total quercetin becomes available for estimation. Therefore, further extraction procedures were carried out in 2N HCl.

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Thus a simple, highly sensitive and more specific extraction cum HPLC procedure for the quantitative determination of nQ in sera as well as in different tissues of mice was reported here. This study deals with the additional steps of extraction of nanotized quercetin, which enhances the detection by two to three folds. Thus we feel that this study not only has the novelty, but has the utility. This method has been found to simple, rapid and more sensitive estimation of other flavonoids in mice as well as in humans. Our studies have verified that the decreased particle size of nQ increased bioavailability as well as aqueous solubility and thus it can be suggested that it is safe, risk free and counter drug suitable for treatment of many diseases that causes inflammatory response.

Chapter 4

Therapeutic potential of Nano Q in OVA induced BALB/c mice of allergic asthma.

CHAPTER 4

THERAPEUTIC POTENTIAL OF NANO Q IN OVA INDUCED BALB/C MICE OF ALLERGIC ASTHMA.

4.1 Introduction

Asthma is a major cause of morbidity characterized by variable and recurring symptoms like airway hyporesponsiveness, tissue remodeling, bronchospasm and chronic airway inflammation. The other common features of asthma are coughing, congestion in chest, wheezing, and shortness of breath (Manno et al., 2010; Wippold et al., 2005). Prevalence of asthma is on increase substantially throughout the globe and approximately 250,000 asthma related deaths have been reported each year (GINA., 2011). Allergic asthma is the most prevalent form of asthma that is typically triggered by allergens like pollen, mold, dust mites and foods (Richter et al., 2011; Rosenstreich et al., 2003). Asthmatic individuals need to be extremely cautions as this population is at high risk from hazardous air particles (HAP) including fine particulate matter, tobacco smoke and other air born pollutants (Leikauf et al., 2002). Moreover, asthma is triggered by several intrinsic factors like upper respiratory infection, physical exercise and weather changes as well (Chua et al., 2007; Tillie et al., 2005). The different allergens and extrinsic factors elicit the immunological processes that are characterized by enhanced IgE levels, mast cells number and activation of Th2 lymphocytes. All these together sets 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 (Rosenstreich et al., 2003; Leikauf et al., 2002; Chua et al., 2007; Tillie et al., 2005). Furthermore, chemokines, including thymus and activation-regulated chemokines, play a vital role in regulation of inflammation and IgE synthesis in asthma. Currently, the available treatment of AHR is done by decreasing the inflammation or relaxing airways, however these techniques are not good enough to fully manage 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 (Mainardi et al., 2009). 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, and allergic asthma (Blanc et al., 2006; Riccioni et al., 2007; Mukhtar et al., 1988). Dietary antioxidants intervention may also reduce oxidative stress and prevent or minimize asthmatic symptoms (Blanc et al., 2006). Quercetin is a plant-derived bioflavanoid known to exhibit excellent anti-oxidant and anti-inflammatory properties (Das et al., 1988). It has been suggested as a good candidate for the management of eosinophil-mediated diseases, such as allergic rhinitis and asthma (Sakai et al., 2013). Quercetin relaxes airway smooth muscle through cAMP-mediated pathways and augments ß-agonist relaxation via dual phosphodiesterase inhibition of PLCγ and PDE4 (Townsend et al., 2013). Quercetin lowers the expression of pro-inflammatory cytokines and improves lung function in RV-infected mice (Ganesan et al., 2012). The immediate effect of

quercetin on experimental allergic asthma after single oral dose indicated that it is responsible for bronchodilation, both *in vivo* and *in vitro* (Joskova et al., 2011). Anti-inflammatory effect of quercetin loaded microemulsion (QU-ME) and quercetin suspension (QU-SP) has been studied on murine model of airways allergic inflammation (Rogerio et al., 2010). Potentiating the existing natural bioflavonoid quercetin using nanotechnology may pave the new direction for treating asthma. We investigated well characterized nQ on OVA induced BALB/c mouse model of allergic asthma for studying its effects on 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.

The tranquillizing effects of quercetin on allergic asthma seemed very promising, but its poor water solubility and bioavailability was still a bottleneck. In previous chapter we discussed about the preparation, characterization and in vivo pharmacokinetics of water soluble nQ. The nQ was found to be more stable and soluble in PBS, and sera of BALB/c mice compared to bulk quercetin. Now, 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.

4.2 Methods and Materials

4.2.1 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. BALB/c mice weighing 20–25 g were used.

4.2.2 Animal Treatment Protocol and dose determination of nQ

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 (Park et al., 2009). in brief, mice were immunized intraperitoneally (i. p.) 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 body weight of nQ in 200 µl of PBS each day from 18 to 20 and designated as a OVA+nQ group. The untreated group sensitized with OVA 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 (Park et al., 2009). 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 Fig. 4.1.

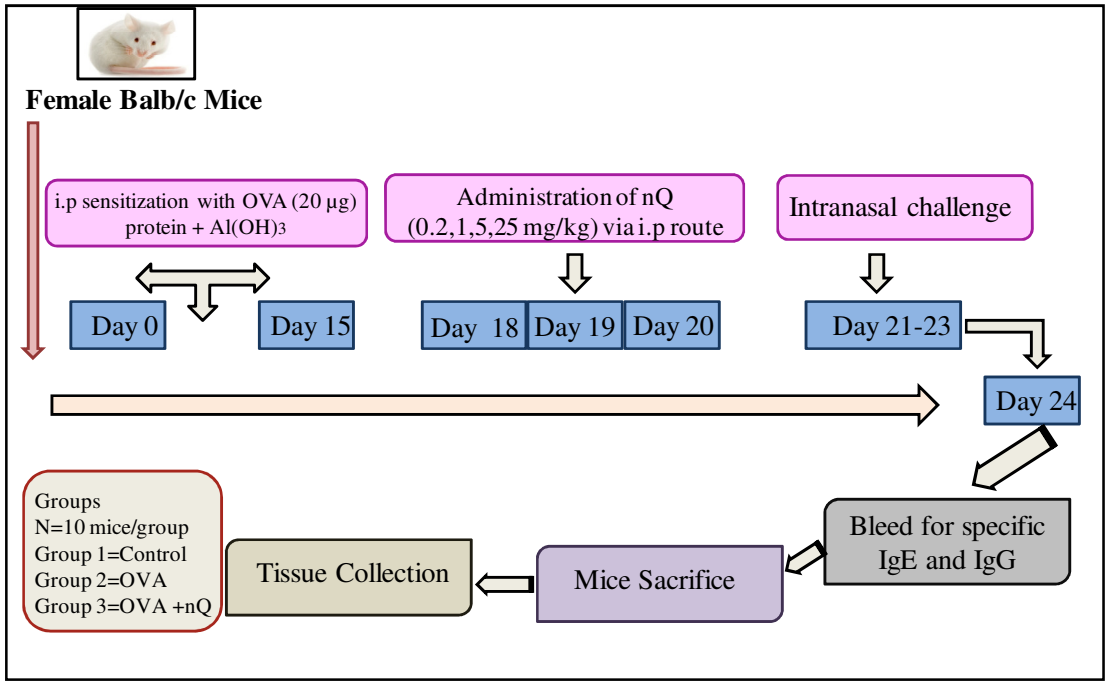

Figure 4.1: Animal sensitization protocol

4.2.3 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 ºC. The plate was washed with PBS washing buffer having 0.05% Tween 20 thrice 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 RT. 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.

4.2.4 OVA Specific IgE and IgG1 Assay

Specific IgE (sIgE), sIgG1 and sIgG2 levels against OVA were estimated by enzyme-linked immunosorbent assay (Polte et al., 2006). Briefly, the wells of microtiter plate (Nunc, Roskilde, Denmark) were coated with 1 µg of OVA in coating buffer (0.05 M Carbonate-Bicarbonate, pH 9.6). After blocking with 3% 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 ºC. Further, the plates were washed and incubated with HRP-conjugated goatanti- mouse IgE (1:1000 v/v) and HRP-conjugated goat anti- mouse IgG1 (1:1000 v/v), respectively, for 3 h at 37 ºC. Color was developed with ortho-phenylene diamine (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). Specific IgE, sIgG1 and sIgG2 levels were measured in triplicate and average value was calculated.

4.2.5 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.

4.2.6 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-m thick sections and stained with Hematoxylin and Eosin. Stained sections were embedded in paraffin, and observed using a Nikon Eclipse TE2000-S microscope.

4.2.7 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.

4.2.8 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-TCGGCATTTTGAACGAGGTC-3, antisense 5 AAAAGCCCGAAAGAGTCTC-3; IL-5: sense 5-TCACCGAGCTCTGTTGACAA-3, antisense 5-CCACACTTCTCTTTTTGGCG-3; and Foxp3: sense 5-GCTCCCGG CCTGGTCTGCTC-3, antisense 5-AGGTGGCGGGGTGGTTTCTGA-3and GAPDH: sense 5-TTCACCACCATGGAGAAGGC-3, antisense 5-GGCATGGACTGTGGTCATGA-3. Cycling conditions were 1 cycle at 50 ºC for 2 min, 1 cycle at 95 ºC for 10 min, and 40 cycles each corresponding to 15 s at 95 ºC and 1 min at 60 ºC. Analysis used the sequence detection software supplied with the instrument. The relative quantification value is expressed as 2 ± DCT, where DCT is the difference between the mean CT value of duplicates of the sample and of the GAPDH control.

4.2.9 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 (Towbin et al., 1979). Proteins from the lungs were isolated according to the method described earlier (Kumar et al., 2013). Goat antimouse 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 ß-tubulin (Santa Cruz Biotechnology, Inc. Santa Cruz, CA) was used as internal control.

4.2.10 Lungs Mast Cells Culture and ß-Hexosaminidase Release Assay

The *ex vivo* relevance of nQ was carried out in the lungs mast cell culture in the control, OVA + nQ and OVA groups. The lungs mast cell culture was performed as per the previously described method (Zaidi et al., 2006). 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, lungs mast cells were stained with toluidine blue (Sigma- Aldrich, St Louis, MO) to confirm its morphology. The ß-hexosaminidase release in the lungs mast cells were studied following the exposure of 10, 20, 30, 40 and 50 µg OVA according to the previously described method (Kumar et al., 2013).

4.2.11 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, western blotting analysis of signalling molecules FcR1, Syk, PKC, p-PKC, c-Yes, c-Fgr, Lyn, PI-3, p-PI-3, PLC-γ2, and p-PLC- γ 2 (Santa Cruz Biotechnology, Inc. Santa Cruz, CA) of mast cells was done. The ß-actin (Santa Cruz Biotechnology, Inc. Santa Cruz, CA) was used as internal control.

4.2.12 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.

4.3 Results

4.3.1 Specific IgE, specific IgG1 and specific IgG2 Level

The levels of sIgE and sIgG1 were found lowest in the OVA group after 1 mg/kg dose of nQ. All the studies on various allergic parameters were therefore carried out using 1 mg/kg dose of nQ as an optimal dose (Fig. 4.2 (A-C)).

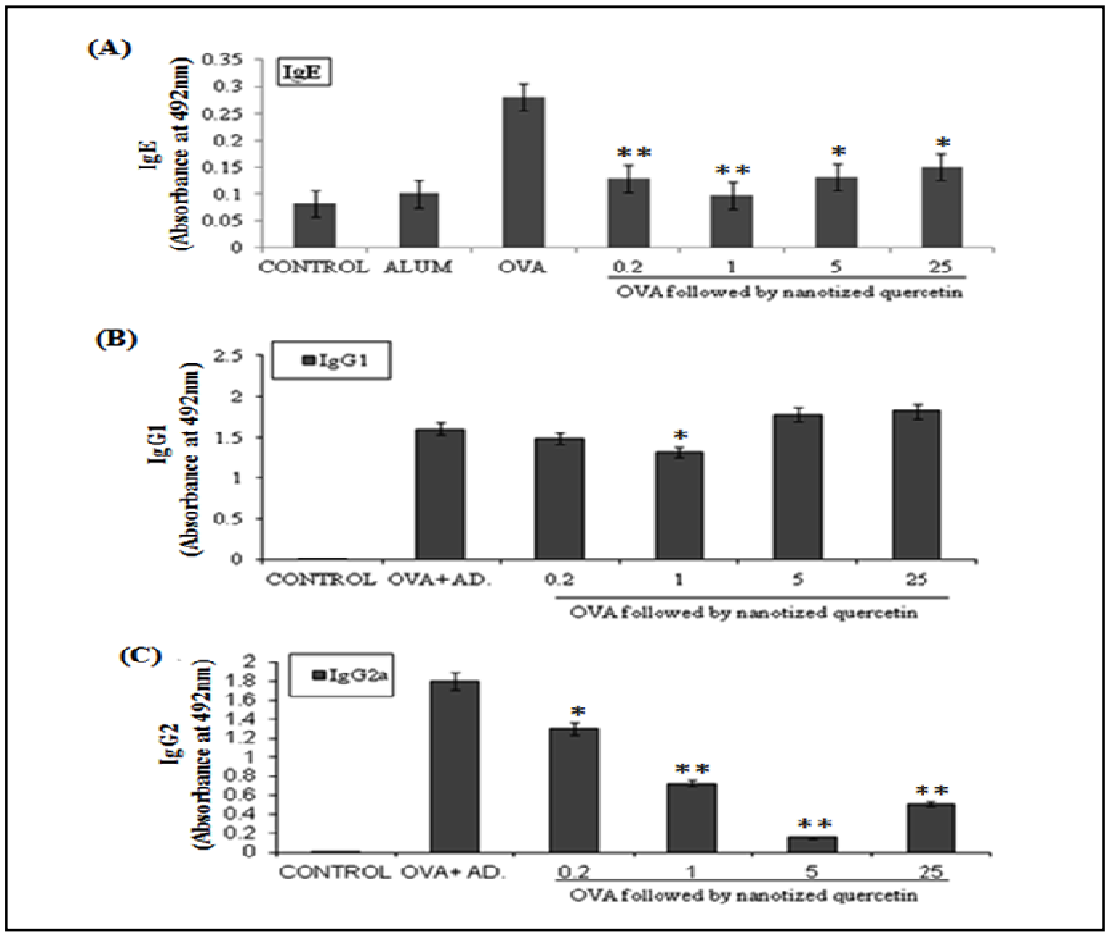

Figure 4.2: The figures above are showing the level of IgE, IgG1, and IgG2 after treatment with different concentration of nQ (0.2, 1, 5, 25 mg/kg) to determine the optimum dose. The data from at least three independent experiments are expressed as the mean ± SEM. Significant difference *p<0.05 and **p<0.01 when compared with OVA. Where ns= non-significant

4.3.2 Total IgE, Specific IgE and Specific IgG1 level

The levels of tIgE, sIgE and sIgG1 are important in the allergic manifestations that were observed to be significantly ($p < 0.05$) lower in the OVA+nQ group at 1 mg/kg dose level when compared to the OVA group as shown in Fig. 4.3(A)–(C).

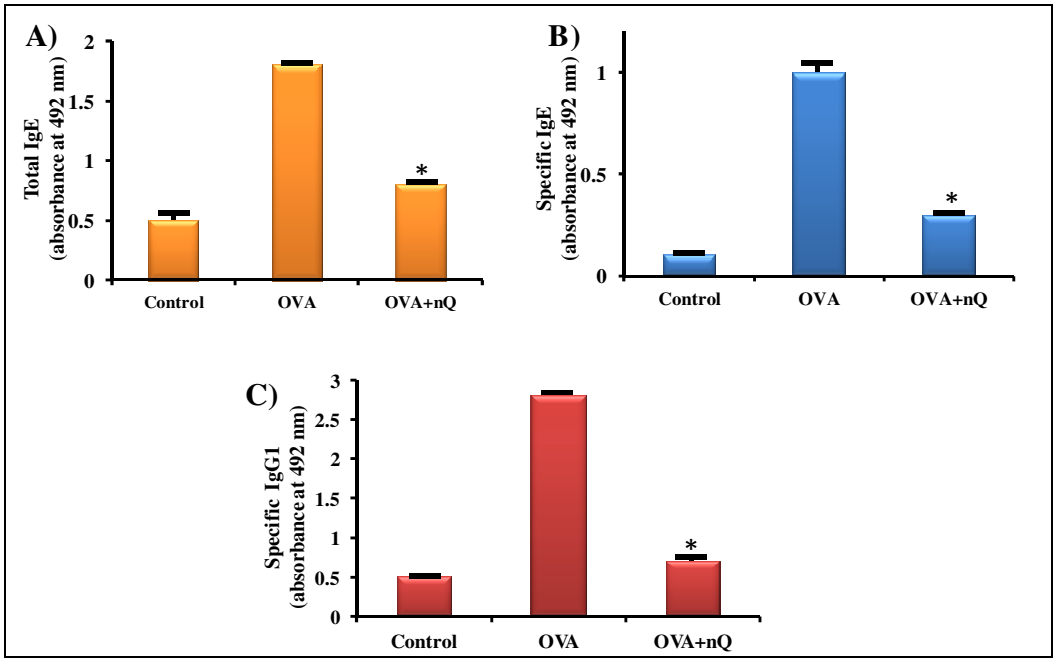

Figure 4.3: Immunoglobulin levels: (A) Level of total IgE in the serum of control, OVA+nQ and OVA; (B) Level of specific IgE in the serum of control, OVA+nQ and OVA; (C) Level of specific IgG1 in the serum of control, OVA+nQ and OVA treated groups. The graphs are showing decreased levels of total IgE, specific IgE and IgG1 in the serum of OVA+nQ treated groups when compared to OVA group; The data from at least three independent experiments are expressed as the mean±SEM. Significant difference *$p<0.05$ when compared to OVA. Where ns = non-significant.

4.3.3 Allergic Mediators level in the Serum

OVA treated mice showed significantly increased levels of PGD2, CysL, MCPT-1, and TSLP (Fig.s. 5(A)–(D), while levels of these mediators returned closer to control in the OVA+nQ group demonstrating efficacy of nQ treatment.

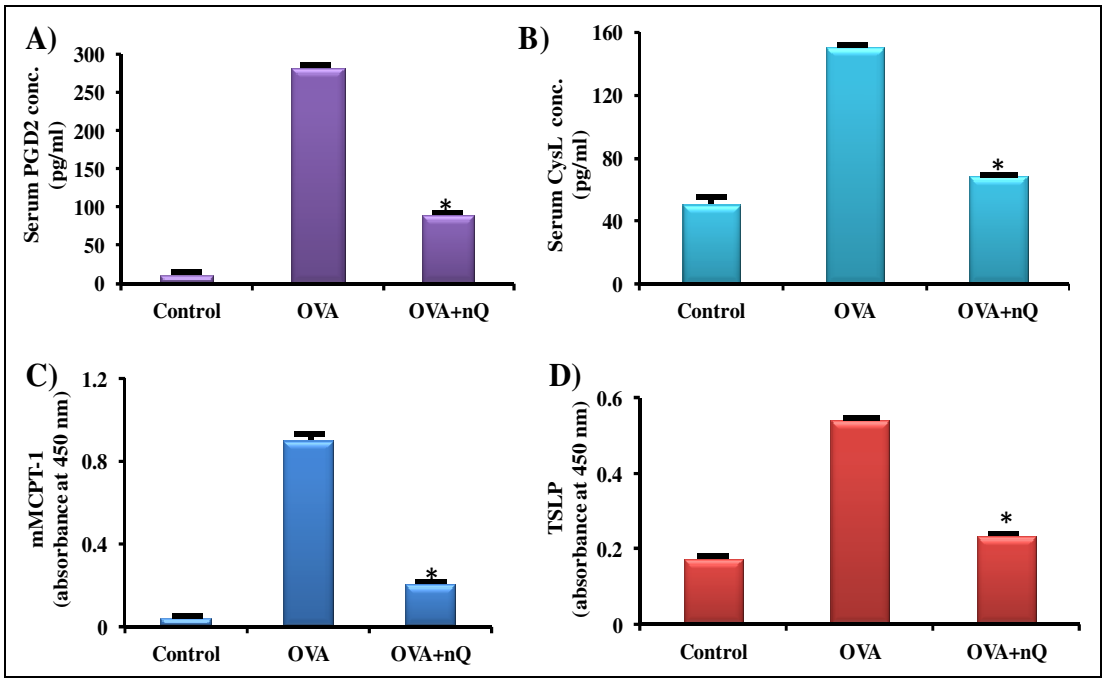

Figure 4.4: Serum concentration of PGD2, CysL, mMCPT-1 and TSLP: The mice of OVA+nQ group showed relatively lower levels of (A) Serum prostaglandin or PGD2 concentration, (B) Serum cysteine leukotriene (CysL) concentration, (C) Serum mouse mast cell protease-1 (mMCPT-1) and (D) Thymic stromal lymphopoietin (TSLP) when compared to OVA group. The data from at least three independent experiments are expressed as the mean±SEM. Significant difference ∗p < 0_05 when compared with OVA. Where ns = non-significant.

4.3.4 Lungs Histopathology

Lungs histopathology of OVA treated mice showed perivascular and peribronchial inflammatory cell infiltrate with mild narrowing of the bronchiolar lumen (Fig. 4.5). 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.

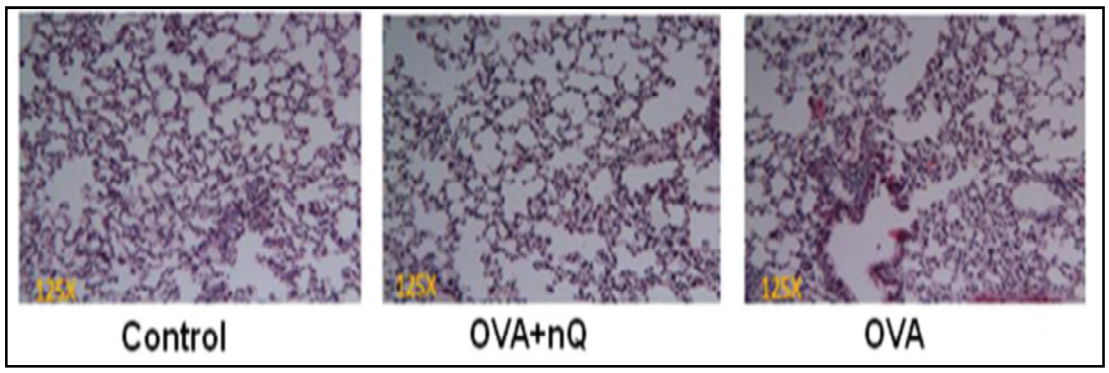

Figure 4.5: shows the lung histopathology of control, OVA+nQ and OVA. The Lung histopathology of OVA mice showed perivascular and peribronchial inflammatory cell infiltrate with mild narrowing of the bronchiolar lumen as well as thickening of alveolar septa throughout the parenchyma. The histology of OVA+NQ seems closer to control.

4.3.5 Mast cell staining and Eosinophils Measurements

In the present study, the mast cell numbers increased in OVA sensitized mice in comparison to control group (Fig. 4.6(A) pointing towards enhanced inflammatory pathways. The number of mast cells was found to be significantly reduced in OVA+nQ group mice suggesting that nQ suppressed asthmatic disorders or other inflammatory responses.

The OVA+nQ group showed significantly reduced eosinophil numbers when compared to OVA (Fig. 4.6(B). Eosinophil numbers in OVA+nQ group were comparable to control level.

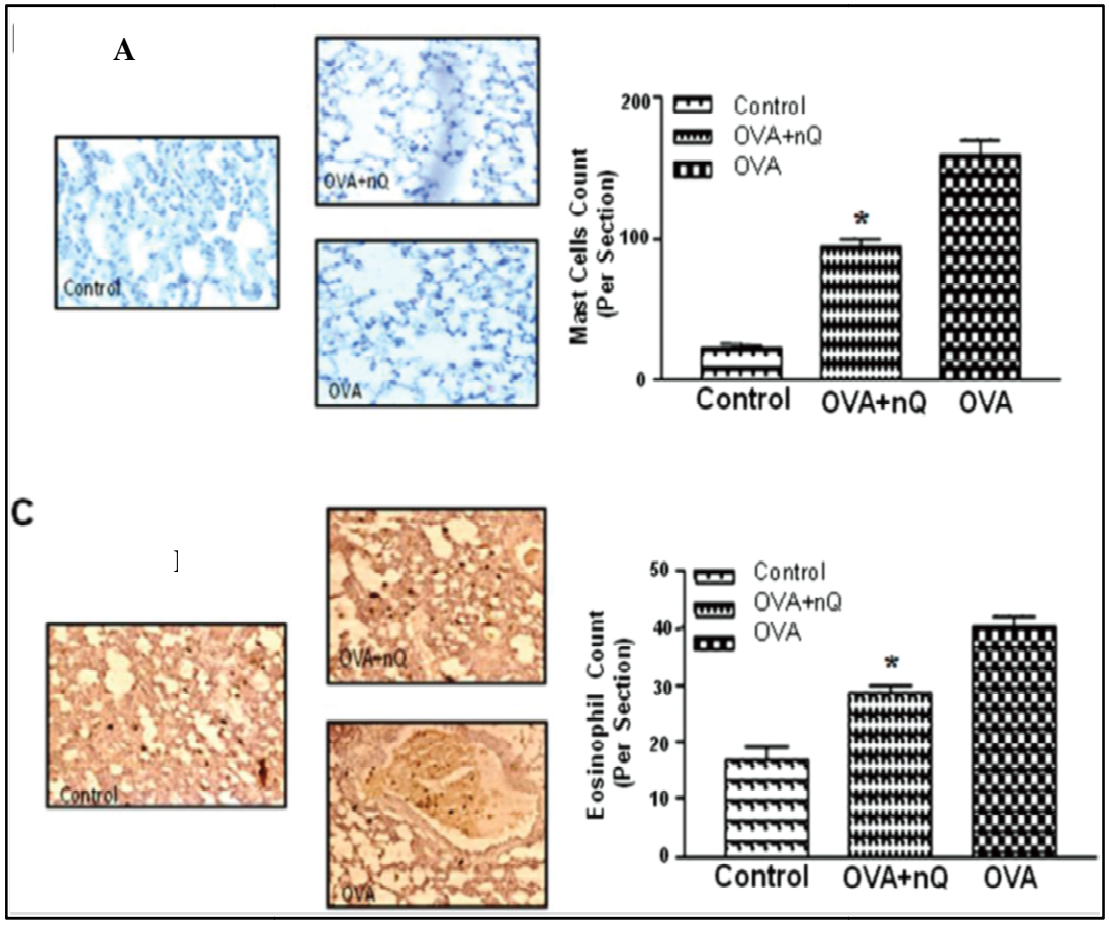

Figure 4.6: (A) and (B) are showing images of mast cell staining with toludine blue and eosinophils counts in lungs tissue. Decreased level of mast cell count along with eosinophils counts was observed in OVA+nQ group as compared to OVA as shown in graph. The data from at least three independent experiments are expressed as the mean±SEM. Significant difference $*p<0.05$ when compared with OVA. Where ns = non-significant.

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

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 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. 4.7(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. 4.7(C)).

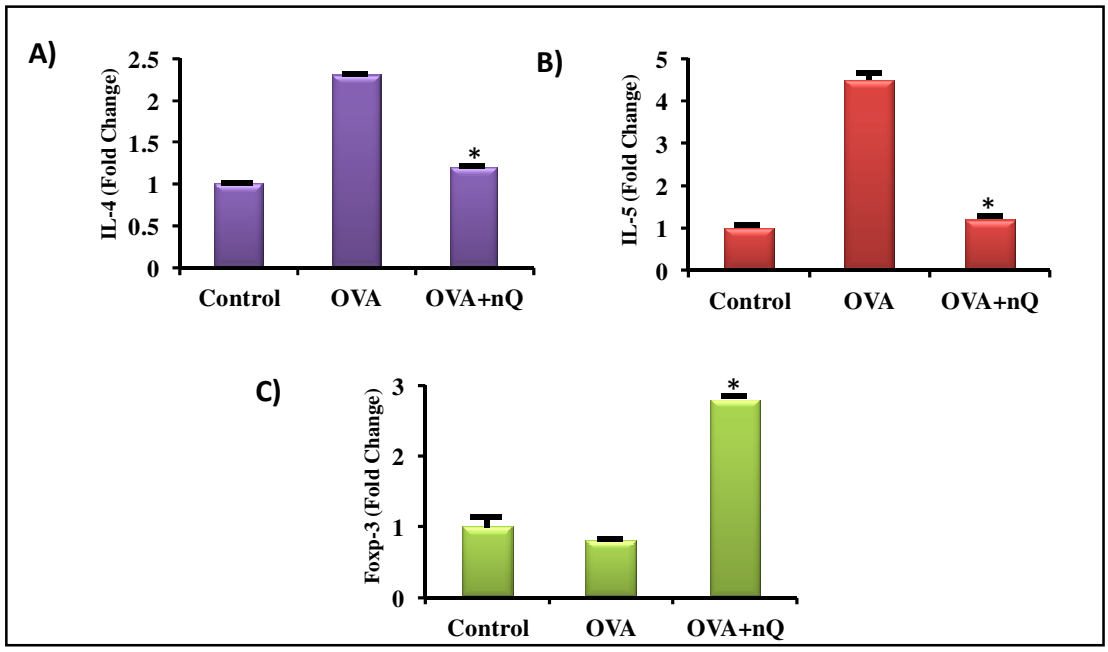

Figure 4.7: Real time PCR: (A), (B) RT PCR results are showing down regulated mRNA expression of Th2 cytokines (IL-4 and IL-5) and (C) Up-regulated expression of transcription factor Foxp3 in the lungs of OVA+nQ group as compared to OVA group. The data from at least three independent experiments are expressed as the mean±SEM. Significant difference ∗p <0.05 when compared with OVA. Where ns = non-significant.

4.3.7 Western Blot Analysis of transcription factors like c-MAF, GATA-3, NFAT and SOCS-3

Furthermore, the Th2 transcription factors c-MAF, GATA-3, NFAT and SOCS-3 were quantified at protein levels. Significantly reduced levels of c-MAF, GATA-3, NFAT and SOCS-3 were noticed in the OVA+nQ group in comparison to OVA group (Fig. 4.8 (A). Figs. 4.8(B)–(F) shows the densitometry analysis of transcription factors observed in western blot studies.

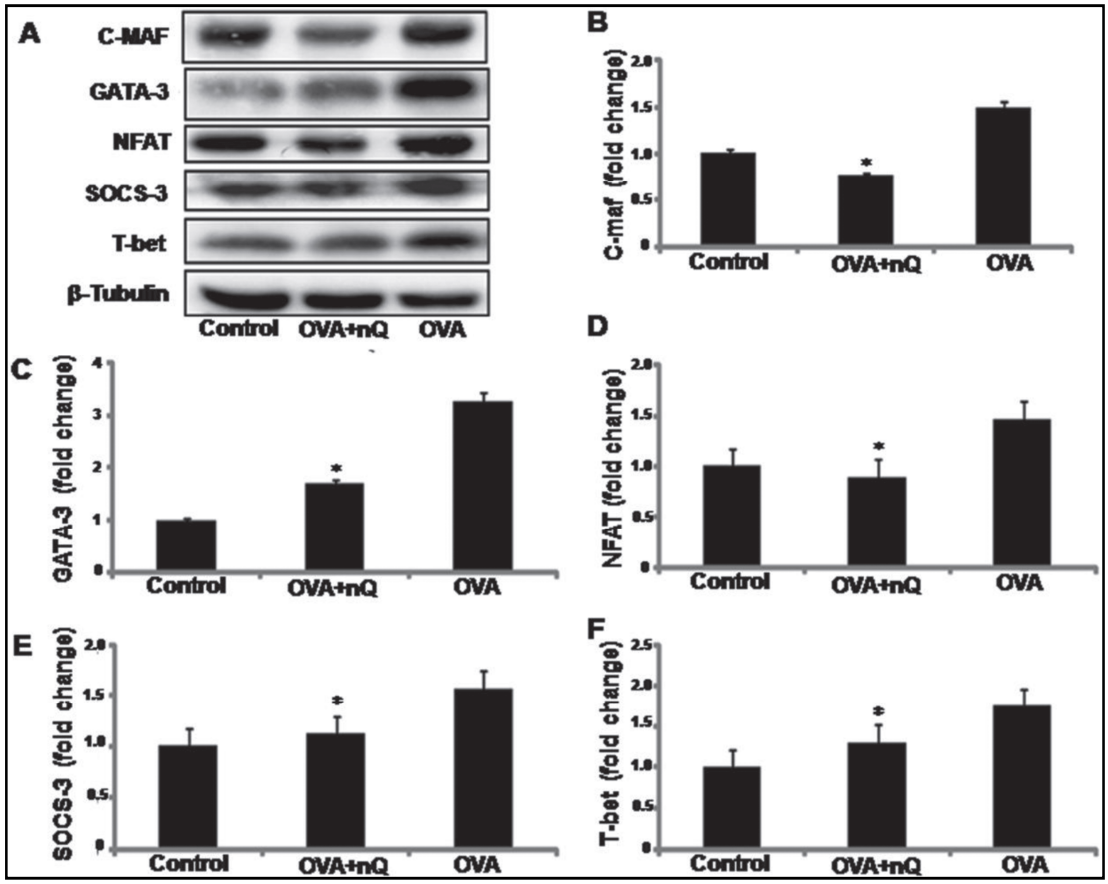

Figure 4.8: Western blots for transcription factors in mast cells: (A) Immunoblot results showed mixed expression of Th2 transcription factor (c-MAF, GATA-3, NFAT, SOCS-3 and T-bet), at the protein level in different groups. (B)–(F) Relative density (in fold terms) of each band measured by densitometry, showing in graph. The data from at least three independent experiments are expressed as the mean±SEM. Significant difference *p <0.05 when compared with OVA. Where ns = non-significant.

4.3.8 *β*-Hexosaminidase Release Assay

In the present study, OVA treatment (10–50 μg) 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. 4.9(A)). Further, mast cells isolated from lungs of OVA + nQ group showed suppressed the release of *β*-hexosaminidase, that suggests effectiveness of NQ even in the *ex vivo* conditions.

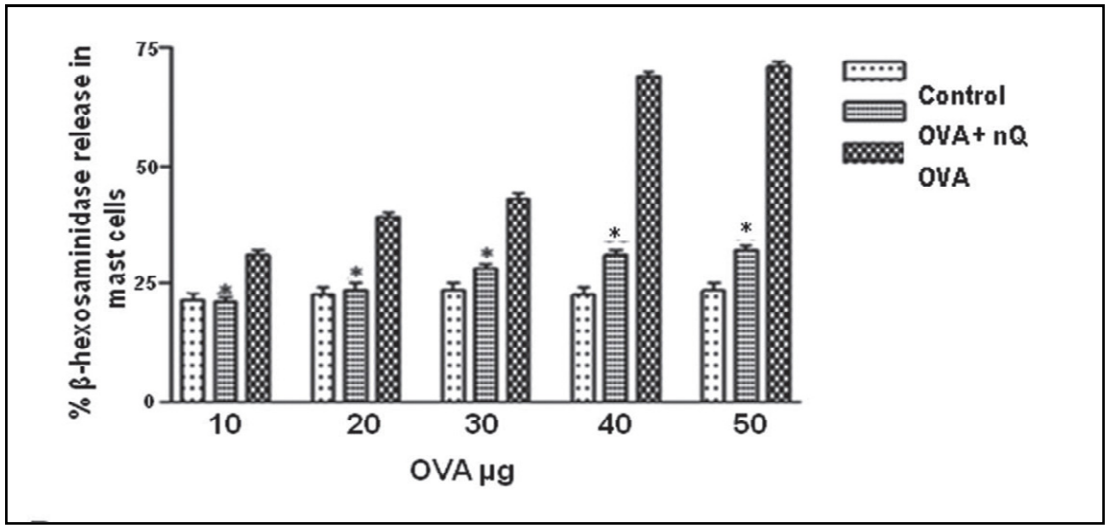

Figure 4.9: ß-hexosaminidase released by lung mast cell in OVA + nQ group as compared to OVA.

4.3.9 Signaling Events in the Mast Cells

Western blot analysis revealed significant increase in the levels of FcϵR1, Syk, PKC, c-Yes, PI-3, phospho-PI-3, PLC-γ2 and phospho-PLC-γ2 proteins in the OVA treated group, while the levels of all these parameters returned close to control in OVA+nQ treated group as can be compared to control (Fig. 4.10) though these levels significantly decresed in comparision to OVA only group in all the parameters. 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 expected to change, as it is only activated in its phosphorylated form on interacting with its substrate (Fig. 4.10). In the present study, enhanced level of Lyn was found in Ova+nQ treated group as compared to Ova treated group. The level of pPKC was up regulated in OVA+nQ treated group.

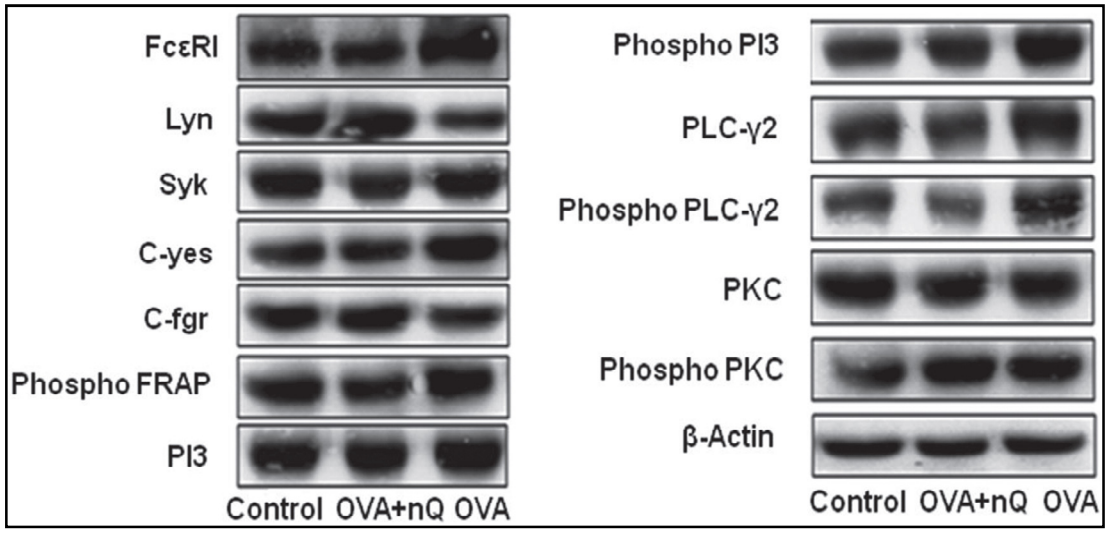

Figure 4.10: Western blots for signaling in mast cells: Immunoblotting of mast cell signaling molecules were carried out using their respective antibody as described in material methods. Immunoblot results showed mixed expression of signaling molecules (FcεR1, PKC, Ppkc, p-FRAP, c-Yes, c-fgr, Lyn, PI-3, p-PI-3, PLC-γ2, p-PLC-γ2 and Syk). The data from at least three independent experiments are expressed as the mean±SEM. Significant difference *p < 0.05 when compared with OVA. Where ns = non-significant.

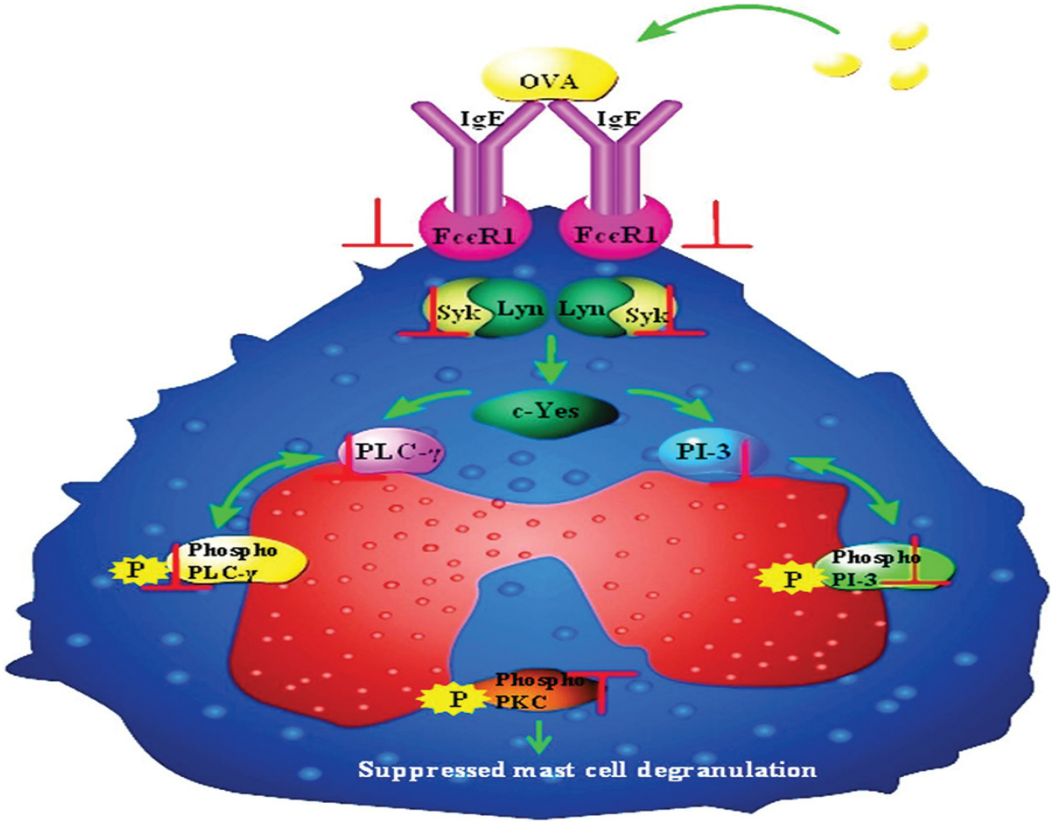

Figure 4.11: Schematic presentation of mast cell signaling.

4.4 Discussion

Enhanced level of OVA specific IgE in mucosal tissues induces accumulation of mast cells with infiltration of eosinophils, basophils and macrophages that 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 (Akdis et al., 1998). Therefore, the levels of IgE and IgG1 have to be evaluated for the pathogenesis of asthma. Animal models do provide a powerful tool in asthma research and to identify and evaluate novel therapeutic targets (Nials et al., 2008). OVA is widely used to induce an IgE response in mouse model of allergic asthma (Nials et al., 2008). 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 (Hamelmann et al., 1999).

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. Earlier studies have shown that the effective dose of bulk quercetin was between 8–16 mg/kg for suppression of allergic asthma (Park et al., 2009), whereas, comparatively very less dose of nQ (1 mg/kg) is required to suppress the asthmatic symptoms effectively. 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 (Carr et al., 1994; Kumar et al., 2012;

Bloemen et al., 2007; Caughey et al., 2007). The PGD2, CysL, mMCPT-1, and TSLP are preformed and newly synthesized allergic mediators found in the activated mast cells that play a pathogenic role in the development of allergic asthma (Carr et al., 1994; Kumar et al., 2012; Bloemen et al., 2007; Caughey et al., 2007; Knight et al., 2000). In this study, the mice of OVA group showed significantly increased levels of PGD2, CysL, MCPT-1, and TSLP (Fig. 4.4 (A)–(D)) while levels of these mediators returned close to control levels in the OVA+nQ group demonstrating efficacy of nQ treatment clearly. Accumulated mast cell followed by OVA exposure may lead to the release of above said mediators, which were efficiently moved 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 (Shishehbor et al., 2010).

Lungs histopathology of OVA treated mice showed perivascular and peribronchial inflammatory cell infiltrate with mild narrowing of the bronchiolar lumen (Fig.4.5(A)). Thickening of alveolar septa throughout the parenchyma was also observed in OVA treated mice compared to OVA + nQ groups. The higher concentration of mast cells including leukocyte infiltration indicated severe pathological changes (Hamelmann et al., 1999). In the present study, the mast cell numbers increased in OVA sensitized mice in comparison to control group (Fig. 4.6(B)) pointing towards enhanced inflammatory pathways. The numbers of mast cells were found to be significantly reduced in OVA+nQ group mice suggesting that nQ may have 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 (Hamelmann et al., 1999). 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, that produce cytokines, chemokines and lipid mediators resulting in a high impact aggravation of airway inflammation (Kay et al., 2004; Walsh et al., 2001). The OVA+nQ group showed significantly reduced eosinophil numbers when compared to OVA (Fig. 4.6(C)). Eosinophil numbers in OVA+nQ group were even 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 (Possa et al., 2013; Moqbel et al., 1994; Schmid et al., 2002; Molet et al., 2001; Garcia et al., 1996; Costa et al., 1993) . Several flavonoids including apigenin in OVA-induced asthma model have demonstrated the ability to inhibit OVA-induced increase in eosinophil counts (Li et al., 2013). 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 is characterized by the presence of large numbers of CD4+ T cells that produce Th2 cytokines, which orchestrate the inflammation associated with asthma (Akbari et al., 2001; King et al., 2005).

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 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 (Fig. 4.7(A)–(B)) demonstrating once again effectiveness of NQ treatment which brought enhanced mRNA levels closer to normal. An up-regulated expression of Foxp3 was observed in the OVA+nQ group while it was reduced in the OVA group over controls (Fig. 4.7(C)). The over expression of Foxp3, a 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 of mice showed the lowering of IL-5 levels (Rogerio et al., 2007). The results of nQ treatment to OVA+nQ group showed decreased levels of IL-4, IL-5 (as observed in RT-PCR) and GATA3, that are in agreement to previous studies for bulk quercetin where levels of histamine, MMP-9, GATA-3 were also found to be significantly decreased. These observations suggest that the activated Th1 response played a dominant role in an OVA+ nQ group of mice (Rogerio et al., 2007). Furthermore, the Th2 transcription factors c-MAF, GATA-3, NFAT and SOCS-3 were quantified at protein levels. Significantly reduced levels of c-MAF, GATA-3, NFAT and SOCS-3 were noticed in the OVA+nQ group in comparison to OVA group (Fig. 4.8(A)). Fig 4.8(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 (Rogerio et al., 2007). 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 upregulated 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, ß-hexosaminidase enzyme release following IgE activation is one of the significant marker of the mast cell degranulation (Alvarez et al., 2009). ß-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 μg) enhanced the release of ß-hexosaminidase in a dose dependent manner, whereas the release of ß-hexosaminidase was found to be much reduced in OVA+nQ treated group in comparison to OVA treated group (Fig 4.9(A)).

Further, mast cells isolated from lungs of OVA + nQ group showed suppressed the release of ß-hexosaminidase that suggests that even in the *ex vivo* condition nQ is equally effective. Western blot analysis revealed significant increase in the levels of FcϵR1, Syk, PKC, c-Yes, PI-3, phospho-PI-3, PLC-γ2 and phospho-PLC-γ2 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 4.9(B)). There is no 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 expected to change, as it is only activated in its phosphorylated form on interacting with its substrate (Fig 4.9(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 (Akdis et al., 1998). IgE moves to mast cells/basophils and bind to the FcϵR1 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) (Moon et al., 2008; Nishizumi et al.,

1997). 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 FcϵRI (Nishizumi et al., 1997; Kawakami et al., 2000; Hernandez et al., 2004; Xiao et al., 2005).

Several studies have demonstrated that Lyn has inhibitory effect on the mast cells degranulation as well as proliferation (Hernandez et al., 2004; Xiao et al., 2005; Odom et al., 2004; Hernandez et al., 2004). Further, Lyn Kinase also inhibits the basophil proliferation, GATA-3 expression and induction of Th2 cell differentiation (Charles et al., 2009). 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 includes 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 that result in mast cell degranulation and allergic reaction in the OVA induced murine model (Parravicini et al., 2002; Nishizuka et al., 1992; Ozawa et al., 1993). PKC, is a key enzyme of the T-cell receptor mediated signaling pathway, that regulates T-cell differentiation and T-cell mediated immune response (Ohayon et al., 2010). Studies have revealed that PKC is involved in both Th2 as well as Th1 type immune responses (Salek et al., 2004). PKC is only activated in its phosphorylated form, thus, we have analyzed only the activated form of PKC was analyzed 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 (Gong et al., 2005; Dehne et al., 2009).

In present study, there is occurs a shift of OVA induced Th2 response towards Th1 response after treatment of nQ, therefore, the microenvironment of the cells may have changed towards Th1 response. Higher expression of pPKC in OVA+nQ group may also be responsible for Th1 gene expression that can be observed in the case of T-bet at mRNA level in nQ treated group. Fig 4.10, schematically shows the probable mechanism related to the effect of nQ on mast cell degranulation like Fc□R1, 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 kinds of nanomedicines have been taken up to cure asthma, including various antioxidants but, nanotized quercetin poses several advantages over other nanoparticles that supports normal respiratory health, cardiovascular health as shown in Fig 4.10. Schematic presentation of mast cell signaling, promotes balanced blood pressure, reduce stress, improve upper respiratory conditions, provide nutritional support for overall health (Townsend et al., 2013; Weng et al., 2012; Perez et al., 2010; Egert et al., 2009; Edwards et al., 2007; Cheng et al., 2012; Long et al., 2013).

Taken together, nQ has the potential to suppress asthmatic conditions along with several other health ailments that makes it an effective therapeutic option.

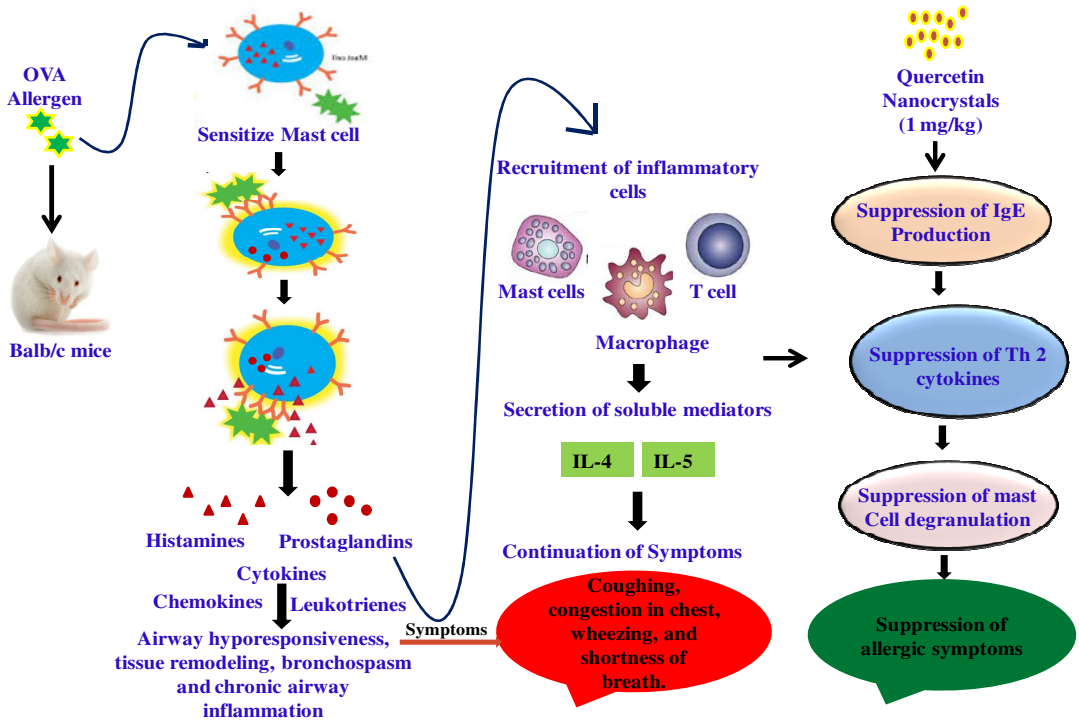

Figure 4.12 Mechanism of anti-allergic effect of Quercetin nanocrystals in OVA induced asthma model

Chapter 5

Therapeutic potential of Nano Q in OVA induced BALB/c mice of food allergy

CHAPTER 5

THERAPEUTIC POTENTIAL OF NANO Q IN OVA INDUCED BALB/C MICE OF FOOD ALLERGY

5.1 Introduction

Food allergies as well as their associated clinical manifestations have long been known to be serious health concern with an alarming prevalence. The most awful form of food allergy, known as food induced anaphylaxis (FIA) has been recognized as a severe life threatening allergic reaction that is rapid in onset and may cause death. Intestinal mast cells, basophils, eosinophils, macrophages and neutrophils play a pathogenic role in the development of FIA (Finkelman et al., 2007). The clinical features of FIA include generalized hives, itchiness, flushing, angioedema, runny nose, swelling of the conjunctiva, abdominal pain, diarrhea, vomiting, myocardial infarction, dysrhythmia, or cardiac arrest and hypothermia immediately after high dose of food allergen exposure followed by systemic allergic reactions (Simons et al., 2009; Simons et al., 2010). Almost, all the body's organ systems including skin (80–90%), respiratory (70%), gastrointestinal (30–45%), heart, vasculature (10–45%), and central nervous system (10–15%) are targeted by FIA hence it is also termed as systemic anaphylaxis (Sajjad et al., 2014). The systemic anaphylactic reactions are governed by two pathways named as classical and alternate pathway. The classical pathway involves the IgE antibody which is a ligand for high affinity receptor FcεR1 expressed on mast cells, basophils, and

eosinophils (Charles et al., 2001; Tsujimura et al., 2008) whereas alternate pathway mediated by IgG1, FcγRIII, FcγRIV, basophils, macrophages, Neutrophil, platelet-activating factor (PAF) (Finkelman et al., 2007; Tsujimura et al., 2008; Jonsson et al., 2011). In the systemic anaphylaxis, small dose of allergen results in a classical pathway while large dose activates alternative pathways (Strait et al., 2002). The cure of food allergic manifestation is currently lacking and the only suggestion given by the clinicians is to avoid allergenic food to get relief from its pathophysiology. Therefore, efforts are going on to produce efficient therapy for the permanent cure of food allergy and its associated secondary complications like asthma, rhinitis and dermatitis without any side effects.

Quercetin (3,31,41,5,7-pentahydroxyflavone), is one of the most abundant polyphenols which belongs to flavonoid family. It is found in variety of fruits and vegetables (Mlcek et al., 2016). The richest source of quercetin are onion and apples in human diet, however it is also present in considerable amounts in variety of herbs (tea, broccoli) fruits (berry crops, grapes) and wine (Tsanova et al., 2013; Jeszka et al., 2015; Martelo et al., 2014; Yoo et al., 2013). Dietary intake of quercetin in the United States is around likely 25 mg per individual. Flavonols especially quercetin, exhibit a wide spectrum of biological activities related to its anti-allergic, anti-inflammatory, anticarcinogenic, antibacterial, wound healing, antispasmodic, anticoagulant, antitumor and antioxidant properties (Chirumbolo et al., 2011; Sato et al., 2015; Hertog et al., 1992; Rice et al., 1995; Bahorun et al., 2006; Jantan et al., 2015; Marzocchella et al., 2011). Quercetin has been demonstrated to have potent inhibitor of lipid peroxidation and free radical scavenger, having superoxide anions and hydroxyl radicals scavenging activities (Cook et al., 1996). In recent years,

researchers have focused on the anti-inflammatory action of quercetin with reference to inhibition of inflammatory mediators as well as Th-2 cytokines production. In addition, to this quercetin also inhibits nuclear factor activation (NF-kappaB), mast cell activation and suppression of eosinophilic inflammation. In a study carried out by Wei et al (2012) the effects of quercetin together with kaempferol and isoflavones has been found to regulate mucosal immunity during hypersensitivity reaction. Shiseboar et al (2010) found reduction in plasma histamine level and anaphylactic reaction in peanut induced IgE mediated food allergy in a wistar model after quercetin treatment for weeks. The rats treated with quercetin at a dose of 50 mg/kg body weight upto four weeks were observed with suppression in plasma histamine levels, vascular permeability, systemic anaphylaxis scores, and total serum Immunoglobulin E levels. Inspite of several beneficial pharmacological effects, role of quercetin in food allergy remains almost unexplored primarily because of low bioavailability.

Clinically food allergic manifestation may lead to bronchial asthma, allergic rhinitis and atopic dermatitis (Kumar et al 2013, Verma et al 2013 and Misra et al 2010), However, no work has been done undertaken on food allergy except avoidance. In India, several groups have started to work on quercetin such as its protective role against idiopathic lung fibrosis (Verma et al., 2013) and quercetin loaded nano emulsifying drug delivery system against cancer (Jain et al., 2014). To the best of our knowledge, no work has been carried out on treatment of food allergies using either bulk or quercetin nanocrystals. Therefore, the objectives of the present study were to investigate the anti-allergic potential of bulk and nano quercetin in OVA induced BALB/c mice of food allergy.

5.2 Materials and Methods

5.2.1 Equipment, reagents, and preparation of quercetin nanocrystals

Quercetin, bovine serum albumin (BSA) and reagents for sodium dodecyl sulfate– polyacrylamide gel electrophoresis (SDS–PAGE) were purchased from Sigma Chemical Company, St. Louis, MO, USA. 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, USA. All the other chemicals used were of the highest purity available from other commercial sources. The nano particulate quercetin was prepared and characterized as described in our previous chapter (Gupta et al., 2016).

5.2.2 Animals maintenance

Healthy BALB/c mice (female, 6-8 weeks old, and 22 [±3] g) were procured from the CSIR-IITR animal facility (Lucknow, India). All mice were housed in a pathogen-free environment maintained at 22±2ºC with a relative humidity of 50-60% and 130-325 lux diffused light (12 hours light: dark cycle). Mice were provided ad libitum access to standard rodent chow and filtered water. Mice (n=40) were divided into the 4 groups, namely Control (PBS), OVA (Positive Control), OVA +Bulk Quercetin (BQ) and OVA +Nano Quercetin (NQ). All protocols herein are in accordance with guidelines of the CPCSEA (Ministry of Environment and Forests, Government of India) and the Institutional Animal Ethics Committee ((Reference no.: IITR/IAEC/06/2016-48/17). For the in vivo study, all mice in each group underwent bleedings to permit measures of serum IgE and IgG1 levels. Blood was collected from retro orbital plexus of mice at different decided time interval. Tissue

samples were taken after the last challenge to study the remaining allergic parameters.

5.2.3 Group designation and Animal Treatment Protocol

OVA with alum sensitized mice were injected with 15 mg/kg/day of bulk quercetin intraperitoneally in 100 µl of PBS to each mice daily from day 28-38 and designated as a OVA+BQ group. OVA with alum sensitized mice were injected 2.5 mg/kg/day of nano quercetin intraperitoneally in 100 µl of PBS to each mice daily from day 28-38 and designated as a OVA+NQ group. The group sensitized with OVA and 1 mg alum ($Al(OH)_3$) on day 0 and 15 but not treated with BQ and NQ was designated as OVA group. After 1 hour of treatment with bulk and nano quercetin in OVA + BQ and OVA + NQ group, and positive control treated with OVA only, all the three groups were challenged orally with 50 mg OVA. The group treated with saline only was designated as Control group (Fig. 5.1).

5.2.3.1 OVA sensitization and challenge protocol

To induce food allergy, BALB/c mice were immunized with 50 µg ovalbumin (OVA) intraperitoneally (i.p.) in 1 mg alum on days 0 and 14 as previously described (Kinney et al. 2015) and depicted in graphical presentation of animal protocol (Fig. 5.1). Two weeks later, mice were challenged orally with 50 mg OVA in 250 µl phosphate buffered saline (PBS) once a day on 6 alternating days. One hour after the 6th challenge, the development of intestinal anaphylaxis was ascertained according to the method described earlier with slight modifications (Li et al., 1999) and mice were sacrificed and tissue samples (intestine) were taken to study the other allergic parameters.

5.2.3.2 Quercetin treatment

To ensure the therapeutic efficacy of BQ as well as NQ, OVA-primed and challenged mice was intraperitoneally treated with 10, 15 and 20 mg/kg of bulk quercetin (BQ) and 1, 2.5, 5 and10 mg/kg of nanotized quercetin (NQ) in PBS from day 28 to day 38 of the treatment, at various times as shown in Fig. 1. The assay of specific IgE and IgG1 were carried out for selection of optimal dose of NQ (2.5 mg/kg) and BQ (15 mg/kg). A graphical presentation of the animal treatment protocol is shown in Fig. 5.1.

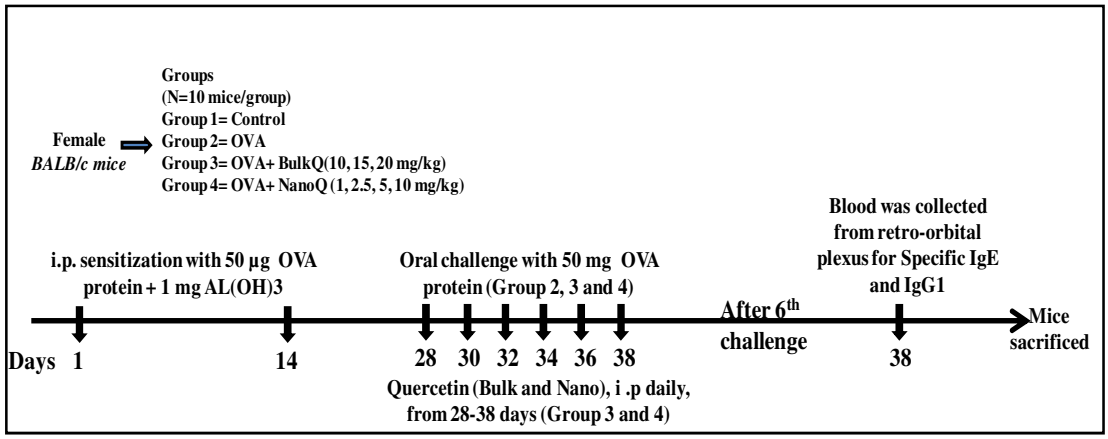

Figure 5.1: Graphical presentation of animal treatment protocol

5.2.4 Anaphylactic symptoms and rectal temperature

After last challenge anaphylactic symptoms were scored in mice (n= 5/group) according to the method described earlier (Li et al., 1999). In brief, mice were scored according to appearance of clinical symptoms after challenge i.e. without symptoms (Score 0); scratching and rubbing around the snout and head (Score 1); puffiness around the eyes and snout, pilar erection, diarrhea, and reduced activity or standing still with an increased respiratory rate (Score 2); wheezing, labored respiration, and

cyanosis around the mouth (Score 3); symptoms as in no-3 with loss of consciousness, tremors, and/or convulsion (Score 4); and death (Score 5). Rectal temperature was monitored 20 min after challenge using a digital rectal thermometer (Bioseb, France).

5.2.5 Total Serum IgE Assay

Total IgE (tIgE) was estimated with the Optia mouse IgE kit (BD Biosciences, San Diego, CA, USA) according to the manufacturer's instructions. The absorbance was read at 450 nm using an ELISA plate reader (Biotek, Power Wave XS2). Total IgE levels were measured in triplicate and average value was calculated.

5.2.6 OVA Specific IgE and IgG1 Assay

Specific IgE (sIgE) and sIgG1 levels against OVA were estimated by indirect enzyme-linked immunosorbent assay (ELISA) using earlier described methods (Polte et al., 2006). Briefly, the wells of microtiter plate (Nunc, Roskilde, Denmark) were coated with 1 μg of OVA in coating buffer (0.05 M Carbonate-Bicarbonate, pH 9.6) and left overnight at 4°C. After removing the OVA solution the nonspecific binding sites were blocked using 3% BSA in PBST. After blocking with 3% bovine serum albumin (BSA), the wells were incubated with diluted sera (1:50 v/v for specific IgE and 1:500 for specific IgG1 respectively) of Control, OVA, OVA+BQ and OVA+NQ 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 TMB substrate and the reaction was stopped by adding 5 N H_2SO_4 after 20 min. The absorbance was read at 450 nm using an ELISA plate

reader (Biotek, Power Wave XS2). Specific IgE and specific IgG1 levels were measured in triplicate and average absorbance value has been given as results.

5.2.7 Measurement of Allergic Mediators in the Serum

Allergic mediators involved in food allergy like mouse mast cell protease-1 (mMCP-1), mouse thymic stromal lymphopoietin (TSLP) and histamine levels were determined in the sera of treated mice using commercially available EIA and ELISA kits (Histamine EIA kit, Cat No. 512031; mMCP-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.

5.2.8 Th1/Th2 Cytokine analysis

Quantification of serum cytokines (Th1/Th2) was carried out using Cytometric Bead Array (CBA) Kit (BD Biosciences, San Diego, CA). Samples were prepared for cytokine analysis as directed by the kit manufacturer and analyzed on the same day. CBA-FCAP array software was used to calculate the concentration of cytokines present in the samples on the basis of standard curve obtained for each cytokine.

5.2.9 Semi quantitative polymerase chain reaction (PCR) for FcεRI, GATA-3, SOCS-3, NFAT and cMAF

A semi-quantitative PCR analysis of various transcription factors like FcεRI, GATA-3, SOCS-3, NFAT and cMAF in the intestine was carried out using gene-specific primers in Control, OVA, OVA+BQ and OVA+NQ groups. The primer

sequences taken were as follows: FcεRI: sense 5′-TCGGCATTTTGAACGAGGTC-3′, antisense 5′-AAAAGCCC GAAAGAGTCTC-3′; GATA- 3: sense 5′-TCTCACTCTCGAGGCAGCATGA-3′, antisense, 5′-GGTACCAT CTCGCCGCCACAG-3′; SOCS-3: sense, 5′-TTTCCCCTTCTGCCTTCTCT-3′; antisense, 5′-TGGTGCTGGGGGTAGTAGAC-3′; NFAT sense 5′-GCTCCCGGCCTGGTCTGCTC-3′, antisense 5′-AGGTGGCGGGGTGGTTTCTGA-3′; and GAPDH: sense 5-TTCACCACCATG GAGAAGGC-3, antisense 5-GGCATGGACTGTGGTCATGA-3; c-MAF: sense 5′-TGCCTGCTCTTACTGACTGG-3′, antisense 5′-CTGGGAAGTGGGTGCAGTTA-3′. Total RNA from the intestine was isolated and cDNA was prepared using high capacity cDNA reverse transcriptase kit (Applied Biosystem, Foster city, CA). After completion of PCR cycles, 8 μL of PCR products was analyzed on 2% agarose gel electrophoresis. The density of each band was estimated by the Genetool software (Syngene Bioimaging System, Cambridge, UK). The GAPDH was taken as an endogenous control and its respective densitometry values were used for normalizing different mRNA cytokines in the intestine, respectively. Normalized values were used for plotting the bar graphs.

5.2.10 Histopathological studies and Alcian blue staining for mucin in intestine

The intestine was taken for histological analysis from Control, OVA, OVA+BQ and OVA+NQ treated groups. Tissue sections were stained with hematoxylin and eosin for microscopic examination (125× and 500× magnifications). To investigate the presence of sulphated mucin in gastrointestinal epithelium, the mice were challenged and sacrificed by cervical dislocation and intestine were collected from control, OVA+ BQ, OVA+ NQ and OVA groups. Tissues were fixed

in 10% formalin in saline for 24 h at RT, cut into 3–5-μm thick sections. To perform Alcian blue staining method the sections were kept in xylene for 20 minutes and then hydrated in serially diluted ethanol (100%, 90%, 80%, 70%) each for 5 minutes and then rinsed in PBS. Thereafter slides were placed in alcian blue solution for 30 minutes. To remove extra stain sections were dipped in 3% acetic acid and rinsed with PBS. Stained sections were counterstained with saffranine for 1 min and again dehydrated with xylene for 5 minutes. The stained sections were mounted with DPX and the slides were observed in microscope.

5.2.11 Immunoflourescence Histochemistry for IL-4 and IL-5

Furthermore, histological expressions of IL-4 and IL-5 levels were analyzed in the intestine tissues by immunoflourescence histochemistry. The Goat anti mouse IL-4 and Rabbit anti mouse IL-5 (Santa Cruz biotechnology, Inc., USA) and Alexa flour 596 and FITC conjugated anti-goat/anti-rabbit antibody were used as primary and secondary antibody, respectively. The images were captured by confocal microscope at 200X maginfication (Leica Micosystem, Germany).

5.2.12 Western Blot analysis of Th2 cytokines (IL-4 and IL-5) and mast cell signaling pathway (FcER1, p-Lyn, p-syk, p-PLC, p-PKC)

Western blotting for Th2 cytokines as well as associated transcription factors (GATA-3, T-bet) and key molecules of mast cell signalling pathway was carried out in the intestinal protein isolates. In order to perform, proteins isolation from intestine tissue was carried out using RIPA buffer [100 mg intestinal tissue in 300 µl chilled RIPA buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 20 mM NaF, 100 mM Na3VO4, 0.5% NP-40, 1% Triton X-100, 1 mM PMSF, 10 mg aprotinin/ml, 10 mg leupeptin/ml (pH 7.4)] with the help of a tissue

homogenizer. Thereafter, 300 µl RIPA buffer was added once more and the sample placed at -80 ^{0}C for 20 min. After 20 min, thawing was done followed by centrifugation at 16 000g for 30 min at 4 ^{0}C. The supernatant was then collected and protein was estimated using a bicinchoninic acid assay kit (Thermo Scientific, Pittsburgh, PA; sensitivity 5 µg/ml). Tissue proteins (50 µg/lane) were loaded and then resolved over a 12% SDS–PAGE gel. The protein samples were then electrotransferred onto PVDF membranes (Immobilon-P, Millipore, Darmstadt, Germany) using a semi-dry blotting unit (Amersham Biosciences). For each sample, three gels/blots were generated. Nonspecific binding sites on each blot were blocked with 3% BSA (in PBS-T) and incubation for 2 h at 37 ^{0}C. Each blot was then washed in PBS-T three times before being coated with various antibodies against IL-4, IL-5, GATA-3, T-bet, Fox-P3, FcER1, p-Lyn, p-syk, p-PLC, p-PKC (Santa Cruz Biotechnology; each at 1:1000 dilution) in PBS-T and placed at 37 ^{0}C for 2 h. Thereafter, each blot was washed with PBS-T five times and incubated for 2 h at 37 ^{0}C in a solution of HRP conjugated either anti goat IgG or anti mouse or rabbit (Sigma, 1:2000 dilutions). Subsequently, after five washes with PBS-T, presence of antibodies on each blot was evaluated using an enhanced chemiluminescence system (Pierce, Waltham, MA). Images of the bands were captured using a Syngene gel documentation system equipped in associated with a CCD camera (Syngene). Densitometric analyses were then done using Gene tools software. The ß-actin (Santa Cruz Biotechnology, Inc. Santa Cruz, CA) was used as internal control.

5.2.13 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 and 0.01 was considered to be significant.

5.3 Results

5.3.1 NQ treatment caused a marked suppression in the development of OVA induced anaphylactic symptoms

In the OVA group, 40% mice were scored for most severe anaphylactic score 5 and other 40% mice were noticed with score 4 whereas, 20% mice were observed with anaphylactic score 3. However, mice from OVA+BQ groups noticed with reduced anaphylactic scores as compared to OVA as anaphylactic scores 2, 3 and 4 were recorded in the 40, 40 and 20 % mice in this group. In case of OVA+ NQ treated group, 60% mice were showed score 2 and remaining 40% mice were found to exhibit anaphylactic score 1 as showed in Fig. 5.2 a. In addition, a significant drop (4-5ºC) in body temperature was monitored in the mice of OVA and OVA+BQ groups as compared to control mice as shown in Fig. 5.2 b whereas, such hypothermia was not observed in the mice of OVA+NQ group similar to control group.

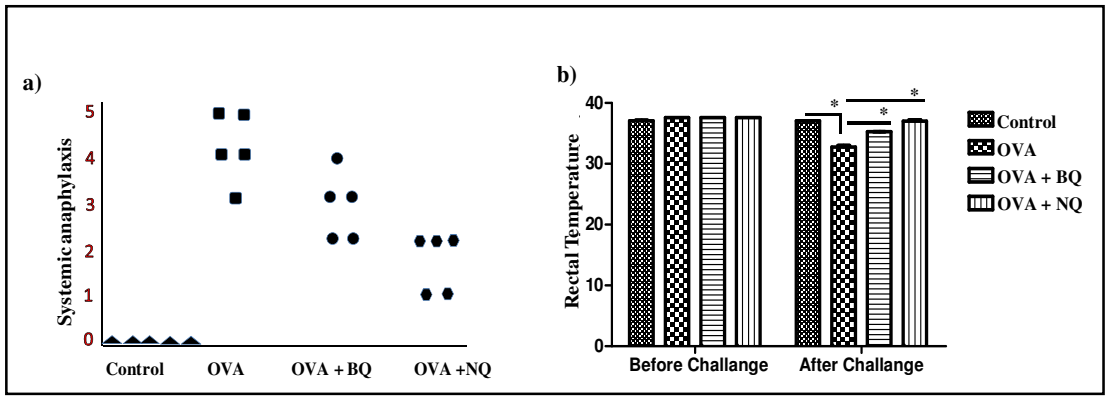

Figure5.2: (a) Anaphylaxis score and (b) Rectal temperature in control, OVA, OVA+BQ, OVA+NQ groups. The data give here is representative of at least three independent experiments. The values are expressed as the mean ± SEM. Significant difference $*p<0.05$ when compared with OVA.

5.3.2 Intraperitoneal administration of NQ attenuates total and OVA specific IgE along with IgG1 production in Balb/c mice

The inhibitory effects of NQ treatment on OVA specific IgE and total IgE production in the serum of Balb/c mice was studied using ELISA technique. The serum levels of total as well as specific IgE were found to be significantly elevated in OVA group as compared to control group whereas mice of OVA+BQ group showed reduced serum levels of both the IgEs in comparison to OVA group as shown in Figs. 5.3 (a and c). However, the same figures demonstrated a more significantly ($p<0.05$) lower IgE production in the sera of OVA+NQ group as compared to OVA as well as OVA+BQ treated groups. Similar to IgE levels, Fig. 3b shows elevated levels of IgG1 in the serum of OVA treated mice as compared to control, while this antibody's production was diminished in the sera of OVA+BQ treated mice when compared to the OVA group. In case of NQ treatment, significantly inhibited production of serum IgG1 was noticed in OVA+NQ group as compared to the OVA and OVA+BQ treated groups. The level of tIgE, sIgE and sIgG1 were found lowest in the OVA+NQ and OVA+BQ group after treatment with 2.5 mg/kg of NQ and 15 mg/kg dose of BQ. Therefore, further studies on various allergic parameters were therefore carried out using 2.5 mg/kg of NQ and 15 mg/kg dose of BQ as an optimal dose.

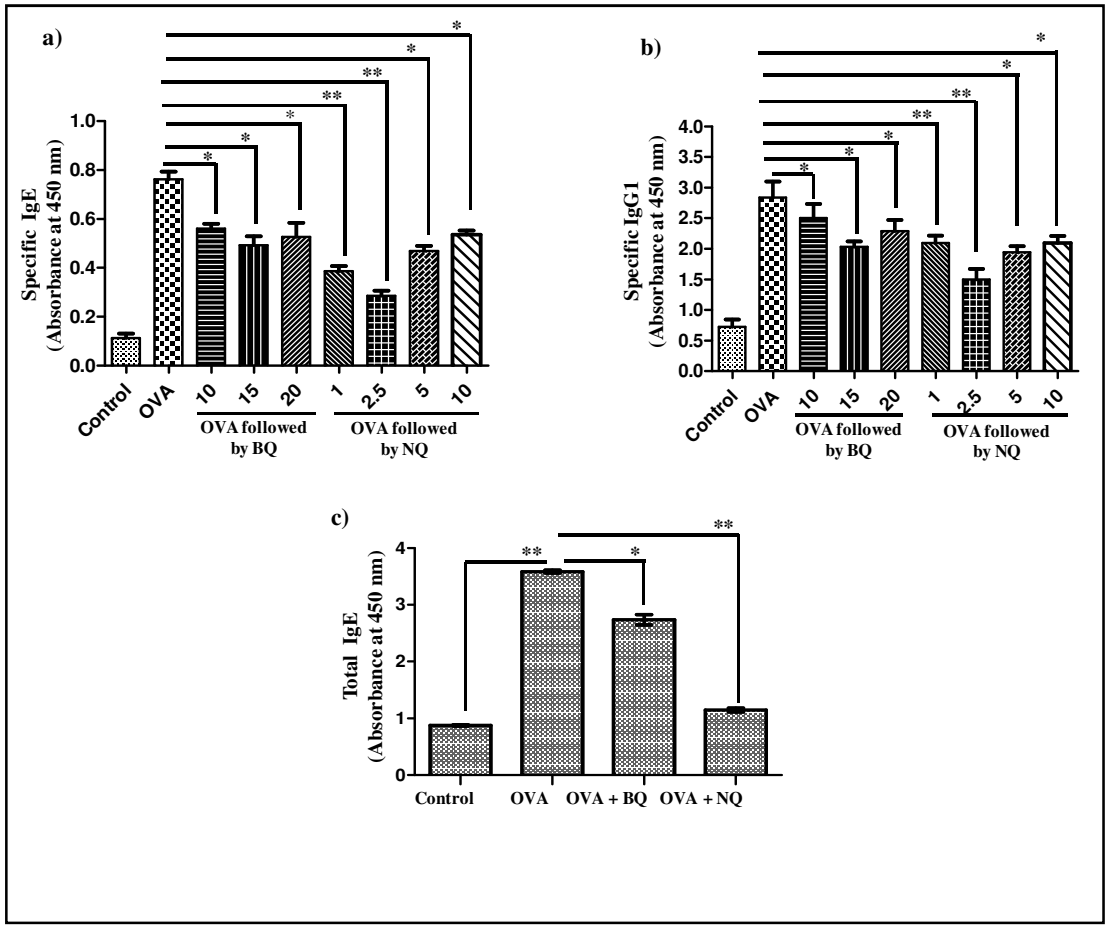

Figure 5.3: Immunoglobulin levels: (a) Level of specific IgE (b) specific IgG1 and (c) total IgE in the serum of control, OVA, OVA+BQ and OVA+NQ; Following graphs are showing decreased levels of total IgE, specific IgE and specific IgG1 in the serum of OVA+NQ treated groups when compared with OVA and OVA+BQ group; Levels of OVA-specific antibodies were expressed as absorbance at 450 nm. Data are expressed as the mean SEM (n = 10 in each group). Results are representative of three independent experiments with similar results. *P < 0.05, **P < 0.01.

5.3.3 Allergic mediators in the sera of NQ treated Balb/c mice

In this study, as expected the mice of OVA treated group showed significantly increased levels of mMCP-1, TSLP and histamine (Figs. 5.4 (a–c), while level 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 allergic mediators, which were significantly reduced after very low dose of NQ treatment along with other associated mediators.

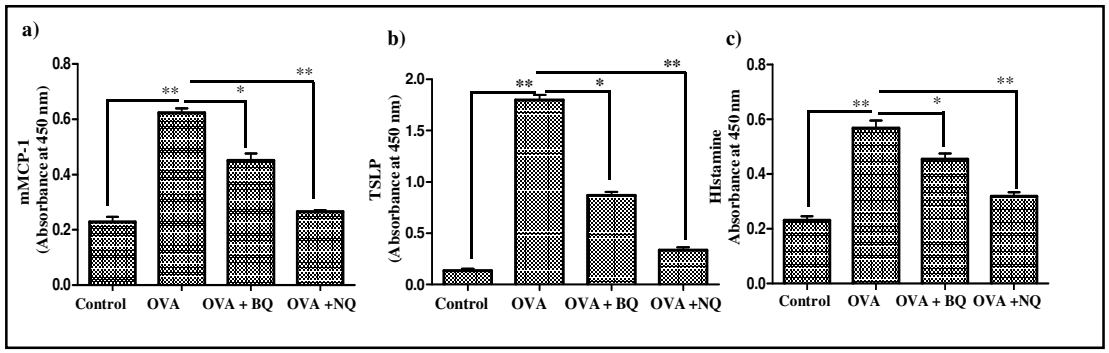

Figure 5.4: Estimation of allergic mediator such as a) mouse mast cell protease-1(mMCP-1), b) TSLP (Thymic stromal lymphopoietin) and c) Histamine in control, OVA, OVA+BQ and OVA+NQ groups. Data are representative of 2 independent experiments. Significant difference $*p<0.05$ and $**P<0.01$when compared with OVA.

5.3.4 Cytokine expression in the sera of NQ treated mice

Cytokines play an important role in immuno-regulation and inflammation. OVA exposure to mice increased the production of pro-inflammatory cytokines IL-4, TNF α, IFN γ and anti-inflammatory cytokine like IL-10. These cytokines have been known to play a pivotal role in the amplification of inflammatory responses and progression of inflammatory disorders like food allergy. Interestingly, the levels of anti-inflammatory cytokines IL-10 and Th-1 cytokine IFN-γ were also elevated in OVA+NQ treated animals, that could be a counter response of the body for damage control. While the level of IL-4, IL-2, IL-6 and TNF-α cytokines returned close to normal in OVA+NQ group (Fig. 5.5 (a-f)).

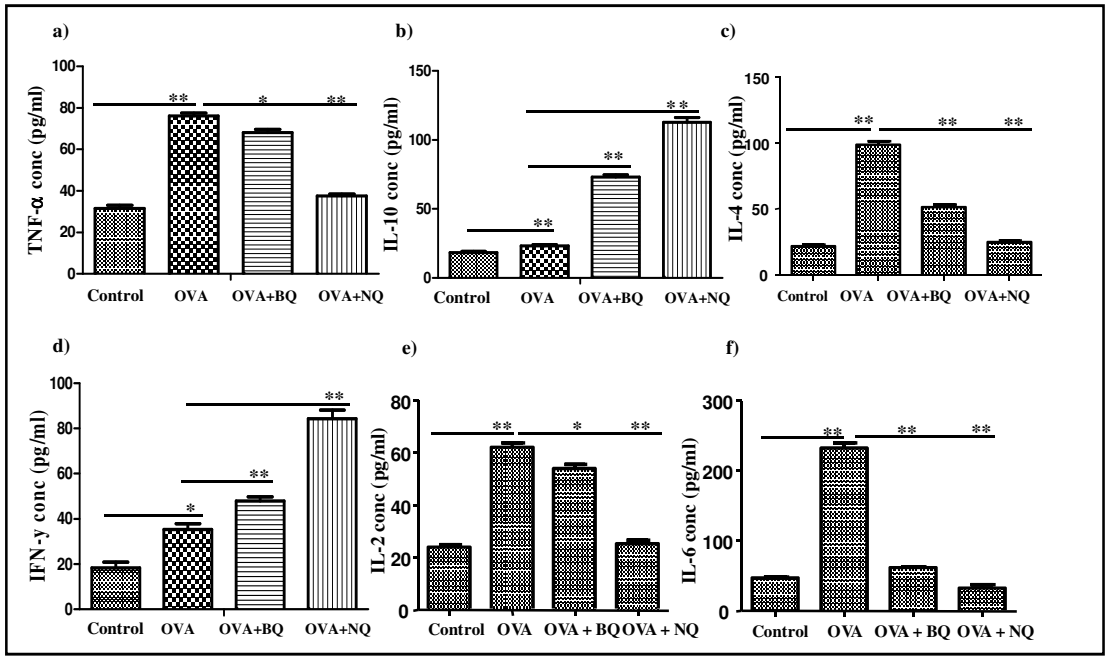

Figure 5.5: The levels of cytokines in serum of treated groups. Effect of OVA on a) TNF- α b) IL-10 c) IL-4 d) IFN- γ e) IL-2 and f) IL-6 levels. The estimation of Th1/Th2 cytokines was carried out using Cytometric Bead Array (CBA) Kit. CBA-FCAP array software evaluated the level of cytokines present in the samples on the basis of standard curve obtained for each cytokine. Values represent means ±SD of 5 animals. *p<0.05 and **P<0.01, indicates significant difference from corresponding OVA.

5.3.5 Semi quantitative PCR showing downregulated expression of FcεRI and Th2 transcription factor in NQ treated group.

In the present study, the mRNA expressions of FcεRI and various transcription factors, GATA-3, SOCS-3, NFAT and cMAF were analyzed by Semi quantitative PCR in all the four groups. The mRNA levels of FcεRI, GATA-3, SOCS-3, NFAT and cMAF were found upregulated in the intestine of OVA and OVA+BQ group that returned towards normal value post NQ treatment in OVA+NQ group when compared to OVA and OVA+BQ group (Fig. 5.6 a-f).

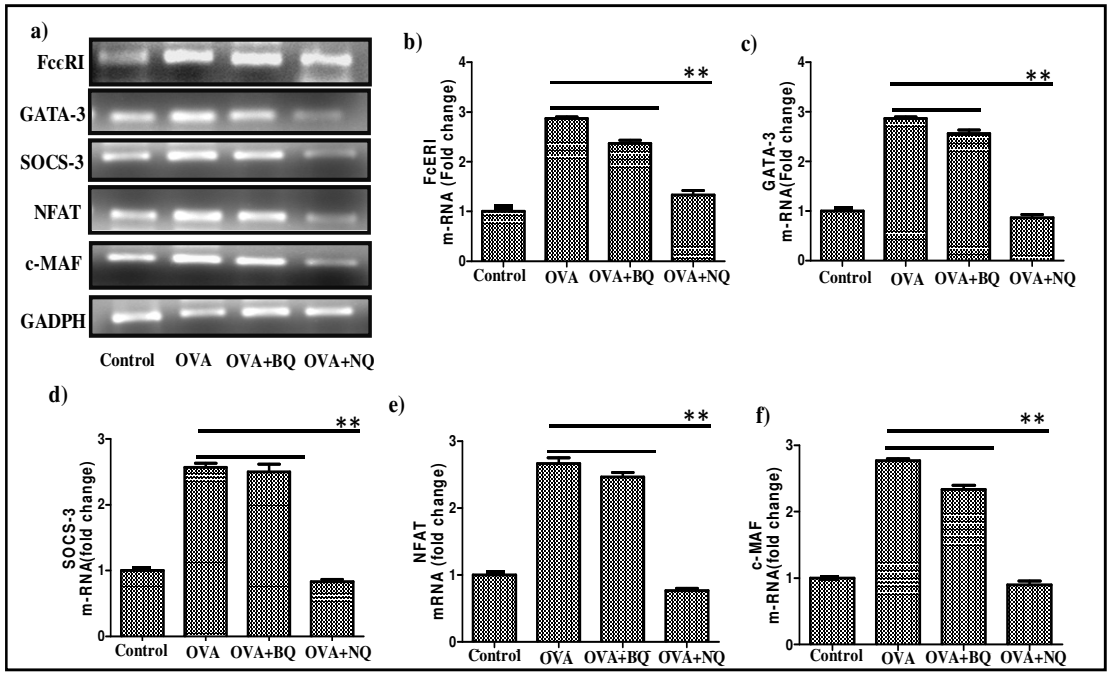

Figure 5.6. mRNA expressions of FcεRI and transcription factors like GATA-3, SOCS-3, NFAT and c-MAF by Semi quantitative PCR in all the four groups. Densitometric analysis was performed and values were normalized with respect to its GAPDH and plotted in terms of fold difference. Values represent means ± SD of 6 animals. *p<0.05 and **P<0.01, indicates significant difference from corresponding OVA.

5.3.6 Therapeutic effect of NQ on OVA induced pathological changes in the intestine of Balb/c mice

Histopathological analysis was carried out in the intestines of Control, OVA, OVA + BQ and OVA + NQ group mice. Histology, as shown in Figs. 5.7 revealed exfoliations of intestina villi, leukocyte infiltrations and increased activity of goblet cells in the intestine of OVA treated mice. Intestine of OVA and OVA + BQ treated group, as shown in Figs. 5.7(b,c) demonstrated degeneration of intestinal epithelium with infiltrations of inflammatory cells. However, no such pathological changes were noticed in the intestine of control and OVA+ NQ groups as seen in Figs. 5.7(a,d)

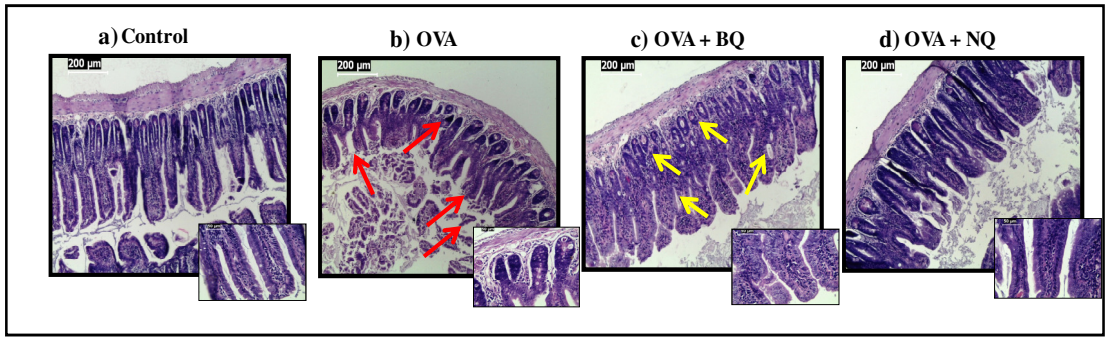

Figure 5.7: a) Histopathological responses induced by OVA administration in intestine. Intestine histology of sensitized/challenged mice: a) Control, b) OVA c) OVA + BQ and d) OVA + NQ. Heavy infiltrations of inflammatory cells (solid red arrows) and loss of mucosal structure (red solid arrows) in OVA treated mice is clearly visible. Increased activity of Goblet cell along with hyperplasia in sub mucosal layer is evident in OVA and OVA + BQ (solid yellow arrows) treated group. Decreased in activity of goblet cells, infiltration of inflammatory cells and normal healthy villi were observed in OVA + NQ treated group. The control group showed normal histology.

5.3.7 Impact of NQ treatment on mucus production in intestine of Balb/c mice

In this result, higher mucus production was observed in the intestine of OVA treated mice as compared to control as showed in Fig. 8b whereas mucus production was significantly reduced in OVA+NQ group followed by OVA+BQ treated group (Fig. 5.8(c-d) as compared to intestine of OVA treated mice.

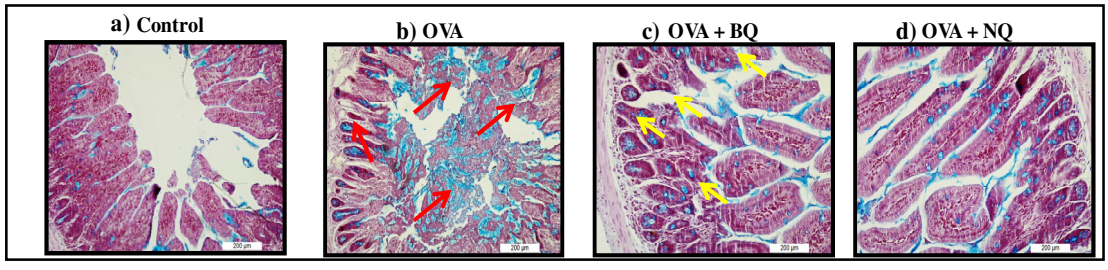

Figure 5.8 Alcian blue staning for mucus production in intestine of control, OVA, OVA+BQ and OVA+NQ groups. Fig. 5.8 b shows higher mucus production in the intestine of OVA treated mice as compared to control, whereas, mucus production was significantly reduced both in OVA+NQ and OVA+BQ treated groups as seen in fig. 5.8 c and d.

5.3.8 Effect of NQ treatment on histological expressions of IL-4 and IL-5 cytokines

Fig. 5.9 (b, f) demonstrated a marked increase in the protein expressions of IL-4 and IL-5 cytokines in the intestinal tissue of OVA treated mice as compared to control. The intestinal tissue of BQ treated mice observed with a reduction in the histological expressions of both the above cytokines as shown in Fig. 5.9 as compared to OVA group. Similar to BQ treatment result, NQ treatment caused further more reduction in the tissue expressions of IL-4 and IL-5 cytokines in the intestine as compared to OVA treated group.

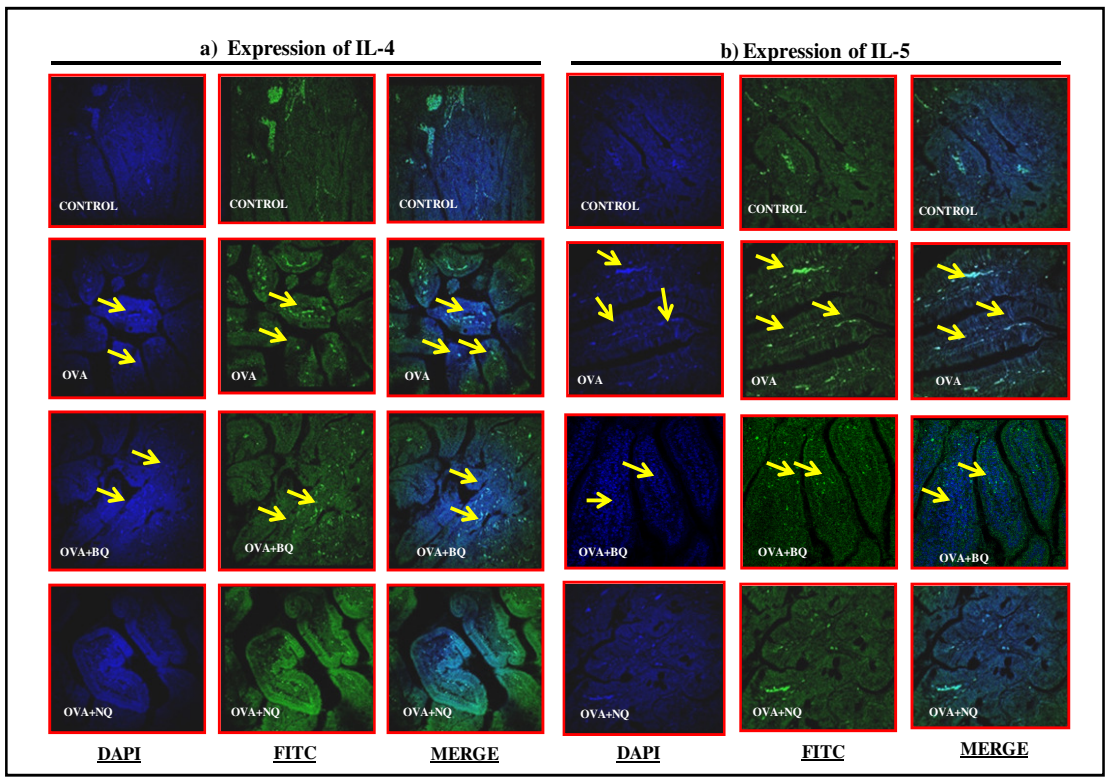

Figure 5.9: Downregulated histological expressions of IL-4 and IL-5 cytokines at the intestinal site of mouse model of food allergy after treatment with BQ as well as NQ quercetin. Intestinal tissues were collected 2 h after the last challenge at day 38 and analyzed for histological expressions of a) IL-4 as well as b) IL-5 levels in intestinal areas of all groups mice (n = 5 mice/ group) using slides having intestinal section. Images of five random fields from five sections of individual mice were captured using confocal microscopy at 200X.

5.3.9 Western Blot Analysis of mast cell signaling proteins

To investigate mast cell signaling pathways, immunoblot analysis of the intestinal proteins isolated from all the treatment groups of mice was carried out. In the OVA treated group, an elevated protein expressions of IL-4, IL-5, GATA-3 were observed in comparison to control group. BQ and NQ treatments were able to significantly reduce the protein expressions of above said cytokines and transcription factors as compared to OVA groups (Fig. 5.10a). Furthermore, markedly upregulated expressions of mast cell surface receptor i.e. FcεRI and its downstream signaling molecules such as phosho (p)-Lyn, p-Syk, p-PKC and p-PLC-γ2 were observed in OVA treated animals when compared to their respective controls. However, these signaling molecules were significantly down-regulated in the intestinal proteins of BQ and NQ treated groups (Fig. 5.10b). In comparison to BQ, NQ treatment more efficiently downregulated the above mentioned signalling molecules associated with Src-kinases and mast cell transduction pathway.

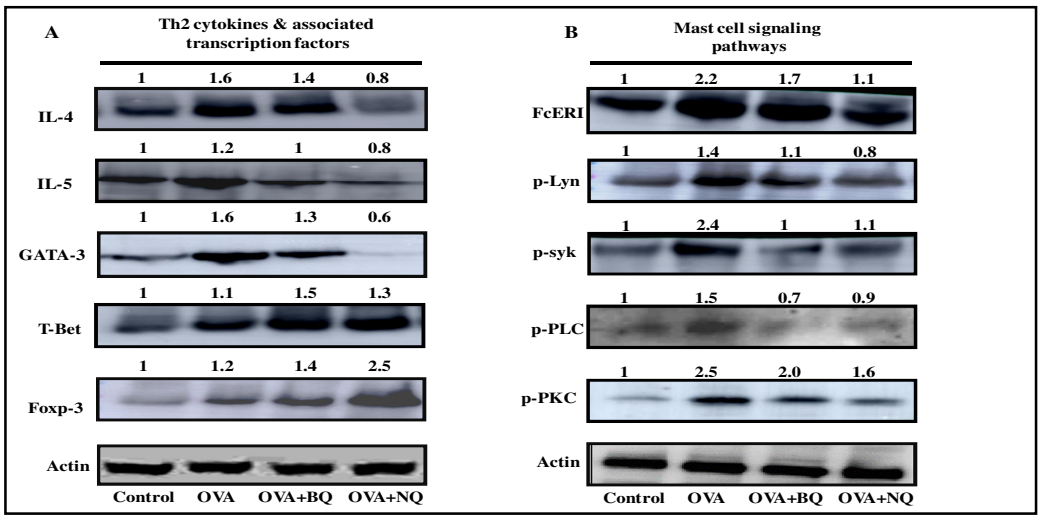

Figure 5.10: Western blots. Inhibition of src kinase for mast cell signalling pathways and transcription factors in the BQ and NQ treated mice with food allergy. Intestinal proteins were isolated from all groups (n=6 mice/ group) 2 hrs after the last challenge and performed western blotting using specific antibodies for Src kinase and mast cell signalling pathways. For each sample, three gels/blots were generated. (a) Western

blotting of IL-4, IL-5, GATA-3, T-bet, Fox-p 3 (b) Western blotting of FcεRI, p-Lyn, p-Syk, , p-PKC, and p- PLCγ2.

5.4 Discussion

Of late, flavonoid quercetin has emerged as a promising therapeutic agent to overcome the pathophysiology of allergic diseases. However, a number of studies indicate that quercetin does not seem to be effective due to its lower bioavailability (Manach et al., 2004; Erlund et al., 2004; Wiczkowski et al., 2008). The nano-formulation of quercetin have been tried to resolve this issue and positive results are being reported in terms of enhanced bioavailability and better efficacy [Gupta et al., 2018]. There are several approaches such as micro-emulsion, suspension; nano encapsulation and through liposome one can try to improve the bioavailability, stability and therapeutic efficacy of flavonoids (Patel et al., 2011). In our previous study, we prepared nanotized quercetin (NQ) with much greater bioavailability and enhanced therapeutic efficacy as compared to bulk quercetin at a very low dose of NQ (1 mg/kg) that was capable of attenuating allergic immune responses in asthmatic mouse model (Gupta et al., 2016). Based on this study, we hypothesized that nanocrystals of quercetin treatment during allergic sensitization and challenge would inhibit the development of anaphylaxis and other food allergic symptoms in OVA induced BALB/c mice. We sought to investigate the therapeutic potential of bulk as well as nanotized quercetin in OVA induced food allergy model of female BALB/c as this strain of mice readily display Th2 responses and favors the higher IgE antibody production (Kumar et al, 2013).

Based on study of Kinney et al (2015), that polyphenol curcumin inhibits the anaphylaxis, we believe that the treatment of allergic sensitization using quercetin

and its nanocrystals would inhibit the development of intestinal anaphylaxis to OVA allergens. In order to examine whether BQ and NQ treatments inhibit anaphylactic symptoms, OVA sensitized and challenged mice were intraperitoneally treated with 15 mg/kg BQ and 2.5 mg/kg of NQ in PBS daily from first challenge with OVA and continued till last challenge throughout the experiment as shown in animal sensitization protocol in group 3 and 4.

A marked increase in the levels of total and specific IgE was evident in sera of OVA sensitized and challenged mice. BQ (15 mg/kg) had a partial inhibitory effect on the production of total as well as sIgE and sIgG. However, treatment with NQ significantly inhibited the production of total as well as sIgE and sIgG1 confirming the protective effect of quercetin against IgE mediated immune responses in murine/mice model. The IgE and IgG mediate the systemic anaphylactic reactions via production of various allergic mediators and cytokines such as histamine, tumor necrosis factor-α (TNF-α), and mouse mast cell protease (Peavy et al., 2008). Similar to the study carried out by Li et al. (2000), we also observed that in group 2 i.e OVA sensitized mice when challenged with high dose of OVA showed anaphylaxis signs and symptoms along with a significant drop in body temperature. NQ treated mice demonstrated a significant reduction in anaphylactic symptoms while keeping the internal body environment in a steady state, on the other hand BQ did not afford a significant effect on same parameters. The data suggest that NQ came to the action via inhibition of both IgE as well IgG production and consequently caused a marked reduction in the anaphylactic features. The enhanced anti-allergic activities of NQ in terms of immunoglobulin production is attributed to small size aqueous dispersibility and increased bioavailability as compare to BQ.

Moreover, we also observed the reduced serum levels of proinflammatory and newly formed mediators like MCPT-1, TSLP and histamine in the mice treated with BQ as well as NQ after OVA challenge. These allergic mediators on antigen exposure inducts food allergic manifestations and development of anaphylactic symptoms (Johnston et al. 2014; Pearce et al., 1984) by recruitment and activation of other circulating leukocytes including mast cells, eosinophils, basophils and neutrophils, as well as monocytes and Th2 cells. This cascade typically results in onset of of allergic symptoms like development of diarrhea, muscle spasm, mucus production and intestinal inflammation (Wang et al., 2016; Hua Xie et al., 2005). It was noticed that NQ throttles the above said mediators efficiently as compared to BQ.

Furthermore, we also quantified the cytokine profile in the serum of all group's mice. Many growth factors like cytokines and chemokines are also released in the series of food allergic manifestation. Generally in allergic diseases, the role of CD4+T cell, IL-4, TNF-α, IL-10, and IFN-γ (33) is critical (Rhodes et al., 2001). Interleukins released by activated Th2 cells (e.g., IL-4 and IL-13) facilitates the production of IgE antibodies. These antibodies, in turn, attached to other effector cells (mast cells, basophils, eosinophils) that possess specific receptors for IgE (Busse et al., 1995) which induces Th2 mediated allergic response, allergic diarrhea, mast cell intestinal activation , expansion and increase susceptibility to anaphylaxis in OVA induced food allergy (Bartnikas et al., 2013). The levels of Th2 cytokines i.e IL-4 have been found to be enhanced in OVA induce murine model of food allergy.

Nair et al (2002) showed that Quercetin upregulates significant gene expression and production of Th-1-derived interferon (IFN-γ), as well as down

regulating Th-2-derived IL-4 production by normal peripheral blood mononuclear cells. Chirumbolo et al (2010) shown that quercetin reduces the increased levels of IL-4, cytokine production in ovalbumin-sensitized and challenged mice. Kawai et al (2007) demonstrated that the inhibitory effect of flavonoid quercetin on IL-4 expression owes through their inhibitory action on activation of nuclear factors of activated T cells and AP-1 (activator protein-1). Min et al (2007) demonstrated that quercetin at 100 μM conc inhibits release of IL-6, IL-8 and TNF-α by 82%, tryptase release by 79%–96%, and histamine release by 52%–77% as well as pro-inflammatory cytokines in human mast cell line.

In current study, NQ has significantly reduced the levels of IL-4, IL-10 and TNF- α at a very low dose i.e 2.5 mg/kg as compared to BQ (15 mg/kg) and remarkable reduction in the symptoms of food allergy. NQ also promotes the induction of Th1 cytokine including IL-2 and IFN-γ and leading to the differentiation of T cell into Th1 phenotype corroborating the previous observations as discussed in above paragraph.

Pathology of intestine of OVA treated mice showed heavy infiltrations of inflammatory cells and loss of mucosal structure with increased activity of goblet cells along with hyperplasia in sub mucosal layer. Exfoliation of intestinal cells along with mild activity of goblet cells also seen in OVA +BQ treated group. While the OVA + NQ group showed similar histology of intestinal tissue as of control group, bestowing the effect of NQ against food allergy. The results establish that NQ conferred the more efficient therapeutic potential in OVA induced food allergy as compared to BQ.

The gastrointestinal tract (GI) mucosal immune system plays an important role in body response to the food allergen on oral exposure. The immune system of GI is comprises of three layer of defense i.e intestinal epithelial barrier, the lamina propria and the gut-associated lymphoid tissue (GALT), where, intestinal epithelial barrier consist of enteroendocrine cells, goblet cells, and paneth cells. The goblet cells secrets mucus which covers the epithelial surface and prevents it against antigens, toxins, pathogens and enteric microbiota, while selectively permeable to absorption of nutrients, electrolytes and water. The mucin is the main constituents of mucus that can be detected by alcian blue staining to measure the mucus production and hyperactivation of goblet cells (Spicer et al.,1960). Oral treatment with microemulsion of quercetin has been shown to decrease the NFKB activation, and the mucus production in the lung of mice (Rogerio et al., 2010). We have shown that NQ efficiently restored the homeostasis between goblet cell and mucus production in GI tract.

Therapeutic administrations of NQ attenuate the symptoms of food allergy in BALB/c mice, promising that it could be the powerful weapon against the inflammatory diseases. Treatment with NQ significantly decreased the level of Th2 transcription factors like GATA-3, NFAT, and SOCS-3 along with Th2 cytokines like IL-4 and IL-5. On the other hand, the level of Th-1 transcription factor i. e. T-Bet and T-regulatory cells like Foxp-3 were over expressed indicates the possibility of shift towards Th-1 from Th-2. The parallel decrease in the expression of FcεRI receptor, Th-2 transcription factors and Th-2 cytokines indicated that one of the primary targets of quercetin is mast cells. It is well established that mast cell is the cell responsible for the majority of food allergic manifestations along with

anaphylactic events caused by secretory products of mast cells like preformed mediators (histamine, tryptase, carboxypeptidase A, and proteoglycans), synthesis of arachidonic acid metabolites (prostaglandins, leukotrienes), and platelet-activating factor (PAF), and delayed-phase (2–6 hours) generation of cytokines (TNF-α) and chemokines resulting from increased gene expression. These mediators, taken as a whole, are capable of producing all of the clinical manifestations of anaphylactic reactions (Johnston et al., 2014; Pablos et al., 2016); Metcalfe et al., 2009). Among these, majority of the pathogenic mediators are produced by mast cells following the early signaling events i.e. Fc□RI-Lyn-Syk signaling (Metcalfe et al., 2009).

Therefore, study of inhibiting potential of NQ on Fc□RI-Lyn-Syk signaling was our subject of interest. In the present study, NQ treatment after OVA challenge in sensitized mice, almost completely inhibited the protein expressions of mast cells cascading pathway including Fc□RI, p-Lyn, p-Syk as well as their downstream signalling molecules such as p-PKC and p-PLCγ2. However, the inhibiting efficacy was also observed in case BQ treatment but it was lower than noticed with NQ. From this result, we concluded that NQ has the potential to inhibit the clinical manifestations associated with food allergy and anaphylaxis by regulating the activation of mast cell signalling pathways and consequently inhibited production of allergic mediators.

These results indicate that NQ attenuated food allergic complications at much lower dose compared to BQ and may be implicated as a potential drug for the treatment of patients with food allergy.

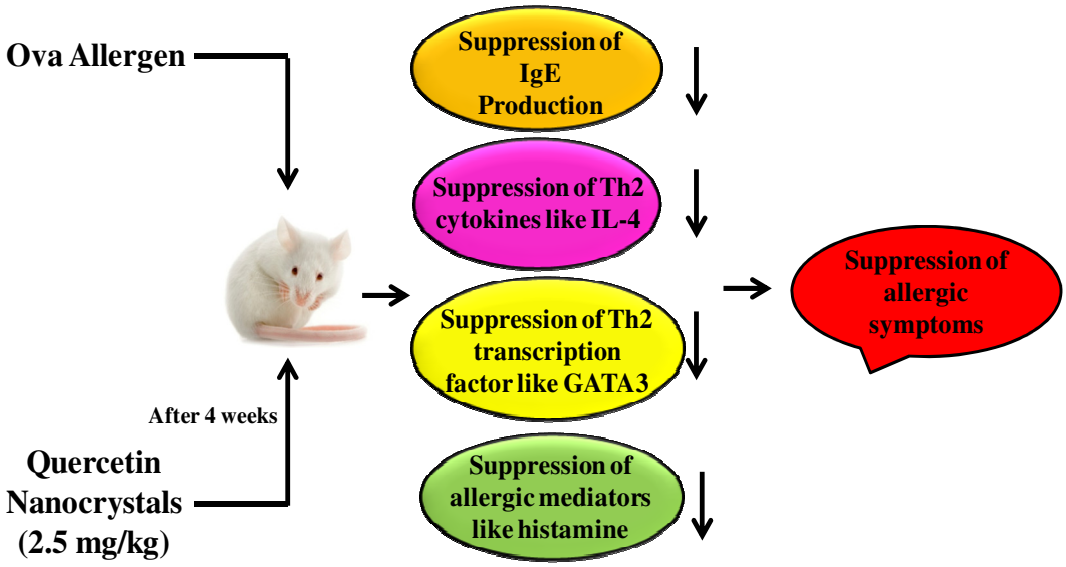

Figure 5.11 Mechanism of anti-allergic effect of Quercetin nanocrystals in food allergy

Summary

SUMMARY

Phytochemicals like flavonoids has a surprisingly wide range of beneficial properties, including antioxidant, antibacterial, antifungal, anti-inflammatory and antiallergic activity. Among polyphenolic compounds, quercetin received more attention due to its potential therapeutic effects but requires enhanced bioavailability and dispersibility. Therefore, by considering various experimental approaches we design a study where firstly we develop quercetin nanocrystals by nanotization and then investigated its *in vivo* pharmacokinetics and therapeutic potential targeting allergic asthma and food induced intestinal anaphylaxis in murine.

Chapter 1 summarizes different types of allergies alongwith their underlying mechanism, quercetin structure, its bioavailability and pharmacokinetics and the role of quercetin in allergic diseases like food allergy, asthma, atopic dermatitis, allergic rhinitis and arthritis. The available literatures prove that quercetin has a potential to combat with pathophysiology of many diseases. But, so far, there have been no reports on therapeutic efficacy quercetin and nanotized quercetin efficacy in the treatment of asthma and food allergy for an efficient cure.

In Chapter 2, we have successfully demonstrated the enhanced bioavailability, solubility and counteractive efficiency of quercetin by synthesized quercetin nanocrystals (NQ). The prepared quercetin nanocrystals were characterized with FTIR, UV, DLS, and TEM. The FTIR spectrum showed 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. Further, the optical properties of bulk and nano quercetin were studied using UV-VIS absorption spectroscopy. 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 changes in nQ compared to bulk quercetin. TEM images showed the formation of nearly spherical monodispersed nQ with size of 16.95±1.3nm. The zeta potential of the nQ (−44.1±6.82 mV) was found to be higher compared to zeta potential of bulk quercetin (−24.9±6.05 mV) corroborating the stability of nQ in water. Therefore, it may be assumed that therapeutic pathways of NQ should not get affected compared to bulk on the basis of functionality. Further, the pharmacokinetics of nQ was studied *in vivo* using well dispersed solution of nQ in aqueous media. 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 was found to be poorly soluble in aqueous media**.** 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 its concentration at different time points by HPLC**.** The stability of nQ at different pH was in following order: 2.0 > 8.0 > 7.4. The results clearly indicate that the bioavailability of nQ increased for a longer time compared to bulk quercetin.

Chapter 3 illustrates simple extraction cum RP-HPLC method for the quantification of nanotized quercetin in biological samples to understand the pharmacokinetics and biodistribution of NQ following intravenous administration. The method involves extraction of NQ from serum and various tissues with 2N HCl and its comparison with available DMSO: MeOH extraction method. Results suggest that amount of NQ at different time intervals in the serum and tissues was found 2-3 fold higher with HCl extraction than DMSO:MeOH extraction method suggesting that HCl extraction is necessary to account for bound NQ with protein. This study deals with the additional steps of extraction of NQ, which enhances the drug detection two to three times. Thus, we believe that this work not only has novelty, but also has the utility. The proposed method has been also found to provide simple, rapid and more sensitive estimation of other flavonoids in mice as well as in humans. Our studies confirmed that decreased particle size of NQ increased its bioavailability as well as aqueous solubility and thus it can be suggested that it is safe, risk-free and counter drug suitable for treatment of many diseases that cause inflammatory response.

The tranquillizing effects of quercetin on allergic asthma are promising, but its poor water solubility and bioavailability is still a bottleneck. In chapter 4, an ovalbumin (OVA) sensitized BALB/c mice asthma model was used to investigate the potential of quercetin nanocrystals (NQ) on relieving asthma aggravation. 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 (sIgE) production, anaphylaxis signs and type 1

skin test. 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. The NQ also significantly modulated the expression of Th2 cytokines like IL-4 and IL-5, which are responsible for IgE class switching. It strongly suppressed the allergic mediators like prostaglandin D2, TSLP, mMCPT-1 and cysteinyl leukotrienes in OVA+NQ group and suppressed the degranulation/secretion of different chemical mediators from activated mast cells. 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. The levels of FcR1, 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. 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 and may play an important role in providing the most effective cure for asthma that is currently lacking. 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.

Chapter 5 is devoted on the use of nano quercetin, which has enhanced bioavailability and water dispersibility, for the treatment of food allergy. Here, synthesized quercetin nanocrystals (NQ) with the size of 10–30 nm were used to

investigate the potential of NQ on relieving food allergic complications in ovalbumin (OVA) sensitized BALB/c mice food allergy mode. Treatment with NQ on OVA sensitized mice exhibited significant anti-allergic potential at much lower dose of NQ (2.5 mg/kg) as compared to bulk quercetin (BQ=15 mg/kg). The treatment with BQ and less doses of NQ, significantly resulted in reduced production of OVA specific immunoglobulins (sIgE and sIgG1), allergic mediators (MCP-1, Histamine, TSLP), Th2 cytokines and less anaphylaxis signs upon oral challenge. In addition, NQ treatment significantly inhibited intestinal mucin production and reversed the intestinal histopathological responses similar to the control group. Apart from this, NQ also markedly caused a down-regulation of gene as well as protein expressions of Th2 cytokines along with associated transcription factors. These results indicate that NQ attenuated food allergic complications at much lower dose compared to BQ and may be implicated as a potential drug for the treatment of patients with food allergy.

Conclusively, the results of the present study prove a unique position of quercetin nanocrystals in the treatment of allergic disorder like food allergy and asthma. Naturally occurring quercetin cannot achieve its optimum therapeutic potential, but nanocrystals of quercetin comes out with great potential for treating food allergy as well as asthma and has been categorized as having preclinical evidence of efficacy. The increased bioavailability represents a new paradigm shift in food induced allergy treatment without any safety and efficacy issues.

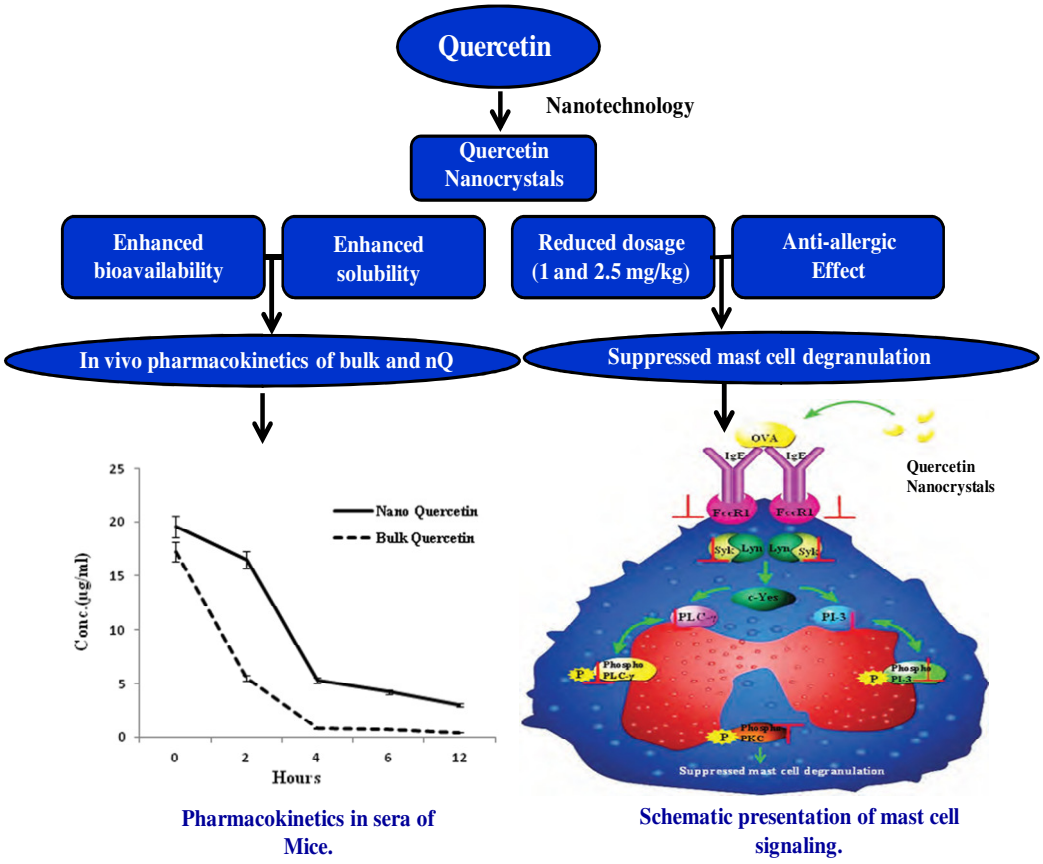

Figure 6.1 Graphical presentation of enhanced aqueous solubility of quercetin nanocrystals and mechanism against allergic manifestations.

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Annexure

Protocols:

1. Preparation and Lyophilisation of Quercetin Nanocrystals

1. Briefly, nQ (2% to 10% w/w) was prepared by mixing bulk quercetin in Milli-Q water (Millipore Corporation, Billerica, MA) having pH 6.8 and resistivity of 18 M with Tween 80 as a stabilizer (0.5 to 2.5 w/w).
2. Prepared solution was subjected to high energy ultra-sonication to obtain quercetin nanocrystals.
3. Thus obtained nQ was freeze dried at −80 ºC in a 20 ml flask using lyophilizer (Allied frost lyophilizer FD-5, New Delhi India) overnight.

***2. In-Vivo* Pharmacokinetics of nQ**

1. BALB/c mice weighing 20–25 g were divided into two groups (n = 10)
2. 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.
3. Each mouse of group 1 and group 2 was injected with bulk quercetin and nQ (30 mg/kg) respectively via a lateral tail vein,
4. The blood from these mice was collected from retro orbital plexus at 0, 2, 4, 6 and 12 hour after injection.
5. Serum was isolated from the blood samples of treated mice and the concentration of delivering bulk and nQ were determined by HPLC.

3. Specific IgE/IgG1 estimation by indirect ELISA method

1. One µg of OVA protein in 100 µl coating buffer [carbonate buffer (pH 9.6)]was coated onto wells of microtiter plates (Maxisorp; NuncTM Immunomodule, Roskilde,Denmark) and left overnight at 4 0C.

2. The wells were then washed with PBS-T (PBS þ0.05% Tween-20). A solution (200 µl) of 3% BSA in PBST (w/v) was then added to each well as blocking buffer and the plates were incubated at 37 0C for 2 h.
3. The plates were then washed and incubated with diluted sera from the sensitized mice.
4. Samples of sera were diluted (using PBS) 1:20 for the IgE and 1:1000 for the IgG1 analyses.
5. All samples were run in triplicate.
6. The plates were incubated overnight at 4 ºC and then washed five times with washing buffer (PBS-T). HRP-anti-IgE and -IgG1 solutions (each at 1:1000) were then added to dedicated wells
7. The plates were incubated 2 h at 37 0C.
8. After five washings, 100 µl substrate solution (5 mg o-phenylenediamine in 10 ml substrate buffer and 10 µl H_2O_2) was added to each wells and the plates were incubated for 30 min at 37 0C in dark.
9. Stop solution. (50 µl of 5 N H_2SO_4) was added to each well.
10. The absorbance (OD) in each then measured at 492nm in an XS2 ELISA plate reader (Biotek Power Wave, Winooski, VT).
11. Only the absolute OD values were used for comparisons among the serum samples.

4. Immunoflourscence histochemistry

1. Tissues sections were placed onto poly-L-lysine coated slides during the cutting process.

2. The sections were circled with an Immunopen pen (Calbiochem, Billerica, MA) and theslides then baked at 60 ºC for 30 min.
3. Slides were placed in xylene for 5 min.
4. Thereafter, the tissues were hydrated in decreasing concentrations of ethanol (absolute, 90%, 70%, 50%) and distilled water.
5. Antigen retrieval was then carried out by incubation in 0.01 M citrate buffer [containing 0.05% Tween-20 (pH 6)] at 90 ºC for 20 min, after which the slides were rinsed in PBS-T.
6. Residual peroxidase activity in the samples was quenched by placing the slides in 3%hydrogen peroxide solution in distilled water for 20 min.
7. The sections were then rinsed thrice with PBS-T and nonspecific binding sites were blocked by incubation in 3% BSA (in PBS-T) for 30 min at 37 ºC.
8. After 2-3 washing with PBS-T, the sections were incubated (2 h, 37 ºC) in PBS-T containing a 1:200 dilution of primary and 1:1000 dilution Alexa flour 594 and FITC conjugated secondary antibody.
9. Further, Prolong gold antifade reagent with 40, 6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI; Invitrogen, Carlsbad, CA, USA) was used as mounting media.
10. Images were captured by confocal microscope.

5. Mast cells counts

1. The sections of the tissues from all groups (3-5µm) were rehydrated in xylene, xylene+ethanol and 100, 90, 70 and 50% ethanol in a serial manner.
2. Sections were washed with PBS and stained with toluidine blue dye for 30 min.

3. Following washing in the running tap water gently, dehydration was carried out using 50, 70, 90 and 100% ethanol, xylene+ethanol and xylene, respectively.
4. Slides were mounted with DPX and images were captured by microscope.

6. Western blotting

1. Tissue protein extraction
2. Proteins from each tissue sample were carried out using RIPA buffer and a tissue homogenizer.
3. Thereafter, 300 µl RIPA buffer was added once more and the sample placed at -80 ºC for 20 min.
4. After 20 min, thawing was done followed by centrifugation at 16,000g for 30 min at 4 ºC.
5. The supernatant was then collected and protein concentration was estimated using a bicinchoninic acid assay kit.

7. Western blotting analysis

1. Tissue proteins (50 µg/lane) were loaded and then resolved over a 12% SDS–PAGE gel.
2. The samples were then electrotransferred onto PVDF membranes using a semi-dry blotting unit.
3. For each sample, three gels/blots were generated.
4. Nonspecific binding sites on each blot were blocked with 3% BSA (in PBS-T) and incubation for 2 h at 37 ºC.
5. Each blot was then washed in PBS-T five times before being coated with above mentioned antibodies (each at 1:200 dilution) in PBS-T+ 3% BSA and placed at 37 ºC for 2 h.

6. After each blot was washed with PBS-T five times, each blot was incubated for 2 hr at 37 ºC in a solution of HRP-conjugated goat anti-mouse IgG (1:1000 dilution).
7. After five washes with PBS-T, presence of antibodies on each blot was evaluated using an enhanced chemiluminescence system (Pierce).
8. Images of the bands were captured using a Syngene gel documentation system equipped in associated with a CCD camera.
9. Densitometric analyses were then done using Gene tools software.
10. The ß-actin/ß-tubulin was chosen as endogenous control.

8. Hematoxylin and Eosin staining

1. Sections were deparrafinized in Xylene I, Xylene II for 10 min each followed by immersing

   in rehydrating alcohol series 100%, 90%, 70% and 50% for 5min each.
2. Rinse slides in dH2O for 10min and pretreated with 0.01 mol/l sodium citrate buffer (pH

   6.0) in a microwave for 10min.
3. Allow slides to cool at RT for at least 10 min and stained the sections in hematoxylin

   solution for 2-4 minutes.
4. Wash the slides in tap water for 1-2min, until sections turn blue (“bluing”).
5. Differentiate sections in 70% ethanol - containing 1% HCl for 5 seconds. This removes excess dye, allowing nuclear details to emerge.
6. Wash the slides in tap water for 2-3min until blue.

7. Stain the slides in Eosin solution for 4-5min and wash again with tap water for 2-3min.
8. Dehydrate the sections with alcohol series 50%, 70%, 90% and 100% for 5min each, cleared in xylene and mounted with DPX.

1. Chemicals and Reagents

1. Western blotting reagents:

1.1. RIPA Lysis Buffer Composition (500ml):

- 50mM Tris-HCl, (pH-7.4) (25ml of 1M)
- 150mM NaCl (15ml of 5M)
- 2mM EDTA (2mlof 0.5M Stock) (Sigma, # E5134-500G)
- 1% NP-40 (5ml)
- 0.5% Sodium deoxycholate acid (2.5g)
- 0.1% Sodium dodecyl sulfate (SDS) (0.5gm)
- 50mM Sodium fluoride (NaF) (1.05gm)

1.2 RIPA Buffer (1ml) for lysate preparation:

- 150µl Sodium fluoride (NaF)
- 10µl Sodium orthovanadate
- 50µl Protease Inhibitor Cocktail P8340 (Sigma- Aldrich)
- 10µl Phosphatase Inhibitor Cocktail P2850 (Sigma-Aldrich)
- 5µl PMSF (50mg/ml stock in isopropanol)

1.3 5X Loading Buffer:

- 0.156 M Tris (pH-6.8)
- 5% SDS (Sigma, # L4390-1KG)
- 25% Gylcerol

- 12.5% beta-mercaptoethanol (Sigma, # M3148-100ML)
- 0.0025% Bromophenol blue (Sigma, # 18047-5G)

1.4 1M Tris-HCL (pH-6.8):

- 12gm Tris buffer in 200ml of distilled H_2O.

1.5 1.5M Tris-HCL (pH-8.8):

- 54.6gm Tris buffer in 200ml of distilled H_2O.

1.6 10% Sodium dodecyl sulfate (SDS):

- 10gm SDS in 100mldistilled H_2O.

1.7 30% Acrylamide (100ml):

- 29gm Acrylamide (Sigma, # A9099-1KG)
- 1gm Bis-acrylamide (Sigma, # T8656-500G)

1.8 10% Ammonium persulfate (APS):

- 1gm APS (Sigma, # A9164-25G) in 10mldistilled H_2O

1.9 Phosphate buffered saline (PBS) (pH-7.4)(10x) for 1L:

- 80.06gm NaCl
- 2.0gm KCl
- 7.652gm Na_2HPO_4
- 1.92gm KH_2PO_4

1.10 5X running buffer/ Tank buffer for 1L:

- 144g Glycine
- 60.5gm Trizma Base
- 5g SDS

1.11 10X Transfer buffer for 1L

- 140g Glycine

- 32.3gmTrizma Base
- 30% Methanol only for making 1X

1.12 Ponceau S Staining solution (0.1% w/v)

- Ponceau S 1g
- Glacial acetic acid 50ml
- Make up to volume ddH_2O 1000ml

1.13 1% PBST (with Tween-20):

- 0.1% Tween-20 (Amresco M14L-1L) in PBS (1x)

1.14 SDS Gel Composition

- **Resolving Gel:**

1 Gel (10ml)	**6%**	**8%**	**10%**	**12%**	**15%**
H_2O (µl)	5300	4600	4000	3300	2300
30% Acrylamide (µl)	2000	2700	3300	4000	5000
1.5M Tris pH 8.8 (µl)	2500	2500	2500	2500	2500
10% SDS (µl)	100	100	100	100	100
10% APS (µl)	100	100	100	100	100
TEMED (µl)	8	6	4	4	4

- **Stacking Gel**

Chemicals	**For 1 Gel (3 ml)**	**For 2 Gels (5 ml)**
H_2O (µl)	2100	3400
30% Acrylamide (µl)	500	830
1.5M Tris pH 6.8 (µl)	380	630
10% SDS (µl)	30	50
10% APS (µl)	30	50
TEMED (µl)	3	5

Antibodies list:

S. No.	Antibody	Company	Cat. No.
1.	PI-3-Kinase	Santa cruz	SC-1637
2.	p-PKC	Santa cruz	SC-12356
3.	p-PLC-γ2	Santa cruz	SC-101785
4.	p-PI 3-kinase	Santa cruz	SC-12929
5.	SOCS-3	Santa cruz	SC-9023
6.	PLC-γ2	Santa cruz	SC-31751
7.	PKC	Santa cruz	SC-17804
8.	p-Lyn	Cell signalling Tech.	2731S
9.	Lyn	Santa cruz	SC-7724
10.	Syk	Santa cruz	SC-1077
11.	GATA-3	Santa cruz	SC-268
12.	IL-4	Santa cruz	SC-1261
13.	IL-5	Santa cruz	SC-7887
14.	IL-10	Santa cruz	SC-365858
15.	IL-2	Santa cruz	SC-133118
16.	IL-6	Santa cruz	SC-28343
17.	IFN-γ	Santa cruz	SC-373727
18.	TNF-α	Santa cruz	SC-133192
19.	Fc€R1	Santa cruz	SC-33484
20.	Rabbit anti goat IgG-HRP	Santa cruz	SC-2944
21.	Goat anti rabbit IgG-HRP	Santa cruz	SC-2004
22.	Sec. Antimouse IgG	Santa cruz	SC-358923
23.	Fox-P3	Santa cruz	SC-166212
24.	T-bet	Santa cruz	SC-11333
25.	ß-actin HRP mouse monoclonal	Santa cruz	SC-47778

List of Kits and miscellaneous reagents

S. No.	Name	Company	Cat No./Ref No.
1.	Mouse TSLP ELISA Kit	eBioscience	88-7490-22
2.	Mouse MCPT-1 ELISA Kit	eBioscience	88-7503-22
3.	Mouse MCP-1 ELISA Kit	eBioscience	555260
4.	Mouse Histamine ELISA Kit	Cayman	512031
5.	Prostaglandin D2 ELISA Kit	Cayman	512031
6.	TEMED	Sigma- Aldrich	T9281
7.	Bovine Serum Albumin (BSA)	Sigma- Aldrich	A2153-100G
8.	Quercetin	Sigma- Aldrich	Q4951-10G
9.	Tween 80 (Polysorbate 80)	Sigma- Aldrich	P4780-500ML
10.	Ovalbumin	Sigma- Aldrich	A5503-25G
11.	BCA kit	Thermo Scientific	23225
12.	Hematoxylin	BioGenex	HK100-9K
13.	Hydrogen Peroxide	Calbiochem	386790
14.	Prestained Protein Ladder	Puregene	PG-PMT2922
15.	Ribonuclease A	Sigma-Aldrich	R6513-50MG
16.	Tween-20	Amresco	M14L-1L
17.	Goat serum	Abcam	ab7481
18.	Western Blot Stripping Buffer	TaKaRa	T7135A
19.	Triton X-100	Sigma-Aldrich	T8532

List of Instruments:

S. No.	Instrument Name	Company	Model No.
1.	Biological Biosafety Cabinet	Thermo Fischer	Forma Class II, A1
2.	Confocal microscope	Leica TCS	SPE-25
3.	Dry Bath	NU life	NU46380-21
4.	Gel DocAmersham 600	GE Heath Care Life Sciences	29-0834-61
5.	Incubator	Thermo Forma	SN302070-1005
6.	Transmission Electron Microscope (TEM)	JEOL Ltd, Tokyo Japan	JEM-2100
7.	Table Centrifuge	RemiElektroTechnik Limited	REMI CM-8Plus
8.	Light microscope	LEICA	LEICA DM i1
9.	Fluorescence activated cellanalyzer (FACS)	BD Biosciences	FACS Canto
10.	SYNERGY-HT multi well plate Reader	Bio-Tek	B119-100
11.	Sonicator	Sonics Vibra cell, Sonics & Material Inc	V-505
12.	Weighing Balance	METTLER TOLEDO	AB304-S
13.	Vortex	Tarson SPINIX	3020
14.	Zetasizer Nano–ZS	Malvern Instruments Ltd, Malvern, UK	Model ZEN 360
15.	pH meter	EUTECH instrument pH tutor (Thermo Scientific)	54X002606C
16.	Gel Rocker	GeNeiTM	SLM-GR-100
17.	Mini Centrifuge	Eppendorf	5418R

List of Publications

List of Publications from Thesis

1. **Gupta, K.,** Sharma, A., Gupta, R., Dixit, S., Singh, S.P., Das, M. and Dwivedi, P.D., 2018. Simple extraction cum RP-HPLC method for estimation of nanotized quercetin in serum and tissues of mice. *Pharmaceutical Chemistry Journal*, Vol. 52, Issue 2, pp.175-181.

2. **Gupta, K.,** Kumar, S., Gupta, R.K., Sharma, A., Verma, A.K., Stalin, K., Chaudhari, B.P., Das, M., Singh, S.P. and Dwivedi, P.D., 2016. Reversion of asthmatic complications and mast cell signalling pathways in BALB/c mice model using quercetin nanocrystals. *Journal of Biomedical Nanotechnology*, Vol. 12 Issue 4, pp.717-731.

List of publication apart from Thesis

1. Gupta, R.K., **Gupta, K.** and Dwivedi, P.D., 2017. Pathophysiology of IL-33 and IL-17 in allergic disorders. *Cytokine & growth factor reviews.*

2. Gupta, R.K., **Gupta, K.,** Sharma, A., Das, M., Ansari, I.A. and Dwivedi, P.D., 2018. Maillard reaction in food allergy: Pros and cons. *Critical Reviews in Food Science and Nutrition*, Vol. 58, Issue 2, pp.208-226.

3. Gupta, R.K., **Gupta, K.,** Sharma, A., Das, M., Ansari, I.A. and Dwivedi, P.D., 2016. Health risks and benefits of chickpea (Cicer arietinum) consumption. *Journal of Agricultural and Food Chemistry*, Vol. 65, Issue 1, pp.6-22.

4. Kumar, S., **Gupta, K.,** Das, M. and D Dwivedi, P., 2013. Recent Advancements in the Therapeutics of Food Allergy. *Recent Patents on Food, Nutrition & Agriculture*, Vol. 5, Issue 3, pp.188-200.

5. Kumar Gupta, R., Kumar, S., **Gupta, K.,** Sharma, A., Roy, R., Kumar Verma, A., Chaudhari, B.P., Das, M., Ahmad Ansari, I. and Dwivedi, P.D., 2016. Cutaneous exposure to clinically-relevant pigeon pea (Cajanus cajan) proteins promote TH2-dependent sensitization and IgE-mediated anaphylaxis in Balb/c mice. *Journal of Immunotoxicology*, Vol.13, Issue 6, pp.827-841.

6. Sharma, A., Kumar, S., Gupta, R.K., Roy, R., **Gupta, K.,** Verma, A.K., Chaudhari, B.P., Das, M. and Dwivedi, P.D., 2014. Elucidation of immediate type I reactions in native and GM mustard (Brassica spp.). *Food Research International*, Vol. 64, pp.810-821.

7. Gupta, R.K., Raghav, A., Sharma, A., **Gupta, K.,** Mandal, P., Tripathi, A., Ansari, I.A., Das, M. and Dwivedi, P.D., 2017. Glycation of clinically relevant chickpea allergen attenuates its allergic immune response in Balb/c mice. *Food Chemistry*, Vol. 235, pp.244-256.

8. Verma, A.K., Sharma, A., Kumar, S., Gupta, R.K., Kumar, D., **Gupta, K.,** Giridhar, B.H., Das, M. and Dwivedi, P.D., 2016. Purification, characterization and allergenicity assessment of 26 kDa protein, a major allergen from Cicer arietinum. *Molecular Immunology*, Vol. 74, pp.113-124.

Abstracts in Conference

1. **Gupta K,** Gupta R K, Dwivedi P D. Reversion Of Asthmatic Complications And Mast Cell Signalling Pathways In BALB/C Mice Model Using Quercetin Nanocrystals. 5th Pedieiatric Allergy and Asthma Meeting 2017 at London, England, 26-28 October, 2017, Clinical and Translational Allergy 2018, 8(Suppl 2):P113.

2. **Gupta K,** Raghav A, Gupta R, Chaudhari B.P., Das Mukul, Dwivedi P. D. Elucidations of atopic dermatitis induced by mung bean (vigna radiata l. Millsp) proteins in allergic patients and Balb/c mice. "The Asia Pacific Association of Pediatric Allergy, Respirology and Immunology (APAPARI) 2013 Congress", Bangkok, Thailand, October 2-4, 2013.

3. Gupta R K, **Gupta K,** Dwivedi P D. Cutaneous Exposure to Clinically-Relevant Pigeon Pea (Cajanus Cajan) Proteins Promote TH2-Dependent Sensitization and IgE-Mediated Anaphylaxis in Balb/C Mice. 5th Pedieiatric Allergy and Asthma Meeting 2017 at London, England, 26-28 October 2017.

4. Gupta R K, Sharma A, **Gupta K,** Dwivedi P D. Purification, characterization and allergenicity assessment of a clinically relevant allergen from Cicer arietinum: putative alpha dioxygenase fragment. 2nd International Toxicological Conclave, CSIR-IITR, Lucknow, Novembor 15-16, 2016.

5. Gupta R K, Sharma A, **Gupta K,** Dwivedi P D. Red gram (Cajanus cajan) proteins induce symptoms of atopic dermatitis in BALB/c mice following intraperitoneal route of exposure. Clinical and Translational Allergy 2017, 7 (Suppl 1):PP003.

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SIMPLE EXTRACTION CUM RP-HPLC METHOD FOR ESTIMATION OF NANOTIZED QUERCETIN IN SERUM AND TISSUES OF MICE

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In recent years, several studies have focused on antioxidant, anti-inflammatory, and anti-cancer activities of quercetin (3,3′,4′,5, -pentahydroxyflavone). The nanotization of quercetin was shown to enhance its therapeutic efficacy due to smaller particle size. In the present study, an additional step was added to simple extraction cum RP-HPLC method for the quantification of nanotized quercetin (nQ) in biological samples to understand the pharmacokinetics and biodistribution of nQ following intravenous administration. The proposed method involves extraction of nQ from blood serum and tissues of mice with 2N HCl in comparison to well-known DMSO:MeOH mix method. The HCl extraction was found to be 2 – 3 times more efficient than DMSO:MeOH mix method. Results showed that the amount of nQ at various time intervals in the serum and tissues was 2 – 3 fold greater for HCl extraction than for DMSO:MeOH mix method, suggesting that HCl extraction must take into account nQ bound with protein. The reversed-phase HPLC was used for nQ detection, which showed the nQ retention time of 3.2 min. The limit of detection of nQ in blood serum was found to be 0.1 μg/mL. The proposed method was also validated in terms of linearity, precision, and accuracy.

Keywords: quercetin; pharmacokinetics; HPLC; extraction method.

1. INTRODUCTION

Quercetin is a multipurpose plant flavonoid widely distributed in plants and involved in UV filtration, symbiotic nitrogen fixation and floral pigmentation [1]. It is an aglycone form of glycoside flavonoid (e.g., rutin, quercetin), a non-sugar compound remaining after replacement of a glycosyl group of glycoside by hydrogen bond [2]. It forms an essential part of human diet by means of various sources such as apples, onions, grapefruit, tea, green vegetables, and beans. Research data demonstrate that 25 mg is the average daily dietary intake of quercetin by an individual in the United States [3]. Like other flavonoids, it has low retention and high excretion. Quercetin exhibits a variety of pharmacological activities, including anti-inflammatory, anticancer, antioxidant, wound-healing, and anti-allergic [4 – 9]. Although quercetin has attracted tremendous attention, its poor aqueous solubility and low bioavailability is still a bottleneck.

Among the bioavailability studies of polyphenolic compounds, quercetin received more attention due to its potential therapeutic effects but required enhanced bioavailability and dispersibility. Since bulk quercetin can be rapidly metabolized from plasma and other organs, its effect vanishes quickly. The pharmacokinetics of quercetin in humans has been extensively studied and it was found that quercetin has a low plasma concentration, poor tissue absorption, and rapid metabolism [10]. Thus, in order to provide an effective treatment with quercetin, its low bioavailability needs to be improved. In order to improve the bioavailability as well as therapeutic efficacy of quercetin, many new formulations have been developed, e.g., by complex forming with cyclodextrins and liposomes [11, 12]. Om one hand, a new formulation can provide improvement, but may be associated with risk of nephrotoxicity [13]. On the other hand, use of liposomal formulations was restricted because of the problem of stability during storage, since the life span of liposome-en-

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Article

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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 (sIgE) 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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Review

Peptide based immunotherapy: A pivotal tool for allergy treatment

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ABSTRACT

Immunotherapies with T-cell epitope peptides have shown a promising impact over allergic diseases as a potential therapeutic tool in in vitro and in vivo conditions. It is recognized as an effective treatment with long lasting clinical effects and subsequent reduction of the allergic inflammatory reactions. In this review, we have summarized the role of peptide based immunotherapy and emphasis has also been given to the recent advancement in pollen, cat, hymenoptera venom, and food allergy.

Contents

1. Introduction

Allergy is a serious, life threatening health concern due to the imbalances in the immune system provoked by environmental substances in the susceptible individuals. Immunoglobulin E (IgE) is the most important immunoglobulin involved in the allergic reactions. Most of the allergic reactions are IgE mediated or type I hypersensitivity reactions.

Abbreviations: IgE, Immunoglobulin E; MHC, Major Histocompatibility Complex; ASIT, Allergen Specific Immunotherapy; APCs, Antigen Presenting Cells; DCs, Dendritic Cells.

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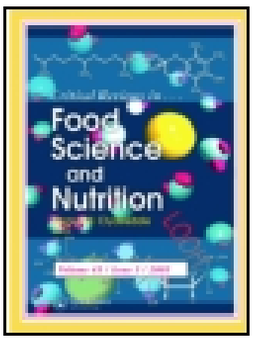

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Maillard reaction in food allergy: Pros and Cons

Rinkesh Kumar Gupta, Kriti Gupta, Akanksha Sharma, Mukul Das, Irfan Ahmad Ansari & Premendra D Dwivedi

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Running Title: Impact of Maillard Reaction in food allergy

Abstract-

Food allergens have a notable potential to induce various health concerns in susceptible individuals. The majority of allergenic foods is usually subjected to thermal processing prior to their consumption. However, during thermal processing and long storage of foods, Maillard reaction (MR) often takes place. The MR is a non enzymatic glycation reaction between the carbonyl group of reducing sugars and compounds having free amino groups. MR may sometimes be beneficial by damaging epitope of allergens and reducing allergenic potential, while exacerbation in allergic reactions may also occur due to changes in the motifs of epitopes or neoallergen generation. Apart from these modulations, non enzymatic glycation can also modify the food protein(s) with various type of advance glycation end products (AGEs) such as Nε-(carboxymethyl-)lysine (CML), pentosidine, pyrraline and methylglyoxal-H1 derived from MR. These Maillard products may act as immunogen by inducing the activation and proliferation of various immune cells. Literature is available to understand pathogenesis of glycation in the context of various diseases but there is hardly any review that can provide a thorough insight on

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Review

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Health Risks and Benefits of Chickpea (*Cicer arietinum*) Consumption

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ABSTRACT: Chickpeas (CPs) are one of the most commonly consumed legumes, especially in the Mediterranean area as well as in the Western world. Being one of the most nutritional elements of the human diet, CP toxicity and allergy have raised health concerns. CPs may contain various antinutritional compounds, including protease inhibitors, phytic acid, lectins, oligosaccharides, and some phenolic compounds that may impair the utilization of the nutrients by people. Also, high consumption rates of CPs have enhanced the allergic problems in sensitive individuals as they contain many allergens. On the other hand, beneficial health aspects of CP consumption have received attention from researchers recently. Phytic acid, lectins, sterols, saponins, dietary fibers, resistant starch, oligosaccharides, unsaturated fatty acids, amylase inhibitors, and certain bioactive compounds such as carotenoids and isoflavones have shown the capability of lowering the clinical complications associated with various human diseases. The aim of this paper is to unravel the health risks as well as health-promoting aspects of CP consumption and to try to fill the gaps that currently exist. The present review also focuses on various prevention strategies to avoid health risks of CP consumption using simple but promising ways.

KEYWORDS: *chickpeas, allergens, isoflavones, protease inhibitors, phenolic compounds*

INTRODUCTION

Health concern caused by legume consumption is a growing subject in developed as well as developing countries.[1] Several studies demonstrating both adverse and beneficial health impacts have been attributed to legume intake. Among leguminous foods, chickpeas (*Cicer arietinum* L.; CPs), also called garbanzo beans, are one of the oldest valuable sources of protein and a main source of human nutrition. CPs produce their offspring from grains and have mainly two varieties, namely, Kabuli and Desi types. CPs are used in several ways, such as whole seeds as well as seeds split in two, which is called "dal", and many other types of traditional, fermented, deep-fried, sweetened, and puffed products, especially in India, Spain, and some other developing Mediterranean countries. CPs are the world's third most essential food legume as they are currently grown on about 11.5 million hectares (ha) across the world, with a total production of 9 million tons.[2] The energy value produced by the Kabuli variety is greater (365 kcal/100 g) than that of Desi variety grains (327 kcal/100 g).[3] Energy is often measured as gross energy or as a caloric value (kcal/100 g) and refers to the amount of energy contained in a food. The WHO in 2003 recommended high consumption of low energy containing foods that are rich in nonstarch polysaccharides present in CPs, other vegetables, and fruits. Furthermore, beans, lentils, and CPs are recommended as an important part of a healthy diet for all Australians as they contain fiber, necessary vitamins, and minerals. CPs are also a rich source of protein, and hence, are included in the meat and fish groups of Australian food.[4] According to the "Dietary Guidelines for Americans", an intake of 3 cups of legumes including CPs in a week is recommended for those who consume approximately 2000 kcal/day energy value.[5] The "Mediterranean diet" has been adopted as a pattern of eating in the olive-growing areas of the Mediterranean region such as Cyprus, Croatia, Spain, Greece, Italy, Morocco, and Portugal. This kind of diet is based on the consumption of food products based on plants. The Mediterranean diet recommends the consumption of legumes in more than two portions/week to minimize the risk factor and rate of cardiovascular diseases, cancer, as well as the incidence of age-associated diseases.[6] According to the U.S. Department of Agriculture, National Nutrient Database, one cup of cooked CPs provides 269 calories, 45 g of carbohydrate, 15 g of protein, 13 g of dietary fiber, and 4 g of fat. Moreover, dietary consumption of six food legumes, namely, dry beans, chickpeas, cowpeas, lentils, fava beans, and pigeon peas, comes to about 6.8 kg/year per capita in the world.[2]

Despite immunological and toxicological responses, CPs have several valuable health benefits concerning the management of several diseases. This review primarily focuses on the beneficial as well as deleterious health effects of CP consumption together on the same platform. This paper provides an overview of health benefits and possible health troubles coupled with descriptions of nutraceutical and toxic components of CPs.

POTENTIAL HEALTH BENEFITS CAUSED BY NUTRACEUTICAL COMPONENTS OF CHICKPEA

CPs are an excellent food choice due to their health-promoting components, including vegetable proteins, complex carbohydrates,

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Recent Advancements in the Therapeutics of Food Allergy

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Abstract: Food allergy is a health complication induced by certain food in the susceptible individuals. Due to lack of permanent cure and the global prevalence, the preventive approach is highly required for food allergy. Recently published patents have shown significant improvements in the food allergy research. In this review, an attempt has been done to highlight the recently developed patents related to the detection of allergens in food mixture. Also, patents regarding treatment options like use of herbal therapy, antihistamines, pre-, pro and synbiotics, nanocarriers, hypoallergens and several immune molecules towards amelioration of food allergy have been reviewed in this article.

Keywords: Allergen, food allergy, histamine, hypoallergen, probiotics.

1. INTRODUCTION

Food is a basic requirement for the fulfillment of calorie demand of the body. However, if food causes immune provocation, in spite of providing nutritional support, the situation becomes very difficult for host [1]. Food allergy is a type 1 hypersensitivity reaction mediated via IgE in the susceptible individuals [2]. The susceptibility of any individual to certain food depends on several factors like environmental set up, genetic predisposition and dietary habits [3]. But, to researchers, it is still an enigma to find out the most appropriate reason for the prevalence of food allergy. The absence of or partially impaired oral tolerance has been frequently observed in the patients of food allergy [4]. Deficiencies in micronutrients especially zinc and iron, as well as vitamin D, in the elderly may also contribute to the development of allergies [5]. The dietary habit is a frequently observed reason behind the food allergy prevalence. In the USA, peanut allergy is prevalent while in Japan, rice allergy has been reported frequently [6]. Similarly, in Indian subcontinent, allergies from red gram, kidney bean, green gram and chickpea have been well documented [7-9]. In Mediterranean region, the prevalence of chickpea and lentil allergies have been reported frequently [10]. Currently, the incidence of food allergy has been reported to be on increase from the several parts of the world including developed and developing countries. Approximately, 90% of all food allergies occur due to the exposure of eight foods often referred to as the "Big-8" that includes foods like milk, egg, fish, crustacean shellfish, tree nuts, peanuts, wheat and soybean. As per the USA food allergen labeling act (FALCPA) there should be information regarding the presence of "Big-8" in the processed foods [11]. In spite of the global prevalence of food allergy, there is a growing need to find out the new therapeutic agents against food allergy. Unfortunately, there is no known permanent cure for the food allergy, so far. Clinicians always suggest avoidance of certain food/s for which patient is susceptible. Several therapeutic options like oral, sub lingual immunotherapy and allergen specific immunotherapy have been taken under consideration for food allergy but, still the best option is yet not known. Recently, several patents have come to light related to the diagnostics and therapeutics of food allergy. In this review, attempts have been made to highlight the patents on detection of food allergens in processed foods and treatment of food allergy using antihistamines, herbal extracts, pre- pro- and synbiotics, nanocarriers and hypoallergens.

2. DETECTION OF FOOD ALLERGENS IN FOOD MIXTURES

There is hardly any treatment that can be used to cure food allergy, completely. Therefore, it is only prevention that can control the prevalence of allergic reactions. Allergen-free compositions have been proved to be useful to prevent prevalence of allergic reactions in the susceptible patients. In processed foods, some hidden allergen(s) may be present that can exacerbate the allergenic reactions in the susceptible patients therefore; it is required to identify the hidden allergen and its proper labeling should be ensured. Several methods have been reported for the identification of hidden allergen within a food and among them in enzyme-linked Immunosorbant assay (ELISA) and polymerase chain reaction (PCR) are most efficient [12]. Along with, semi quantitative PCR, real-time PCR (qPCR) has also been used to obtain a more précised detection of allergens in a food [13]. Further, proteomic techniques have also been used to investigate allergens in a mixture of foods [14]. The term "allergenomics" is used to describe allergen-targeted proteomics, used to elaborate detailed information of allergens from plant source [15]. Gold immunochroma-

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RESEARCH ARTICLE

Cutaneous exposure to clinically-relevant pigeon pea (*Cajanus cajan*) proteins promote T_H2-dependent sensitization and IgE-mediated anaphylaxis in Balb/c mice

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ABSTRACT

Epicutaneous (EC) sensitization to food allergens may occur when the skin has been lightly damaged. The study here tested whether cutaneous exposure to pigeon pea protein(s) may cause allergic sensitization. BALB/c mice were either orally gavaged or epicutaneously sensitized by repeated application of pigeon pea crude protein extract (CPE) on undamaged areas of skin without any adjuvant; afterwards, both groups were orally challenged with the pigeon pea CPE. Anaphylactic symptoms along with measures of body temperature, MCPT-1, TSLP, pigeon pea-specific IgE and IgG_1, myeloperoxidase (MPO) activity, T_H2 cytokines, T_H2 transcription factors (TFs) and filaggrin expression were determined. Mast cell staining, eosinophil levels and histopathological analysis of the skin and intestines were also performed. In the epicutaneously-sensitized mice, elevated levels of specific IgE and IgG_1, as well as of MCPT-1, TSLP, T_H2 cytokines and TFs, higher anaphylactic scores and histological changes in the skin and intestine were indicative of sensitization ability via both routes in the pigeon pea CPE-treated hosts. Elevated levels of mast cells were observed in both the skin and intestine; increased levels of eosinophils and MPO activity were noted only in the skin. Decreased levels of filaggrin in skin may have played a key role in the skin barrier dysfunction, increasing the chances of sensitization. Therefore, the experimental data support the hypothesis that in addition to oral exposure, skin exposure to food allergens can promote T_H2-dependent sensitization, IgE-mediated anaphylaxis and intestinal changes after oral challenge. Based on this, an avoidance of cutaneous exposures to allergens might prevent development of food anaphylaxis.

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Introduction

Anaphylaxis, a potentially fatal allergic reaction that can occur rapidly, is characterized by many symptoms, including troubled breathing, hives, swelling, tightness of the throat, nausea, abdominal pain, diarrhea, dizziness, fainting, low blood pressure, rapid heartbeat and sometimes cardiac arrest (Simons et al. 2007). Hospitalization due to food-related anaphylaxis is a growing concern (8.8% per annum) as the overall prevalence of food allergy is 3–4% in adults and 8% in children (Sicherer & Sampson 2010). Systemic anaphylaxis to food results from IgE and IgG-mediated sensitivity (reviewed in Finkelman et al. 2007). However, IgE and IgG antibodies to food(s) can exist in individuals after ingestion without causing anaphylactic symptoms, suggesting that factors other than IgE and IgG may be required (Hill et al. 2007). With food-related anaphylaxis, a major issue is how individuals get sensitized to allergens. Is the oral route only one for sensitization to food allergens? While most people develop oral tolerance after ingesting food, in some cases, exposure to foods occurs through routes other than ingestion. These alternative routes may be important for sensitization as evident by case report of food allergy following occupational allergen exposure (Hoffman & Guenther 1988). Epidemiological data also suggest that sensitization to peanut proteins occur in children after application of peanut oils to inflamed skin (Lack et al. 2003).

Clinically, food allergy is frequently associated with atopic dermatitis (AD). Environmental exposure to allergens in the context of an impaired skin barrier (as seen in AD) due to mutations in the filaggrin gene are thought to promote a cutaneous sensitization that could lead to development of food anaphylaxis (FA) (Sicherer and Leung 2015). Interestingly, in a previous study, some pigeon pea-allergic patients were found to have AD-like symptoms such as urticaria (Misra et al. 2010). This suggested that, besides oral, an epicutaneous (EC) route could be a route for sensitization that could ultimately give rise to food-related anaphylaxis.

Legumes are major culprits in food-related anaphylaxis. Several reports have reflected the role of legumes, including peanuts, chickpeas, red kidney beans, green gram and broad beans in provoking anaphylactic symptoms in Balb/c mice (Misra et al. 2010; Verma et al. 2012; Kumar et al. 2013). Food-dependent exercise-induced anaphylaxis to chickpea and soya bean allergens have been reported in sensitized individuals (Orhan & Karakas 2008; Adachi et al. 2009; Ciccarelli et al. 2014). The latter 2014 study

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Purification, characterization and allergenicity assessment of 26 kDa protein, a major allergen from *Cicer arietinum*

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ABSTRACT

Chickpea (CP), a legume of the family Fabaceae, is an important nutrient-rich food providing protein, essential amino acids, vitamins, dietary fibre, and minerals. Unfortunately, several IgE-binding proteins in CP have been detected that are responsible for allergic manifestations in sensitized population. Therefore, the prevalence of CP induced allergy prompted us towards purification, characterization and allergenicity assessment of a major ~26 kDa protein from chickpea crude protein extract (CP-CPE). Purification of CP 26 kDa protein was done using a combination of fractionation and anion exchange chromatography. This protein was further characterized as "Chain A, crystal structure of a plant albumin" from *Cicer arietinum* with Mol wt 25.8 kDa by Liquid chromatography-tandem mass spectrometry (LC–MS/MS) analysis. Further, allergenic potential of purified 25.8 kDa protein was assessed using *in vivo* and *in vitro* model. Purified protein showed IgE-binding capacity with sensitized BALB/c mice and CP allergic patient's sera. Enhanced levels of specific and total IgE, MCP-1, MCPT-1, myeloperoxidase, histamine, prostaglandin D2, and cysteinyl leukotriene were found in sera of mice treated with CP ~26 kDa protein. Further, expressions of Th2 cytokines (i.e. IL-4, IL-5, IL-13), transcription factors (*i.e.* GATA-3, STAT-6, SOCS-3) and mast cell signaling proteins (Lyn, cFgr, Syk, PLC-γ2, PI–3 K, PKC) were also found increased at mRNA and protein levels in the intestines of mice treated with CP ~26 kDa protein. In addition, enhanced release of β-hexosaminidase, histamine, cysteinyl leukotriene and prostaglandin D2 were observed in RBL2H3 cell line when treated (125 μg) with CP 26 kDa protein. Conclusively, *in vivo* and *in vitro* studies revealed the allergenic potential of purified CP 26 kDa protein. Being a potential allergen, plant albumin may play a pivotal role in CP induced allergenicity. Current study will be helpful for better development of therapeutic approaches to prevent the allergenicity in CP sensitized individuals.

1. Introduction

Allergic reactions are consequences of abnormal immune response to common and harmless substances, present in the environment. A substance that causes allergic reaction is known as allergen. In last two decades, the prevalence and severity of allergic diseases are increasing worldwide and food allergy constitutes a major part of this increase (Sicherer and Sampson, 2010). Generally, the food allergy may be defined as an adverse health effect that arises from a specific immune response on exposure to a given food (Boyce et al., 2010). Moreover, food induced allergy is characterized by induction of Th2 cells and production of IL-4 cytokine that further acts upon naive T-cells and causes their differentiation into allergen specific Th2 cells to secrete Th2 cytokines including IL-4, IL-5, IL-13 which assist B-cell responses and induce the production of allergen specific IgE (McKenzie et al., 1998). Further, various transcription factors like GATA-3, STAT-6, SOCS-3, C-MAF also play an important role in skewing of immune response towards Th2 reactions thereby provoking allergic manifestations by enhancing the production of Th2 cytokines (Kumar et al., 2012).

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Elucidation of immediate type I reactions in native and GM mustard (*Brassica* spp.)

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ABSTRACT

Mustard, a widely consumed spice can provoke allergic manifestations in mustard sensitive individuals. The aim of this study is to explore the allergenicity potential of GM mustard varieties (GM-V2 and GM-V4) having increased carotenoid content and compare it with the native (Varuna) and commercially available variety (Urvashi). Mustard protein sensitized (GM and non-GM) BALB/c mice sera were used to identify the allergenic proteins by IgE immunoblotting. Immunoglobulin levels, mouse mast cell protease-1, monocyte chemotactic protein and histamine were measured in serum. The levels of Th1/Th2 transcription factors GATA-3, T-bet, SOCS3, STAT 6 and c-maf in intestinal proteins of all groups were detected by immunoblotting and PCR. Major IgE-binding proteins of 21, 29 and 33 kDa were found in all mustard varieties. The enhanced levels of Th2 cytokines IL-4, IL-5 and IL-13 and transcription factors GATA-3 and SOCS-3 were observed. The increased levels of MCP-1, MCPT-1 and histamine were also evident in commercial, native, GM-V2 and GM-V4 varieties of mustard treated groups. Conclusively, all these finding indicate that introduction of GM mustard varieties with increased carotenoid content did not cause any increase in allergenicity as compared to its native counterpart and therefore can be safe from allergenicity point of view.

1. Introduction

Globally, the incidence of allergic diseases is on the rise and food allergies constitute a major part of this increase (Harwanegg & Hiller, 2004). The overall prevalence of food allergy is 3–4% in adults and 8% in children (Sicherer & Sampson, 2010). According to US National Institute of Allergy and Infectious Diseases (NIAID 2010) sponsored guidelines, food allergy is an "adverse health effects that arise from specific immune responses on the exposure of a given food" (Boyce et al., 2010). The incidences of food allergy vary among different countries and depend on the consumption habit of a particular country as higher consumption of a particular food may lead to increased sensitization among the susceptible consumers (Dalal et al., 2002).

Oriental mustard *Brassica juncea* (L.) is a member of the Cruciferae (Brassicaceae) plant family. Mustard seeds are used in a variety of food products to enhance flavor and nutritional values. Mustard seeds are composed of protein (23–30%), fixed oil (29–36%), and carbohydrate (12–18%) together with minor constituents including minerals (4%), essential oil (glucosinolates, 0.8–2.3%), phytin (2–3%) as well as phenolic compounds and dithiolethiones. Common foods containing mustard seeds include pickled products, processed meats, seasoning mixes, salad dressings, sauces, and condiments. Foods formulated with mustard seeds are expected to increase in popularity in the future due to its sensory attributes, its high protein content, and its functional properties. However, the widespread use of mustard in foods has raised concerns as mustard can cause IgE-mediated allergic reactions in sensitive individuals (Jorro, Morales, Bras'o, & Pel'aez, 1995). The prevalence of mustard allergy is reported in 1.1% children suffering from food allergies (Morisset et al., 2003; Rance, Dutau, & Abbal, 2000).

Advancements in biotechnology and recombinant DNA technology have resulted in an increasing number of genetically engineered/modified crops. As per different guidelines like Codex Alimentarius, Organisation for Economic Co-operation and Development (OECD), Department of Biotechnology (DBT), India, allergenicity assessment of genetically modified (GM) crops is mandatory prior to their release in the market. Recently, genetic modifications have been introduced in mustard (GM-mustard) by inserting phytoene synthase (*psy*) from *Zea mays* and phytoene desaturase (*crtI*) from *Erwinia uredovora* using Agrobacterium-mediated transformation to increase the carotenoid

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