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ORIGINAL ARTICLE

Chrysin Ameliorates Ovalbumin-Induced Allergic Response in Allergic Rhinitis: Potential Role of GATA-3, T-Box Protein Expressed in T Cells, Nuclear Factor-Kappa B, and Nuclear Factor Erythroid 2-Related Factor 2

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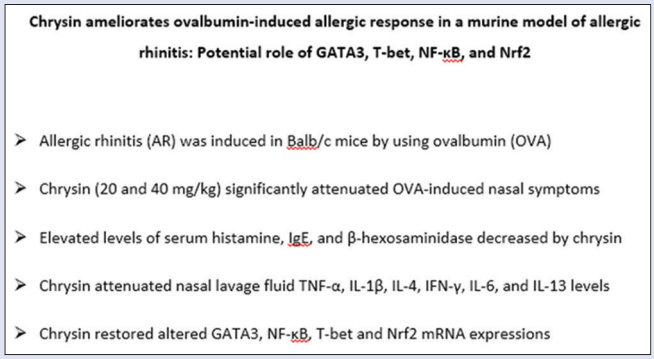
ABSTRACT

Background: Rhinitis is an allergen-induced, immunoglobulin E (IgE)-mediated, chronic immune-inflammatory disease affecting individuals worldwide. Chrysin has been well documented for its anti-allergic potential. **Aim:** This study aimed to determine the efficacy and mechanism of action of chrysin against allergic rhinitis (AR) induced by ovalbumin (OVA) in experimental mice. **Materials and Methods:** Induction of AR was performed in BALB/c mice via intraperitoneal administration sensitization and intranasal challenge with of OVA. Chrysin was concomitantly administered in mice at doses of 10, 20, and 40 mg/kg, p.o. **Results:** OVA challenge caused statistically significant ($P < 0.05$) increase in nasal rubbing, sneezing, and discharge as well as elevated serum histamine, β-hexosaminidase, IgE (OVA-specific and total) levels, whereas chrysin treatment at a dose of 20 and 40 mg/kg significantly ($P < 0.05$) inhibited these biomarkers and thus reduced nasal symptoms. The elevated total and differential cell count, splenic oxido-nitrosative stress, and myeloperoxidase levels after OVA administration decreased statistically significantly ($P < 0.05$) by chrysin. There was a significant increase in the levels of tumor necrosis factor-alpha (TNF-α), interleukin (IL)-4, IL-1β, IL4/interferon-gamma, IL-6, and IL-13 in nasal lavage fluid after OVA challenge, which was inhibited statistically significantly ($P < 0.05$) by chrysin. It also statistically significantly ($P < 0.05$) downregulated spleen GATA-3 and nuclear factor-kappa B (NF-κB), whereas upregulated T-box protein expressed in T cells (T-bet) and nuclear factor erythroid 2-related factor 2 (Nrf2) mRNA expression in spleen. Histological alteration induced in nasal and spleen tissue after OVA challenge was statistically significantly ($P < 0.05$) ameliorated by chrysin treatment. **Conclusion:** Chrysin modulated GATA-3/T-bet pathways and inhibited NF-κB activation, thus attenuating the release of various inflammatory mediators (TNF-α, IL-1β, histamine, IgE, and β-hexosaminidase), Th2 cytokines (ILs), and oxido-nitrosative stress (Nrf2) to exert its anti-allergic potential in experimental AR.

Key words: Allergic rhinitis, chrysin, GATA-3, immunoglobulin E, interleukins, nuclear factor erythroid 2-related factor 2, nuclear factor-kappa B, T-box protein expressed in T cells, tumor necrosis factor-alpha

SUMMARY

- In the present study, we have evaluated the antiallergic potential of chrysin against ovalbumin (OVA)-induced allergic rhinitis in mice. Chrysin at doses of 10, 20, and 40 mg/kg, p. o. was administered in OVA-challenged mice, which showed significant inhibition in OVA-induced nasal symptoms (sneezing, rubbing, and discharge) as well as increased levels of serum histamine, β-hexosaminidase, and immunoglobulin E (IgE) (OVA specific and total). Chrysin also inhibited elevated splenic oxido-nitrosative stress, GATA-3, and nuclear factor-kappa B (NF-κB) mRNA expressions as well as tumor necrosis factor-alpha (TNF-α), ILs, and interleukin (IL)-4/interferon-gamma levels in nasal lavage fluid after OVA challenge. The downregulated splenic mRNA expression of nuclear factor erythroid 2-related factor 2 (Nrf2) and T-box protein expressed in T cells (T-bet) was restored by chrysin. Findings of the present study suggest that chrysin exerts its anti-allergic potential via modulation of GATA-3/T-bet pathways and inhibited NF-κB activation, thus attenuated the release of various inflammatory mediators (histamine, IgE and β-hexosaminidase, TNF-α, and IL-1β), Th2 cytokines (ILs). and oxido-nitrosative stress (Nrf2).



Abbreviations used: AR: Allergic rhinitis; C: Chrysin; GATA-3: GATA binding protein 3 (i.e. Erythroid transcription factor); GSH: Reduced glutathione; IFN-γ: Interferon-gamma; Ig: Immunoglobulin; ILs: Interleukins; MDA: Malondialdehyde; i.e.: Lipid peroxidation; MLT: Montelukast; MPO: Myeloperoxidase; NLF: Nasal lavage fluid; NF-κB: Nuclear factor-kappa B; NO: Nitric oxide; Nrf2: Nuclear factor erythroid 2-related factor 2; OVA: Ovalbumin; SOD: Superoxide dismutase; T-bet: T-box protein expressed in T cells; TNF-α: Tumor necrosis factor-alpha.

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INTRODUCTION

Allergic rhinitis (AR) is widely represented by allergic diseases, mainly affecting the inner lining of the nasal mucosa. AR is a global health

issue that deteriorates 400–500 million individuals' school performance, quality of social life, and work productivity worldwide.[1] Furthermore, AR is associated with significant indirect costs. Rhinitis is mainly characterized by the combination of symptoms including sneezing, nasal congestion, nasal itching, lacrimation of eyes and rhinorrhea followed by respiratory obstruction, which results in pain.[2] Researchers have well documented that a complex interaction of environmental and genetic factors results in the development of AR. Activated mast cells release histamine and cytokines after the induction of AR, which play a vital role in the activation of sensory nerve endings, dilation of blood vessels, and sinusoidal congestion.[3]

Numerous literature have established a direct link between the inflammation of nasal mucosal and elevated production of inflammatory cytokines, including tumor necrosis factor-alpha (TNF-α) and interleukins (ILs, such as IL-4 as well as IL-5, which are released by type-2 T helper (Th2) cells.[2,3] An array of allergens, including cockroaches, dust mites, and molds, stimulate the production of IL-4, which in turn activates B lymphocytes and promotes the release of antigen-specific immunoglobulin E (IgE).[2,3] Further, cross-linking of IgE to its high-affinity immunoglobulin Fc epsilon receptor I present on the surface of mast cell results in mast cell degranulation.[4] During this early-phase response, degranulated mast cell releases various inflammatory mediators, including histamine, cytokines, chemokines, prostaglandins, β-hexosaminidase, and leukotrienes.[5,6] Whereas, in late-phase response, predominant recruitment of eosinophils with overproduction of cytokines such as TNF-α and ILs implicates and maintains allergic inflammatory response.[2,3] Previous studies suggest that growing industrialization in developing countries resulted in increasing number of AR patients.[2,3]

Currently available effective therapeutic options for the management of AR include antihistaminic (brompheniramine and chlorpheniramine), antileukotrienes (montelukast), decongestants (phenylephrine), mast cell stabilizers (cromolyn), and intranasal corticosteroids (budesonide). However, these agents are not only associated with significantly high costs but also provide a promising effect only in a fraction of patients. In addition, long-term administration of these agents is questionable due to their adverse effects, including blurring of vision, dryness of nasal mucous membrane, headache, irritation of throat, and sedation.[7,8] Thus, there is a need of hour for the development of safe and effective therapeutic strategies for the management of AR.

Recently, studies documented the potential of bioactive moieties of plant origin for their potent anti-allergic effects. Animal models play a vital role in the evaluation of various treatment options against the management of AR.[9-11] Murine model of immune response induced by ovalbumin (OVA) is one such model that is well established and widely used for the determination of immunomodulatory mechanism of various therapeutic moieties.[11-13] OVA is a protein allergen that induces IgE-mediated allergic response after its systemic administration followed by sensitization with intranasal challenge in experimental animals. It induces clinicopathological symptoms of rhinitis which include sneezing, rubbing, and nasal discharge, followed by elevated levels of serum histamine, IgE, and inflammatory infiltration.[11-13]

Flavonoids are naturally occurring polyphenolic secondary plant metabolites that are common in regular diet. Chrysin is a plant flavonoid which is commonly found in honey, propolis, and various passion flowers (Passiflora incarnata and Passiflora caerulea). In view of its beneficial medicinal properties, it is widely used in dietary supplements. Research carried out on chrysin over past decades indicated its antioxidant, antiviral, antidiabetic, antihypertensive, anxiolytic, anti-inflammatory, anticancer, nephroprotective, and neuroprotective potential.[14-19] Chrysin inhibited various allergic diseases including OVA-induced hyperresponsiveness through stimulation of T-box protein expressed in T cells (T-bet) and inhibition of GATA-3 expression in murine model of asthma.[15] A study documented that this dihydroxyflavone alleviated the decreased levels of interferon-gamma (IFN-γ) and inhibited serum IgE and inflammatory influx including eosinophils, IL-4, and IL-13.[19] Chrysin inhibited upregulated expressions of IgE, TNF-α, ILs, and nuclear factor-kappa B (NF-κB), thus inducing mast cell stabilization.[14] It exerts its antioxidant potential via inhibition of elevated levels of lipid peroxidation and nitrite concentration (nitric oxide [NO]) in OVA-sensitized rats.[18] However, potential of chrysin on OVA-induced AR has not been determined yet. Thus, the present investigation was undertaken with an aim to evaluate the possible mechanism of action of chrysin against OVA-induced allergic response in experimental animals.

MATERIALS AND METHODS

Drugs and chemicals

Chrysin (purity ≥97%, Sigma-Aldrich Co., St. Louis, MO, USA), OVA (Grade V, Sigma-Aldrich Co., St. Louis, MO, USA), aluminum hydroxide (Sigma-Aldrich Co., St. Louis, MO, USA), and histamine dihydrochloride (Sigma-Aldrich Co., St. Louis, MO, USA), Montelukast (Cipla Limited, Mumbai, India), mouse OVA-specific IgE, total IgE, β-hexosaminidase, TNF-α, IL-1β, IL-4, IL-6, IL-13, and IFN-γ enzyme-linked immunosorbent assay (ELISA) Kit (Bethyl Laboratories Inc., Montgomery, TX, USA), total RNA extraction kit, and real-time polymerase chain reaction (RT-PCR) kit (MP Biomedicals India Private Limited, Mumbai, Maharashtra, India).

Animals

Adult male BALB/c mice (18–22 g) were kept under housing conditions of temperature: 24°C ± 1°C, relative humidity: 45%–55%, dark/light cycle: 12:12 h, food: standard pellet chow, water:filtered (*ad libitum*) throughout the experimental protocol. A time of 09:00 to 17:00 h was considered to carry out all the experiments' protocol (CPCSEA/75/2012) which was approved by the Institutional Animal Ethics Committee (IAEC, Poona College of Pharmacy). Guidelines mentioned by the Committee for Control and Supervision of Experimentation on Animals (CPCSEA), Government of India, were followed to perform all the experiments.

Induction of allergic rhinitis and treatment schedule

A sensitization solution (1 g of aluminum hydroxide and 50 mg of OVA) was used to induce allergies in BALB/c mice. Sensitization was carried out by administrating i. p. injection of 500 µl of sensitization solution on various days, namely, days 1, 3, 5, 7, 9, 11, and 13.[20] After sensitization, the mice were divided randomly into various groups (n = 18 mice/group), namely, AR control (received distilled water [DW, 10 mg/kg, p.o.]), montelukast treated ([10], received montelukast [10 mg/kg, p.o.]), and chrysin (10 or 20 or 40 mg/kg, p.o.). A separate group of mice were maintained which were nonsensitized and divided into two groups (n = 18 mice/group), namely, normal (received DW) and *per se* (received chrysin [40 mg/kg, p.o.]). All the treatments were provided for 7 days (from day 14 to day 21). A previous report used to determine the treatment doses of chrysin (10, 20, and 40 mg/kg).[16,17] Mice were challenged on day 21 with intranasal administration of OVA, and nasal symptoms (nasal rubbing, sneezing, and discharge) were recorded for the next 10 min according to a previously reported method.[20] On day 24 (after interruption of treatment), in a separate group of mice, histamine-induced hypersensitivity (nasal rubbing and sneezing) was determined by intranasal challenge of histamine dihydrochloride (10 µl).[20]

Serum biochemistry

On day 21, blood was withdrawn by a retro-orbital puncture, and the total and differential cell (eosinophils, neutrophils, lymphocytes, and macrophages) counts were determined. The levels of IgE (OVA specific and total), β-hexosaminidase, TNF-α, ILs, and IFN-γ were determined in serum by using reagent assay mouse ELISA kits (Bethyl Laboratories Inc., Montgomery, TX, USA). The levels of serum histamine were determined according to a previously reported method.[21]

Nasal lavage fluid biochemistry

Nasal lavage fluid (NLF) collection was performed according to a previously described method.[22] The levels of TNF-α, ILs, and IFN-γ were determined in serum by using reagent assay mouse ELISA kits (Bethyl Laboratories Inc., Montgomery, TX, USA).

Spleen biochemical analysis

The spleen was isolated from each mouse, and the levels of total protein, superoxide dismutase (SOD), GSH, malondialdehyde (MDA) (MDA content), and NO were estimated according to earlier reported methods.[23-27] The mRNA expressions of GATA-3, T-bet, nuclear factor erythroid 2-related factor 2 (Nrf2), and NF-κB (n = 4) were estimated using real-time polymerase chain reaction (RT-PCR) according to method described elsewhere.[16]

Histological examination

Histopathological analysis of spleen and nasal mucosal tissue was carried out using hematoxylin and eosin (H and E) stain as described previously.[20]

Statistical analysis

GraphPad Prism 5.0 software (GraphPad, San Diego, CA, USA) was used to perform data analysis. Data were expressed as mean ± standard error mean and analyzed by using one-way analysis of variance followed by Tukey's multiple range *post hoc* analysis (for parametric tests) as well as Kruskal–Wallis test for *post hoc* analysis (nonparametric tests). P <0.05 was considered statistically significant.

RESULTS

Body weight and relative spleen weight

Body weight decreased statistically significantly ($P < 0.05$) and relative spleen weight increased statistically significantly ($P < 0.05$) in AR control mice when compared with normal mice. However, montelukast (10 mg/kg) administration statistically significantly attenuated ($P < 0.05$) in OVA-induced alterations in relative spleen weight and body weight as compared to AR control mice. Chrysin (20 and 40 mg/kg) treatment also statistically significantly ($P < 0.05$) decreased relative spleen weight and increased body weight when compared with AR control mice. However, when compared with chrysin treatment, montelukast treatment more significantly ($P < 0.05$) attenuated OVA-induced alterations in relative spleen weight and body weight. There were no significant alterations in relative spleen weight and body weight in *per se* treatment group when compared with normal mice [Table 1].

Nasal symptoms

Administration of OVA statistically significantly ($P < 0.05$) induced nasal symptoms (rubbing, sneezing, and discharge) in AR control mice as compared to normal mice. Treatment with montelukast (10 mg/kg) significantly decreased ($P < 0.05$) OVA-induced nasal symptoms (rubbing, sneezing, and discharge) when compared with AR control mice. Treatment with chrysin (20 and 40 mg/kg) significantly ($P < 0.05$) inhibited OVA-induced nasal symptoms when compared with AR control mice. However, OVA-induced nasal symptoms including rubbing and sneezing reduced more significantly ($P < 0.05$) by montelukast treatment when compared with chrysin treatment [Table 1].

Histamine-induced nasal hypersensitivity

There was a statistically significant increase ($P < 0.05$) in nasal rubbing and sneezing after intranasal administration of histamine in AR control mice as compared to normal mice. However, administration of montelukast (10 mg/kg) statistically significantly attenuated ($P < 0.05$) histamine-induced nasal rubbing and sneezing as compared to AR control mice. Chrysin (20 and 40 mg/kg) treatment also statistically significantly ($P < 0.05$) decreased nasal rubbing and sneezing induced by histamine as compared to AR control mice. When compared with chrysin treatment, montelukast treatment showed more significant ($P < 0.05$) attenuation of histamine-induced increased nasal rubbing and sneezing

Table 1: Effect of chrysin treatment on OVA-induced alterations in body weight, relative spleen weight, OVA challenge induced nasal rubbing, sneezing, and nasal discharge as well as histamine challenge induced nasal rubbing and sneezing in AR mice

Parameters	Treatment						
	Normal	AR Control	MLT (10)	C (10)	C (20)	C (40)	Per se
Body weight (gm)	30.33±1.28	25.33±0.95$^{\#}$	30.33±1.12$^{*,\$}$	25.00±1.61	27.33±0.99$^{*,\$}$	29.00±0.97$^{*,\$}$	30.50±1.06
Spleen wt / Body wt (mg/gm) (X10^{-3})	3.50±0.14	6.02±0.30$^{\#}$	3.66±0.27$^{*,\$}$	6.21±0.39	4.70±0.29$^{*,\$}$	3.83±0.24$^{*,\$}$	3.57±0.16
OVA challenge							
Rubbing (number)	14.17±2.06	73.50±1.52$^{\#}$	24.67±0.88$^{*,\$}$	64.67±1.43	46.67±2.54$^{*,\$}$	29.00±0.86$^{*,\$}$	17.17±1.60
Sneezing (number)	10.83±1.38	39.50±1.34$^{\#}$	14.67±1.33$^{*,\$}$	34.67±1.38	22.50±0.56$^{*,\$}$	20.67±1.05$^{*,\$}$	13.50±1.23
Discharge (score)	0.33±0.21	2.67±0.21$^{\#}$	0.67±0.21$^{*,\$}$	2.33±0.21	1.83±0.31$^{*,\$}$	0.50±0.22$^{*,\$}$	0.17±0.17
Histamine challenge							
Rubbing (number)	17.00±2.93	72.67±2.38$^{\#}$	24.50±3.25$^{*,\$}$	63.00±2.68	45.00±2.35$^{*,\$}$	34.83±3.59$^{*,\$}$	19.67±2.20
Sneezing (number)	11.83±1.96	52.33±2.60$^{\#}$	15.33±2.67$^{*,\$}$	49.17±2.65	30.83±2.57$^{*,\$}$	23.00±1.93$^{*,\$}$	16.17±2.04

Data were represented as Mean±SEM. Data for body weight and relative spleen weight were analyzed by one-way ANOVA followed by Tukey's multiple range test whereas data of OVA and histamine challenge number and score were analyzed by non-parametric Kruskal-Wallis test ANOVA followed by Mann-Whitney's multiple comparison tests. *$p < 0.05$ as compared with AR control group and $p < 0.05 as compared with each other. Figures in parentheses indicate an oral dose in mg/kg.

as compared to chrysin treatment. *Per se* treated mice did not show any significant alterations in nasal rubbing and sneezing after intranasal histamine administration as compared to normal mice [Table 1].

Serum histamine, ovalbumin-specific immunoglobulin E, total immunoglobulin E, and β-hexosaminidase levels

There was a statistically significant ($P < 0.05$) increase in serum histamine, β-hexosaminidase, and IgE (OVA specific and total) levels in AR control mice as compared to normal mice. Administration of montelukast (10 mg/kg) showed statistically significant attenuation ($P < 0.05$) in OVA-induced elevated serum histamine, β-hexosaminidase, and IgE (OVA specific and total) levels in serum as compared to AR control mice. These levels were also statistically significantly ($P < 0.05$) decreased by chrysin (20 and 40 mg/kg) treatment as compared to AR control mice. Moreover, montelukast treatment more significantly ($P < 0.05$) attenuated OVA-induced elevated histamine, β-hexosaminidase, and IgE (OVA specific and total) levels in serum when compared with chrysin treatment [Table 2].

Differential and total cell count

The differential cell (neutrophils, eosinophils, lymphocytes, and macrophages) and total counts statistically significantly ($P < 0.05$) increased after intranasal challenge with OVA in AR control mice as compared to normal mice. Treatment with montelukast (10 mg/kg) statistically significantly decreased ($P < 0.05$) these elevated levels of differential and total cell counts as compared to AR control mice. Treatment with chrysin (20 and 40 mg/kg) also statistically significantly ($P < 0.05$) attenuated OVA-induced elevated total and differential cell counts as compared to AR control mice. However, these elevated total and differential cell count was more statistically significantly ($P < 0.05$) attenuated by montelukast treatment as compared to chrysin treatment. There were no significant alterations in these cell count in *per se* treated mice when compared with normal mice [Table 3].

Splenic oxido-nitrosative stress and myeloperoxidase levels

The levels of splenic MDA, NO, and myeloperoxidase (MPO) were statistically significantly ($P < 0.05$) increased, whereas splenic GSH and

Table 2: Effect of chrysin treatment on OVA-induced alterations in serum histamine, OVA-specific IgE, total IgE, and β-hexosaminidase levels in AR mice

Parameters	Treatment						
	Normal	AR Control	MLT (10)	C (10)	C (20)	C (40)	Per se
Histamine (μg/ml)	61.59±9.19	365.00±9.56$^{\#}$	93.50±10.77$^{*,\$}$	337.70±8.44	246.50±10.45$^{*,\$}$	138.70±8.33$^{*,\$}$	78.31±8.63
OVA-specific IgE (ng/ml)	12.66±1.59	63.63±1.34$^{\#}$	21.14±2.81$^{*,\$}$	59.79±1.41	41.66±1.48$^{*,\$}$	25.23±1.85$^{*,\$}$	17.31±1.37
Total IgE (ng/ml)	90.33±15.71	436.70±14.56$^{\#}$	152.40±14.23$^{*,\$}$	419.00±10.72	321.80±11.44$^{*,\$}$	185.20±14.7$^{*,\$}$	115.40±13.36
β-hexosaminidase (ng/ml)	15.76±1.74	46.28±1.40$^{\#}$	18.26±2.04$^{*,\$}$	43.12±2.28	35.24±2.14$^{*,\$}$	27.61±2.89$^{*,\$}$	19.26±2.26

Data were represented as Mean±SEM and analysed by one-way ANOVA followed by Tukey's multiple range test. *$p < 0.05$ as compared with AR control group and \$$p < 0.05$ as compared with each other. Figures in parentheses indicate oral dose in mg/kg.

Table 3: Effect of chrysin treatment on OVA-induced alterations in total and differential cell count in Nasal Lavage Fluid in AR mice

Parameters	Treatment						
	Normal	AR Control	MLT (10)	C (10)	C (20)	C (40)	Per se
Eosinophils (X 10^4/ml)	4.33±0.49	48.17±1.58$^{\#}$	17.50±0.92$^{*,\$}$	42.00±1.51	29.67±1.17$^{*,\$}$	22.00±1.63$^{*,\$}$	4.83±0.95
Neutrophils (X 10^4/ml)	2.33±0.61	7.67±0.61$^{\#}$	2.83±0.48$^{*,\$}$	6.83±0.60	5.17±0.54$^{*,\$}$	4.33±0.49$^{*,\$}$	2.00±0.52
Lymphocytes (X 10^4/ml)	0.83±0.31	2.67±0.21$^{\#}$	1.50±0.22$^{*,\$}$	2.50±0.22	2.00±0.37$^{*,\$}$	1.33±0.21$^{*,\$}$	0.67±0.33
Macrophages (X 10^4/ml)	27.17±2.90	71.83±2.74$^{\#}$	39.00±2.13$^{*,\$}$	65.83±3.83	48.00±1.86$^{*,\$}$	39.83±2.20$^{*,\$}$	28.33±2.74
Total cells (X 10^4/ml)	34.67±2.79	130.30±3.53$^{\#}$	60.83±2.17$^{*,\$}$	117.20±4.51	84.83±1.82$^{*,\$}$	67.50±2.85$^{*,\$}$	35.83±2.81

Data were represented as Mean±SEM and analysed by one-way ANOVA followed by Tukey's multiple range test. *$P < 0.05$ as compared with AR control group and \$$p < 0.05$ as compared with each other. Figures in parentheses indicate oral dose in mg/kg.

Table 4: Effect of chrysin treatment on OVA-induced alterations in splenic oxido-nitrosative stress and MPO levels in AR mice

Parameters	Treatment						
	Normal	AR Control	MLT (10)	C (10)	C (20)	C (40)	Per se
SOD (U/mg of protein)	1.37±0.11	0.53±0.11$^{\#}$	1.11±0.14$^{*,\$}$	0.69±0.11	0.91±0.07$^{*,\$}$	1.16±0.14$^{*,\$}$	1.32±0.12
GSH (μg/mg of protein)	0.63±0.05	0.15±0.05$^{\#}$	0.44±0.04$^{*,\$}$	0.20±0.04	0.28±0.05$^{*,\$}$	0.28±0.05$^{*,\$}$	0.51±0.04
MDA (nM/ mg of protein)	2.05±0.24	5.36±0.3$^{\#}$	3.13±0.28$^{*,\$}$	5.10±0.29	3.98±0.33$^{*,\$}$	3.38±0.25$^{*,\$}$	2.44±0.27
NO (μg/mL)	4.26±0.39	8.43±0.15$^{\#}$	4.87±0.24$^{*,\$}$	8.04±0.34	6.59±0.25$^{*,\$}$	5.29±0.39$^{*,\$}$	4.66±0.30
MPO (nM/mg of protein)	0.24±0.03	0.49±0.03$^{\#}$	0.32±0.03$^{*,\$}$	0.45±0.03	0.38±0.02$^{*,\$}$	0.34±0.02$^{*,\$}$	0.24±0.01

Data were represented as Mean±SEM and analyzed by one-way ANOVA followed by Tukey's multiple range test. *$P < 0.05$ as compared with AR control group and \$$p < 0.05$ as compared with each other. Figures in parentheses indicate oral dose in mg/kg.

SOD levels were statistically significantly ($P < 0.05$) decreased in AR control mice as compared to normal mice. Montelukast (10 mg/kg) and chrysin (20 and 40 mg/kg) significantly attenuated ($P < 0.05$) altered splenic GSH, SOD, MDA, NO, and MPO levels as compared to AR control mice. However, when compared with chrysin treatment, montelukast treatment showed more significant ($P < 0.05$) inhibition in splenic oxido-nitrosative stress and MPO. Whereas, splenic GSH, SOD, MDA, NO, and MPO levels did not alter significantly in *per se* treated mice as compared to normal mice [Table 4].

Nasal lavage fluid tumor necrosis factor-alpha, interleukins, and interferon-gamma levels

The TNF-α, IL-1β, IL-4, IL-6, and IL-13 levels showed a statistically significant ($P < 0.05$) increase, whereas IFN-γ level showed a significant ($P < 0.05$) decrease in NLF of AR control mice when compared with normal mice. Montelukast (10 mg/kg) treatment significantly ($P < 0.05$) decreased the NLFs TNF-α and ILs levels as well as significantly ($P < 0.05$) increased NLFs IFN-γ level as compared to AR control mice. In addition, chrysin at a dose of 20 and 40 mg/kg also significantly ($P < 0.05$)

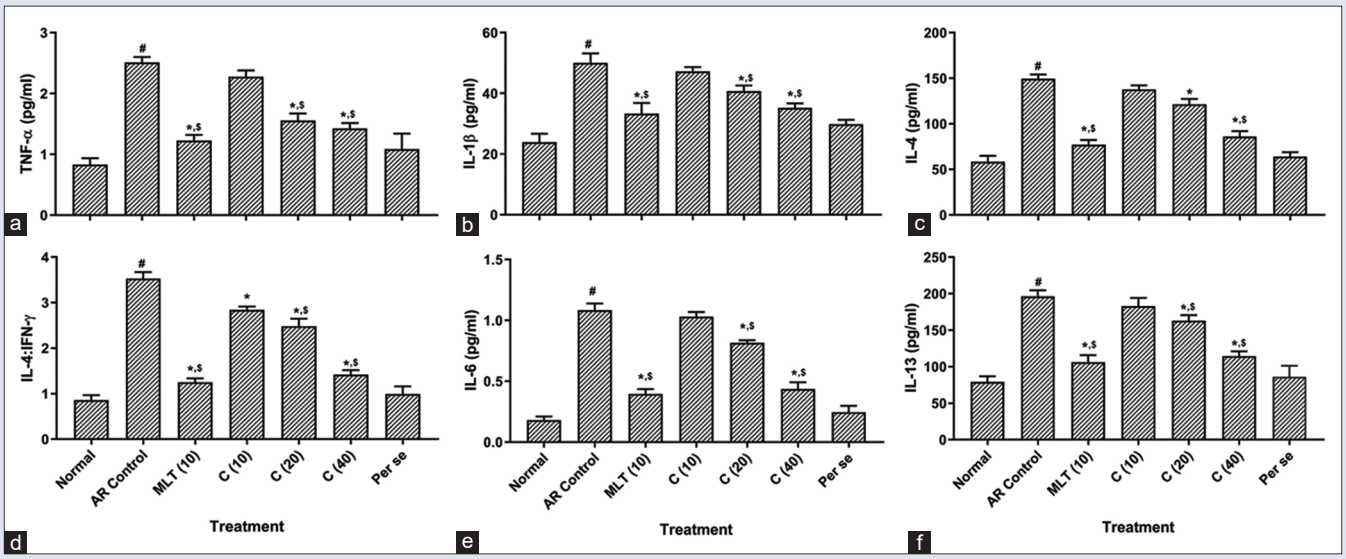

Figure 1: Effect of chrysin treatment on ovalbumin-induced alterations in tumor necrosis factor-alpha (a), interleukin-1β (b), interleukin-4 (c), interleukin-4:interferon-gamma ratio (d), interleukin-6 (e), and interleukin-13 (f) levels nasal lavage fluid in allergic rhinitis mice. Data were represented as mean ± standard error mean and analyzed by one-way analysis of variance followed by Tukey's multiple range test. $^{\#}P < 0.05$ as compared with normal group, $^{*}P < 0.05$ as compared with allergic rhinitis control group, and $^{\$}P < 0.05$ as compared with each other

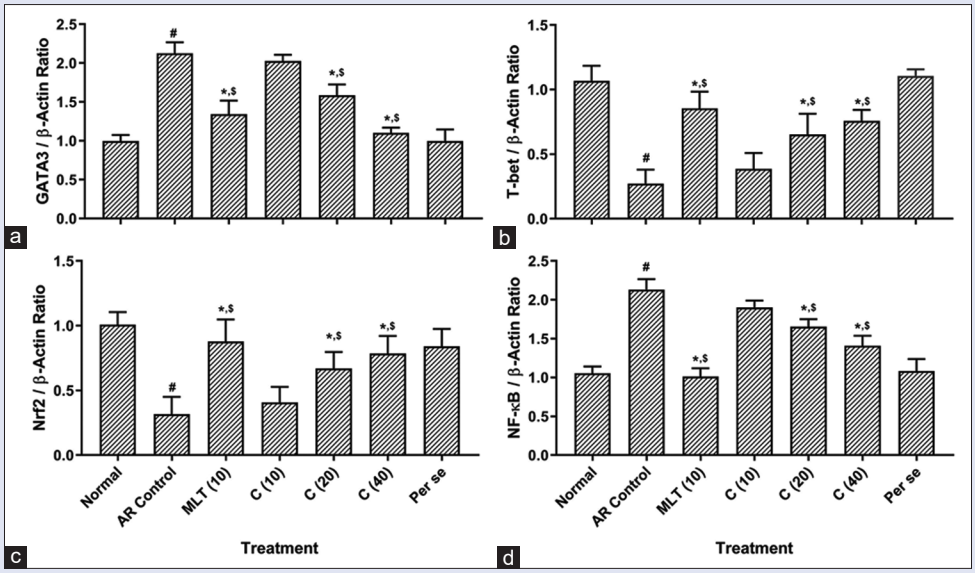

Figure 2: Effect of chrysin treatment on ovalbumin-induced alterations in spleen GATA-3 (a), T-box protein expressed in T cells (b), nuclear factor erythroid 2-related factor 2 (c), and nuclear factor-kappa B (d) mRNA expression in allergic rhinitis mice. Data were represented as mean ± standard error mean and analyzed by one-way analysis of variance followed by Tukey's multiple range test. $^{\#}P < 0.05$ as compared with normal group, $^{*}P < 0.05$ as compared with allergic rhinitis control group, and $^{\$}P < 0.05$ as compared with each other

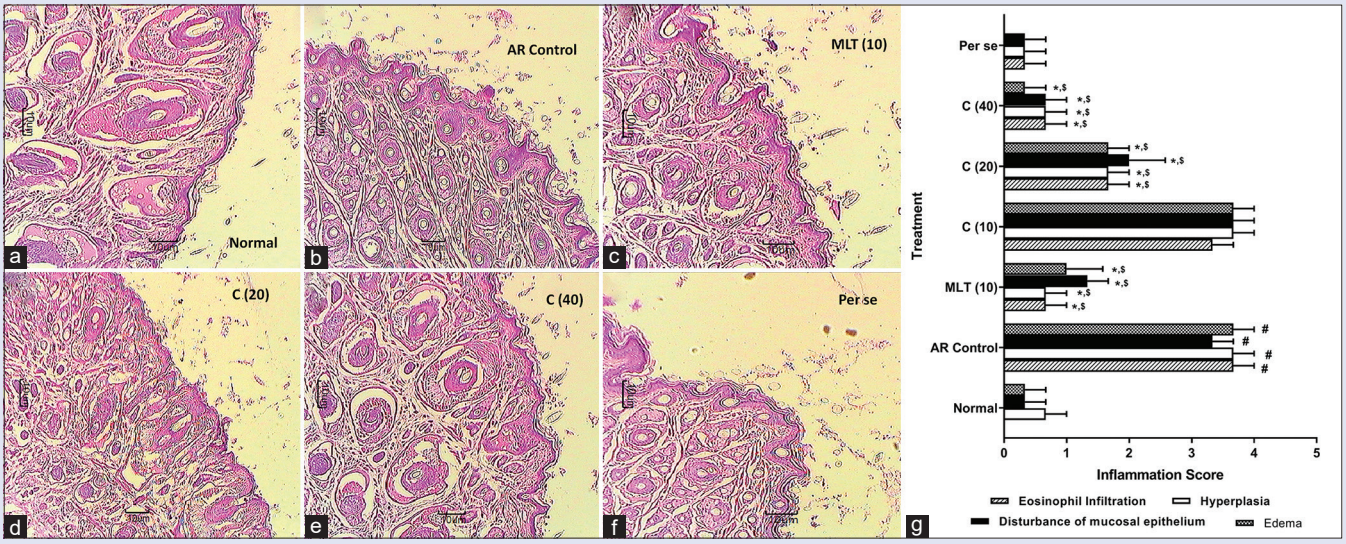

Figure 3: Effect of chrysin treatment on ovalbumin-induced alteration in nasal histopathology in allergic rhinitis mice. Photomicrograph of sections of nasal tissue from normal (a), allergic rhinitis control (b), montelukast (10 mg/kg)-treated (c), chrysin (10 mg/kg)-treated (d), chrysin (10 mg/kg)-treated (e), and chrysin (20 mg/kg)-treated (f) mice (H and E stain). Data were expressed as mean ± standard error of mean and one-way analysis of variance followed by the Kruskal–Wallis test applied for *post hoc* analysis. $^{\#}P < 0.05$ as compared with normal group, $^{*}P < 0.05$ as compared with allergic rhinitis control group, and $^{\$}P < 0.05$ as compared with each other

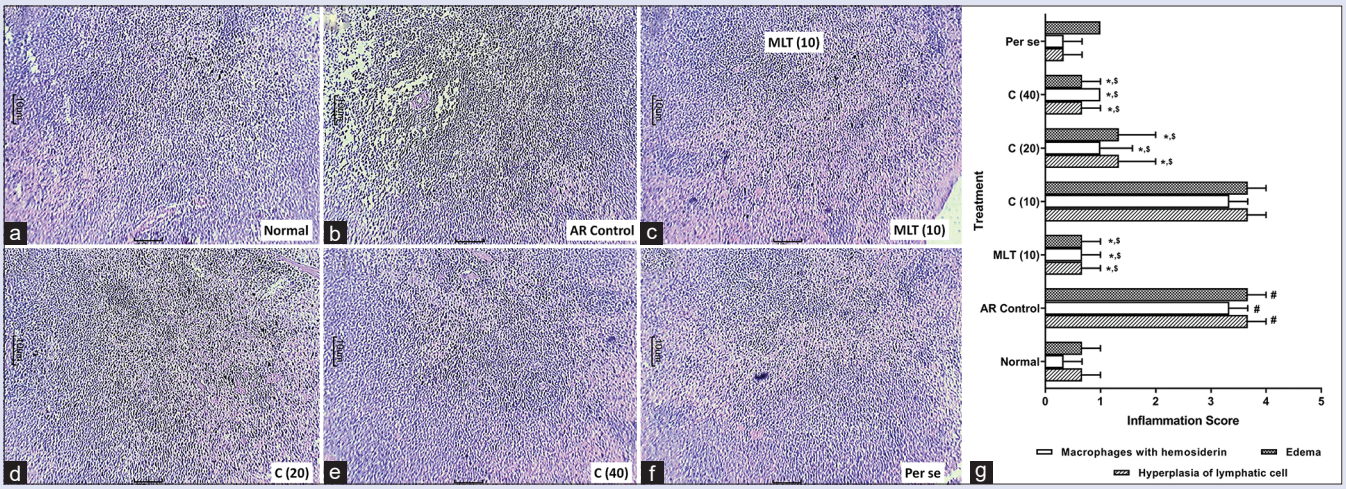

Figure 4: Effect of chrysin treatment on ovalbumin-induced alteration in spleen histopathology in allergic rhinitis mice. Photomicrograph of sections of spleen tissue from normal (a), allergic rhinitis control (b), montelukast (10 mg/kg)-treated (c), chrysin (10 mg/kg)-treated (d), chrysin (10 mg/kg)-treated (e), and chrysin (20 mg/kg)-treated (f) mice. (H and E stain). The quantitative representation of histological score (g). Data were expressed as mean ± standard error of mean and one-way analysis of variance followed by the Kruskal–Wallis test applied for *post hoc* analysis. $^{\#}P < 0.05$ as compared with normal group, $^{*}P < 0.05$ as compared with allergic rhinitis control group, and $^{\$}P < 0.05$ as compared with each other

attenuated OVA-induced alterations in TNF-α, ILs, and IFN-γ levels in NLF as compared to AR control mice. However, when compared with chrysin treatment, montelukast treatment more significantly ($P < 0.05$) attenuated OVA-induced alterations in TNF-α, ILs, and IFN-γ levels in NLF. However, there was no significant change in these levels of Th1 and Th2 cytokines in *per se* treated mice as compared to normal mice [Figure 1].

Splenic GATA-3, T-box protein expressed in T cells, nuclear factor erythroid 2-related factor 2, and nuclear factor-kappa B mRNA expressions

The mRNA expressions of GATA-3 and NF-κB in spleen were significantly ($P < 0.05$) upregulated, whereas splenic T-bet and Nrf2 mRNA expressions were significantly downregulated ($P < 0.05$) in AR control mice when compared with normal mice. Administration of montelukast (10 mg/kg) and chrysin (20 and 40 mg/kg) significantly attenuated ($P < 0.05$) OVA-induced alterations in splenic GATA-3, T-bet, Nrf2, and NF-κB mRNA expressions as compared to AR control mice. However, when compared with montelukast, chrysin treatment showed more significant ($P < 0.05$) downregulation in splenic GATA-3 mRNA expression. Whereas, *per se* treated mice did not show any significant alterations in splenic GATA-3, T-bet, Nrf2, and NF-κB mRNA expressions as compared to normal mice [Figure 2].

Histopathology of the nasal mucosa

Intranasal administration of OVA resulted in significant ($P < 0.05$) alterations in the histological architecture of nasal mucosa in AR

control mice reflected by increased eosinophil infiltration, edema, and disturbances in the mucosal epithelium [Figure 3b] as compared to normal mice. Nasal mucosa from normal mice showed mild inflammatory infiltration, hyperplasia, and disturbances in mucosal epithelium [Figure 3a]. Montelukast (10 mg/kg) significantly inhibited ($P < 0.05$) OVA-induced alterations in nasal mucosal membrane when compared with AR control mice [Figure 3c]. Additionally, chrysin at doses of 20 and 40 mg/kg also significantly ($P < 0.05$) decreased eosinophil infiltration and edema, thus reducing disturbances in mucosal epithelium [Figure 3d and e] as compared to AR control mice. Figure 3f depicts the normal histological architecture of nasal mucosa from *per se* treated mice. [Figure 3g].

Histopathology of spleen

Figure 4a represents the normal histological structure of spleen tissue from normal mice with minimal hemosiderin macrophages and hyperplasia of lymphatic cells. However, spleen tissue from AR control mice showed histological aberrations reflected by statistically significantly ($P < 0.05$) increased hemosiderin macrophages, edema, and hyperplasia of lymphatic cells [Figure 4b] as compared to normal mice. Treatment with montelukast (10 mg/kg) resulted in significant attenuation ($P < 0.05$) of histological aberrations induced in spleen tissue [Figure 4c] after OVA challenge as compared to AR control mice. Whereas, chrysin (20 and 40 mg/kg) treatment also statistically significantly ($P < 0.05$) inhibited OVA-induced elevated hemosiderin macrophages, edema, and hyperplasia of lymphatic cells [Figure 4d and e] as compared to AR control mice. There was no significant histological alteration induced in spleen tissue of *per se* treated mice as compared to normal mice [Figure 4f and g].

DISCUSSION

AR is a chronic immune-inflammatory disease affecting about 20% of the total population worldwide.[2,3] Allergen-induced IgE-mediated allergic response is a characteristic feature of AR. Such typical characteristic allergic response can be mimicked in the OVA-induced murine model of AR where it downstreams immunologic responses via modulation of Th1/Th2 cytokine profile.[2,3] In the present study, we have determined the anti-allergic potential as well as the possible mechanism of action of chrysin against OVA-induced AR in experimental mice. The results of the present study showed that chrysin exhibited anti-allergic effect via balancing Th1/Th2 response, thus inhibiting rhinitis symptoms including sneezing and rubbing in OVA-induced AR mice. This anti-allergic activity of chrysin may be attributed to its inhibitory potential against various inflammatory mediators (histamine, IgE, and β-hexosaminidase, TNF-α, IL-1β, and NF-κB), Th2 cytokines (ILs), GATA-3/T-bet pathways, and oxido-nitrosative stress (Nrf2).

It has been well established that the stimulation of nasal mucosa by an allergen (OVA) follows biphasic response.[2,3,28] In the initial-phase response, activation of mast cells via allergen-IgE reaction releases various inflammatory mediators such as β-hexosaminidase, histamine, prostaglandins, and leukotrienes.[6] This inflammatory influx resulted in elevated nasal symptoms such as nasal discharge, itching, sneezing, and rubbing. Whereas, in the late-phase response after 4–6 h, the accumulation of various inflammatory mediators, eosinophils, mast cells, and basophils resulted in nasal mucosa-aggravated allergic response.[28,29] Furthermore, studies have reported that eosinophil is an essential element in allergic response through modulation of mucosal epithelial barrier.[7,29] Clinically, it has been reported that presence of eosinophil in the inner lining of nasal mucosa plays a vital role in the development and maintenance of allergic response.[29] Additionally, neutrophil contains vicious products, including cytokines, reactive oxygen species (ROS), and NO, which contribute to the induction of allergic response.[29] Recruitment of leukocyte in the NLF is thought to play a vital role in the injury to nasal epithelium via elevated levels of ROS.[29] In the present study, intranasal OVA challenge resulted in elevated response of inflammatory mediators including histamine and β-hexosaminidase followed by inflammatory influx, including eosinophil, neutrophil, and macrophages in nasal mucosa. However, administration of chrysin significantly attenuated elevated levels of histamine and β-hexosaminidase, which might contribute to its anti-allergic potential.

Numerous evidence suggest that T lymphocyte plays a vital role in the recognition and uptake of antigen via antigen-presenting cells including dendritic cells, mast cell, macrophages, and B cells to convert and digest the antigen into short peptides to stimulate naive T cells.[20,28,30] This leads to cross-linking of IgE-Fcε (immunoglobulin Fc epsilon receptor) complexes, which brings about release of histamine and chemokines such as regulated on activation, normal T cell expressed and secreted, eotaxin (CCL11), thymus, and activation-regulated chemokine (CCL17) and macrophage-derived chemokine (CCL22) via mast cell degranulation and basophils.[4,31] It also caused an elevation of IL-4 concentration in NLF, resulting in inflammatory infiltration in nasal mucosa.[7] Along with inflammatory cells, histamine caused stimulation of H_1 receptor on trigeminal nerve endings, leading to induction of nasal symptoms.[20] In the present investigation, OVA administration followed by its challenge led to the induction of nasal discharge, sneezing, and rubbing. The results of the present investigation are in line with the findings of Sakairi *et al*.[31] Hence, to relieve these symptoms, antihistaminic drugs such as chlorpheniramine, fexofenadine, and cetirizine are widely used.[32] It has been reported that along with histamine, mast cells released mediators such as prostaglandins, leukotrienes, and tryptase, which play an equal role in nasal rubbing and sneezing. Hence, the effectiveness of chrysin in sneezing, rubbing, and nasal discharge might be mediated through the inhibition of release of histamine and stabilization of mast cell. A previous investigator also reported the inhibitory potential of chrysin against mast cells via downregulation of IgE level, and findings of the current study are in accordance with the results of the previous researcher.[14]

Recently, it has been reported that elevated level of ROS is also associated with mitochondrial dysfunction, which occurs through induction of oxidative damage in nasal mucosa.[33] A researcher has reported that various toxicants, including OVA, induce oxidative insult in the nasal mucosa via downregulation of total oxidant status and oxidative defense mechanism.[33,34] SOD and GSH are well-documented vital antioxidant enzymes, and the elevated generation of ROS plays a decisive role in diminishing their efficacy.[35-37] ROS combines with NO to produce peroxynitrite ($ONOO^-$), thus promoting lipid oxidation and protein nitration.[38,39] Elevated lipid oxidation (i.e., MDA) interferes with mitochondrial respiratory chain and thus reduces energy production.[40-42] Furthermore, Nrf2, which is a redox-sensing transcription factor, has been suggested to play a vital role in maintaining oxidant/antioxidant balance.[43] Under normal conditions, binding of Keap1 (Kelch-like ECH-associated protein 1) to Nrf2 brings out the degradation of Nrf2 in a ubiquitin-dependent manner, resulting in decrease in its expression. Thus, inhibition of Nrf2 activity contributes to the induction of allergic responses.[34] Administration of OVA showed a significant elevation in the oxido-nitrosative stress revealed by elevated MDA and NO activities with a decrease in GSH, SOD, and Nrf2 activities. However, researchers have shown that flavonoid exhibits free-radical scavenging potential due to the presence of hydroxyl groups in the aromatic ring structures.[44] In the present investigation also, treatment with chrysin may have inhibited generation of free radicals that could be attributed to the presence of

a hydroxyl group in its molecular structure. These, in turn, resulted in an increased level of GSH, SOD, and Nrf2 as well as decreased lipid peroxidation and NO, which ameliorates the OVA-induced AR. Previous researchers also showed the protective effect of chrysin via downregulation of oxidative stress, and result of the present study corroborates with those of previous researchers.[15,16]

It has been well documented that insult into nasal mucosa, triggered by chemical irritants such as OVA, house dust mites, pollen, or molds, is responsible for the release of various inflammatory cells including eosinophils, lymphocytes, macrophages, Th cells, and mast cells.[3,45] These inflammatory infiltrations play important role in allergic response where recruitment and activation of polymorphonuclear neutrophils by the chemokines are implicated in the remodeling of nasal mucosa.[45] It has been reported that neutrophils contain abundant amount of MPO, which interacts with H_2O_2 to form hypochlorous acid that induces cellular toxicity.[43] Furthermore, macrophages and neutrophils have an ability to induce the production of pro-inflammatory and Th2 cytokines that stimulate allergic response.[6,20] Thus, an inflammatory influx in nasal epithelial areas contributes to a range of structural alterations, including loss of mucosal epithelial integrity, hyperplasia, smooth muscle cell hypertrophy, and thickening of basement membrane.[13,46] In agreement with previous reports, OVA mediates nasal injury via inflammatory response reflected by increased levels of MPO and inflammatory influx (eosinophils, lymphocytes, and macrophages) in OVA-induced mice.[13,46] However, administration of chrysin significantly inhibited inflammatory influx and MPO levels, depicting its anti-inflammatory potential. The histological examination supported this finding where nasal and spleen tissue from chrysin-treated animals showed a reduction in the level of inflammatory cells.

TNF-α is recognized as one of the earliest pro-inflammatory endogenous mediators involved in various inflammatory processes, including proliferation, maturation, and migration of inflammatory cells.[47] It is associated with an increase in the levels of chemokines, matrix metalloproteases, and other pro-inflammatory cytokines (ILs), prostaglandin E2, as well as apoptotic factors.[48] It is also responsible for the proliferation and the differentiation of T cells as well as increasing nasal epithelial permeability by disrupting tight junctions, further accelerating inflammation in the nasal mucosa.[3,49] IL-1β and IL-6 are other pro-inflammatory cytokines closely associated with inflammatory response during the pathogenesis of AR.[50] IL-6 plays an important role in modulating T-cell function, proliferation, survival, and development of pathogenic Th2 response.[10] In addition, IL-13 is also responsible for the activation of NO expression in nasal epithelial cells.[49,51] Furthermore, IFN-γ has the ability to activate IL-1β production, which subsequently induces Th1-type immune-inflammatory response by attracting phagocytic cells at the site of inflammation.[31,50] Immune homeostasis is maintained by IFN-γ, and its depleted levels signal the onset of immunoinflammation. Researchers had previously reported elevated levels of TNF-α, IL-1β, and IL-6 in patients with AR.[52] In addition, elevated levels of cytokines (such as TNF-α and ILs) stimulated inflammatory cells to express inducible NO synthase, playing a vital role in the induction of nitrosative stress.[43] Notably, NO has been shown to inhibit the release of IFN-γ, resulting in the induction of Th2 response in macrophages.[53] These results are in accordance with those of previous literature where administration of OVA induces activation of NF-κB, which further stimulates inflammatory response via the release of TNF-α and ILs in experimental AR.[13,46] Administration of chrysin significantly ameliorates elevated pro-inflammatory release (TNF-α and ILs) by inhibiting NF-κB, and these results are in line with the findings of previous investigators.[14,15,18]

Researchers have well established the important role of GATA-3 in the inhibition of Th1 activation and stimulation of Th2-mediated allergic responses.[13,15,30] GATA-3 is a transcription factor belonging to the GATA family, and its elevated expression demonstrated the induction of STAT6 (signal transducer and activator of transcription 6)-dependent Th2 differentiation, whereas suppression of GATA-3 expression represents Th1 cells' differentiation.[13,15,30] Furthermore, T-bet is another transcription factor which is explicitly expressed in Th1 cells and modulates the expression of Th1 cytokines including IFN-γ.[30] During normal physiological conditions, an equilibrium exists between Th1 and Th2 cells, and these crossregulate each other's functions, thus maintaining IFN-γ/IL-4 cytokine balance.[30,49] However, IgE-mediated activation of GATA-3 results in the differentiation of Th cells and thus serves a critical role in the pathogenesis of allergic response. The findings of the present study also revealed that intranasal challenge with OVA induces upregulation of GATA-3 expression in spleen tissue followed by downregulation of T-bet expression in AR control mice. Notably, administration of chrysin downregulated GATA-3 expression and upregulated T-bet expression. This notion is further supported by reduction in the OVA-induced elevated IFN-γ/IL-4 ratio by administration of chrysin. Previous investigators also demonstrated that chrysin attenuated allergic response via direct improvement in the IFN-γ/IL-4 ratio via inhibition of Th2-specific transcription factor GATA-3.[15,18] Findings of the present study are in accordance with these previous investigators, suggesting immunomodulatory potential of chrysin via regulation of Th1/Th2 differentiation in AR.

Currently, montelukast, a CysLT1 receptor antagonist, is an approved therapeutic moiety for the management of AR,[54,55] and in the present investigation, we have implicated it as a positive control. An array of researchers have documented its efficacy against the symptoms of AR, including sneezing, congestion, pruritus, and rhinorrhea.[54-56] It is also effective in the management of congestion-induced sleep difficulty and awakening during night time. However, a recent US Food and Drug Administration report, as well as postmarketing surveillance data, suggested the association of chronic use of montelukast with several psychiatric adverse events, including aggression, hallucination, anxiousness, depression, restlessness, and insomnia.[8] Thus, use of a therapeutic moiety of herbal origin has been suggested as a safe and effective alternative for the management of AR. Indeed, previous reports justify the beneficial effects of various flavonoids from plant origin against AR.[57] Thus, chrysin might be a useful candidate of plant origin against AR clinically.

CONCLUSION

Findings from the present study suggest that chrysin exhibits anti-allergic potential via balancing Th1/Th2 response. Chrysin modulates GATA-3/T-bet pathways and inhibits NF-κB activation, thus attenuating the release of various inflammatory mediators (histamine, IgE, β-hexosaminidase, TNF-α, and IL-1β), Th2 cytokines (IL-4, IL-6, and IL-13), and oxido-nitrosative stress (Nrf2) to exert its anti-allergic potential in murine model of OVA-induced AR.

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Conflicts of interest

There are no conflicts of interest.

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