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ORIGINAL ARTICLE

ANTI-ALLERGIC POTENTIAL OF FISETIN IN A MURINE MODEL OF OVA-INDUCED ALLERGIC RHINITIS VIA INHIBITION OF GATA-3 AND TH2 CYTOKINES

ZHAO L.,[1] KANDHARE A.,[2] MUKHERJEE A.[2] AND SUBHASH BODHANKAR S.[2]
[1]*Department of Otolaryngology, Head and Neck Surgery, The First Affiliated Hospital of Xi'an Medical University Feng Hao West Road, Lianhu District, Xi'an, Shaanxi-710077, China*
[2]*Department of Pharmacology, Poona College of Pharmacy, Bharati Vidyapeeth Deemed University Erandwane, Paud Road, Pune-411038, India*

ABSTRACT

Background and Objectives: Allergic rhinitis (AR) is an IgE mediated immune-inflammatory disease, characterized by sneezing, rhinorrhea, lacrimation, etc. Fisetin is a plant flavonoid, reported to have anti-allergic potential via inhibition of Th2 response. Objective of this study was to evaluate the anti-allergic potential of fisetin against ovalbumin (OVA)-induced experimental allergic rhinitis in BALB/c mice.

Methodology: Mice were sensitization by OVA (500 µl, i.p.) on days 1, 3, 5, 7, 9, 11, 13 and treated with fisetin (10, 20 and 40 mg/kg, p.o.) for 21 days, followed by OVA (5 µl per nostril) challenged on the 21st day.

Results: Administration of fisetin (20 and 40 mg/kg) significantly attenuated (p< 0.05) OVA-induced nasal rubbing, sneezing and discharge, histamine-induced rubbing and sneezing, and hematological parameters as compared to AR control mice. OVA-induced elevated levels of serum histamine, β-hexosaminidase, IgE and IgG1 as well as Th2 cytokines (IL-4, IL-5, IL-13, and IL-17) in nasal lavage fluid was significantly (p < 0.05) attenuated by fisetin. It also significantly inhibit (p < 0.05) up-regulated mRNA expressions of GATA3, IL-4, IL-5 and IL-13 in spleen tissue. Fisetin administration significantly reduced (p < 0.05) histological aberrations induced by OVA in nasal mucosa, spleen, and lungs.

Conclusion: The findings of the present study showed that fisetin exerts its anti-allergic potential via modulation of GATA3 pathway to inhibit the release of Th2 cytokines (IL-4, IL-5, IL-13) and IgE, thus reducing OVA-induced nasal rubbing and sneezing during allergic rhinitis.

Keywords: Allergic rhinitis, Fisetin, GATA3, lgE, Interleukins, Ovalbumin, TNF-α.

INTRODUCTION

Allergic rhinitis (AR) is a chronic immune-inflammatory disorder which is increasing, significantly, worldwide resulting in important social and medical problems.[1] Rhinitis is associated with widespread morbidity, significant treatment costs, impaired work productivity and quality of life. The characteristic features of AR include nasal congestion, sneezing, rhinorrhea, and pruritus of nosea long with eyes. Additionally, it is also associated with various complications including headache, fatigue, sinusitis, sleep disturbance, eustachian tube dysfunction, and cognitive impairment.[2] According to a report by European Community Respiratory Health Survey (ECRHS), the prevalence of AR is 5-22%. While, more than 35% of the European and Australian population suffer from AR, about 26% of Indians are affected with it.[3]

Researchers have well documented that exposure to an array of mediators such as indoor allergens (such as dust mites and stuffed furniture), outdoor allergens (like molds and pollen of grains, grass, trees, weeds, etc.), chemical irritants, tobacco smoke, air pollution, and food cause hypersensitive response which results in Allergic rhinitis.[4]Cumulative data obtained from animal studies have suggested that imbalance in T-helper type 1 (Th1) and T-helper type 2 (Th2) responses cause inflammation and remodelling in nasal mucosa that result in the progress of AR.[5,6] Th2 responses are up-regulated due to elevated production of cytokines including tumour necrosis factor-alpha (TNF-α), interleukins (IL's) (IL-1β, IL-4, IL-5 and IL-6), release of nitric oxide from macrophages, production of reactive oxygen species (ROS), immunoglobulin (IgE), and mast cell.[5,6] These vicious molecules are responsible for synthesis of IgE and its cross-linking with high-affinity IgE receptors (FcεRI) present on mast cell sur-

Corresponding Author: Dr. S. L. Bodhankar
Dept. of Pharmacology, Poona College of Pharmacy, Bharati Vidyapeeth Deemed University, Erandwane, Pune-411038, Maharashtra, India. Email: drslbodh@gmail.com

face. It leads to mast cell degranulation and release of various inflammatory mediators including histamine, cytokines, and leukotrienes.[7,8] The inflammatory influx in the nasal mucosa results in structural aberrations that contribute to congestion, post nasal drip, concurrent sneezing, and nasal discharge leading to development of AR.[9]

Current treatment of AR includes antihistamines, cromolyn sodium, nasal decongestants, glucocorticoids that provide symptomatic relief. However, these medications are associated with side effects such as nausea, vomiting, headache, hypertension, immune system suppression and growth retardation.[1,10] Hence, there is an urgent need to develop strategies for the management of AR using safer and more effective treatment methods of natural origin. Numerous evidences suggest that natural compounds also provide promising effects for asthma treatment.[11,12]

Many herbal medicines have been traditionally known for either their symptomatic relief property or halting disease progression in the treatment of AR. However, the extent of their effectivity and presence of active components lack systematic study and hence scientific evidence. Thus, various animal models including pollen extract, house mite allergen, ovalbumin (OVA), and bee venom have been employed for the development of various therapeutic moieties against AR.[13] Amongst all, murine model of OVA-induced AR is well established, widely used and a reproducible immunological tool that mimics clinicopathological features of AR.[13,14] OVA-induced AR exhibits various characteristics including congestion, redness, sneezing, post-nasal drip, etc. which are the symptomatic hallmarks found in an AR patient.[15] The inflammatory responses include release of lymphocytes, eosinophils, neutrophils, and mast cell with elevated mucus secretion into nasal mucosa. It subsequently results in elevated Th2 response along with an increased level of IL-4, IL-5, IL-13, and IgE that eventually cause the development of persistent nasal mucosal inflammation, remodelling, and AR.

Fisetin (3, 3', 4', 7-tetrahydroxyflavone, **Supplementary Figure 1**) is a naturally occurring plant flavonoid which is widely present in apples, grape seeds, onions, strawberries, cucumbers, and persimmons. Fisetin has been documented to have antioxidant, anticarcinogenic, antiulcer, anticonvulsant, anti-hyperlipidemic, antiarthritic, antidiabetic, antimicrobial, antiviral, cardioprotective, and neuroprotective properties.[16-22] Recently, researchers have also reported the antiasthmatic potential of fisetin against OVA-induced airway hyperresponsiveness via inhibition of lgG, lgE, iNOS, TNF-α, IL-4, IL-5, IL-13, and NF-kB.[19,23] As immune-inflammatory reactions like AR and asthma are two closely related disorders of airway disease, almost 88% of asthma patients have suffered from the symptoms of AR as well.[24] Moreover, the animal models of both the allergic inflammation diseases are produced similarly.[24] But, although the anti-asthmatic potential of fisetin has been well reported, its effect against allergic rhinitis is not yet evaluated. Hence, the aim of the present investigation was to evaluate the anti-allergic potential of fisetin against ovalbumin (OVA)-induced experimental allergic rhinitis in BALB/c mice.

MATERIALS AND METHODS

Drugs and Chemicals

Ovalbumin (Grade V), aluminum hydroxide and histamine dihydrochloride were purchased from Sigma Chemical Co. (St Louis, MO, USA). Sodium sulfate, acetone, alcohol, sulphanilic acid, sodium nitrite, trisodium phosphate was purchased from S.D. Fine Chemicals, Mumbai, India. Sulphanilamides, naphthalaminediamine HCl, phosphoric acid was obtained from Loba Chemie Pvt. Ltd., Mumbai, India. Montelukast (Montecip®, Cipla Limited, India). Mouse OVA-specific IgE, total IgE, total IgG1, β-hexosaminidase, IL-4, IL-5, IL-13, IL-17 and Interferon-gamma (IFN-γ) enzyme-linked immunosorbent assay (ELISA) Kit were obtained from Bethyl Laboratories Inc. (Montgomery, TX, USA). Total RNA Extraction kit and One-step Reverse transcription-polymerase chain reaction (RT-PCR) kit was purchased from MP Biomedicals India Private Limited, India.

Animals

Adult male BALB/c mice (18-22 g) were purchased from National Toxicology Centre, Punae and kept in quarantine for a week at the institute animal house. Groups of ten animals per cage were kept under standard laboratory conditions at a temperature of 24°C ± 1°C, relative humidity of 45–55% and 12:12 h dark and light cycle. The experiments were carried out between 10:00 am and 5:00 pm. Animals had free access to food (Standard chaw pellet, Pranav Agro-industries Ltd., Sangli, India) and water *ad libitum*. Experimental protocols and procedures were approved by the Institutional Animal Ethics Committee of Poona College of Pharmacy, Pune and performed in accordance with the guidelines on animal experimentation recommended by Committee for Control and Supervision of Experimentation on Animals (CPCSEA), Government of India.[25] Animals were brought to the testing laboratory 1 h before the experiments for adaptation purpose.

Induction of AR

Sensitization of BALB/c mice was done on days 1, 3, 5, 7, 9, 11, 13 by i.p. injection containing 500 µl of sensitization solution (50 mg of OVA and 1000 mg of aluminum hydroxide dissolved in 500 ml of saline).[14,26] On day 14, mice were randomly divided into 6 treatment groups (n = 12 mice/group) and treated for the next 7 days (day-14 today-21) as follows:

Group I: *Normal:* Non-sensitized and received a suspension of aluminum hydroxide in saline followed by distilled water (10 mg/kg, p.o.)

Group II: *AR Control:* OVA-sensitized and received distilled water (10 mg/kg, p.o.)

Group III: *Montelukast (10):* [MLT (10)]: OVA-sensitized and received standard drug treatment i.e. montelukast (10 mg/kg, p.o.)

Group IV: *Fisetin (10):* [F (10)]: OVA-sensitized and received fisetin (10 mg/kg, p.o.)

Group V: *Fisetin (20): [F (20)]:* OVA-sensitized and received fisetin (20 mg/kg, p.o.)

Group VI: *Fisetin (40):* [F (40)]: OVA-sensitized and received fisetin (40 mg/kg, p.o.)

On day 21, one hour after the last dose of treatment, mice were challenged with intranasal (i.n.) administration of OVA (5%, 5 µl per nostril) and observations were recorded. During the acute toxicity study, animals did not show any signs and symptoms such as restlessness, respiratory distress, diarrhoea, convulsions and/or coma with graded doses up to 5000 mg/kg body weight.[27] The three different dosages of fisetin, i.e., 10, 20 and 40 mg/kg were selected on the basis of previous studies carried out in our laboratory.[20] The solutions of fisetin were freshly prepared daily by dissolving in distilled water at 1 mg/ml and administered orally for biological evaluations.

Nasal Symptoms in OVA-Induced AR Mice

After OVA challenge, nasal symptoms were evaluated within 10 min period.[26,28] The number of sneezes and nasal itching motions (nasal rubbing) were recorded. Nasal discharge was scored as 0 = no discharge, 1 = the discharge reaches the anterior nasal aperture, 2 = the discharge overshoots the anterior nasal aperture, 3 = the discharge flows out.

Nasal Symptoms during Histamine-Induced Hypersensitivity in OVA-Induced AR Mice

To evaluate effects on histamine-induced hypersensitivity after interruption of the drugs, mice were challenged with histamine dihydrochloride (10 µl per nostril of a solution of 1 µmol/ml in physiological saline) on day 24 of study, and the number of instances of nasal rubbing and sneezing was counted during 10 min period after challenge.[14,26]

Blood Sample Collection from OVA-Induced AR Mice

On day 21, 2 h after OVA challenge, blood specimens were collected from the retro-orbital plexus and serum was obtained by centrifugation at 8350 ×g for 10 min at 4°C. Samples were stored at −20°C until biochemical and hematological measurements.

Hematological Measurements in OVA-Induced AR Mice

Hematological parameters were measured in blood samples using an automated hematologicalanalyzer (BC2800, Golden Harvest Ltd.) with software specific to mice. Parameters such as total leukocyte count (TLC), polymorphonuclear leukocytes (PMN) and platelet count (PLT) were analyzed.

Biochemical Measurement in Serum of OVA-Induced AR Mice

OVA-specific IgE, total IgE, total IgG1 and β-hexosaminidase in serum, whereasIL-4, IL-5, IL-13, IL-17, and IFN-γ in nasal lavage fluid (NLF) were evaluated using respective mouse ELISA quantitation kit (Bethyl Laboratories Inc., Montgomery, TX, USA) as per manufacturer's instructions. Results were evaluated by the P/N value. The test was done in duplicate to avoid false-negative, and false-positive results and the average value was taken for final calculation.[29-31]

Determination of Histamine Level in Serum of OVA-Induced AR Mice

The histamine content of the serumwas measured by the ophthaldialdehyde (OPA) spectrofluorometric procedure. The fluorescent intensity was measured at 460 nm (excitation at 355 nm) using a spectrofluorometer and histamine content was calculated.[32]

Reverse Transcriptase PCR

The levels of mRNA were analyzed in spleen tissue (n = 4) using reverse transcription (RT)-PCR approach as described elsewhere.[33-35] Briefly, single-stranded cDNA was synthesized from 5 µg of total cellular RNA using reverse transcriptase (MP Biomedicals India Private Limited, India) as described elsewhere.[33,36,37] The primer sequences along with its length (no. base pairs) for GATA3, IL-4, IL-5, IL-13, and β-actin are presented in **Table 1**. Amplification of β-actin served as a control for sample loading and integrity. PCR products were detected by electrophoresis on a 1.5 % agarose gel containing ethidium bromide. The size of amplicons was confirmed using a 100-bp ladder (0.5 µg/µL) as a standard size marker. The amplicons were visualized, and images were captured using a gel documentation system (Alpha Innotech Inc., San Leandro, CA, USA). Gene expression was assessed by generating densitometry data for band intensities in different sets of experiments, by analyzing the gel images on Image J program (Version 1.33, Wayne Rasb and, National Institutes of Health, Bethesda, MD, USA) semi-quantitatively. The band intensities were compared with constitutively expressed β-actin. The intensity of mRNAs was standardized against that of the β-actin mRNA from each sample, and the results were expressed as the PCR-product/β-actin mRNA ratio.

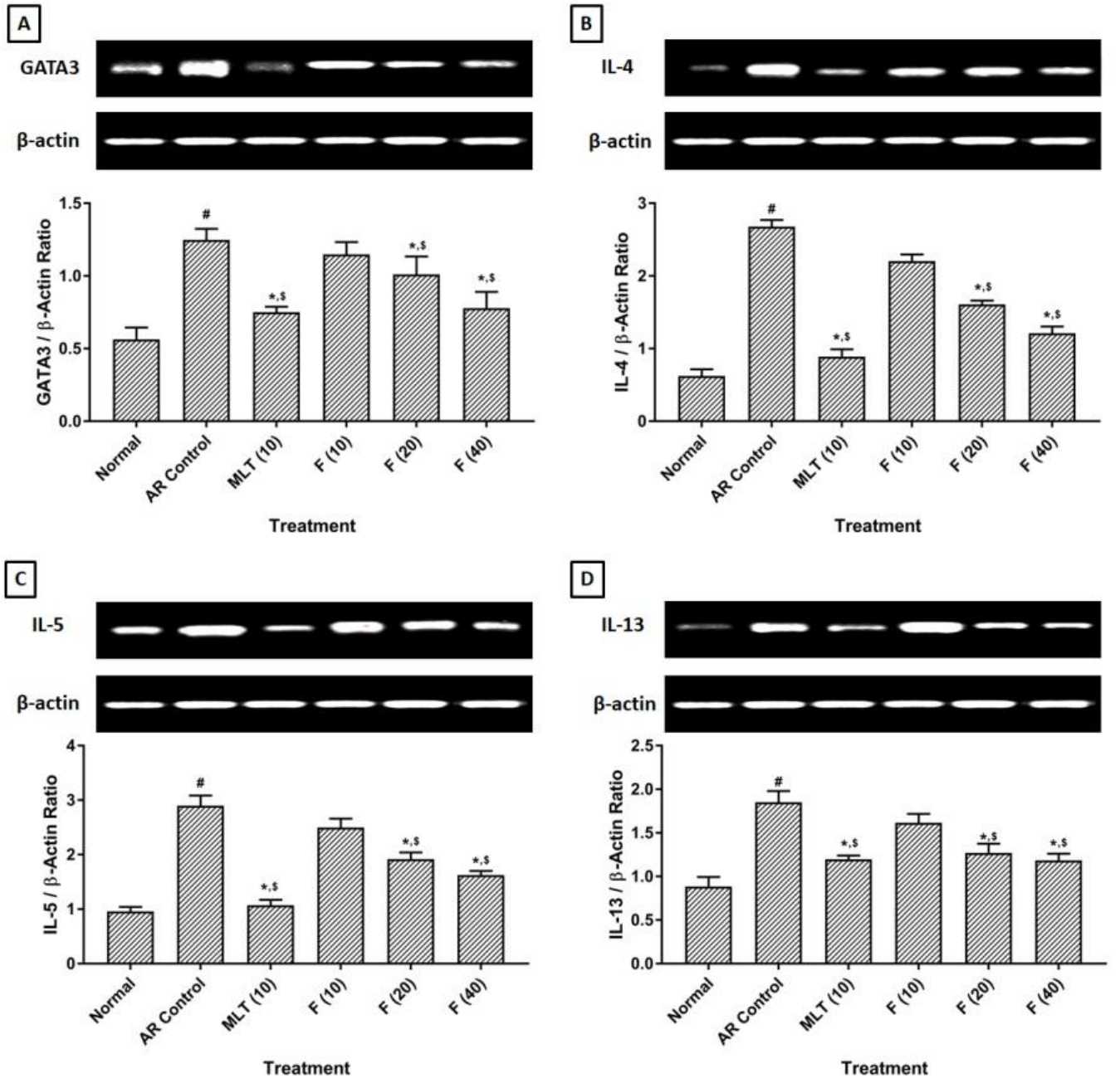

Fig. 1: ***Effect of fisetin treatment on OVA-induced alterations in spleen GATA3 (A), IL-4 (B), IL-5 (C), and IL-13 (D) mRNA expression in AR mice****. Data are represented as Mean ± SEM (n = 4) and was analyzed by one-way ANOVA followed by Tukey's multiple range test. #p <0.05 as compared with normal group, *p <0.05 as compared with AR control group and $p < 0.05 as compared with each other. Figures in parenthesis indicate oral dose in mg/kg. AR: Allergic Rhinitis; HR: Hyperresponsiveness; OVA: Ovalbumin; MLT (10): Montelukast (10 mg/kg) treated; F (10): Fisetin (10 mg/kg) treated; F (20): Fisetin (20 mg/kg) treated; F (40): Fisetin (40 mg/kg) treated; GATA3: GATA binding protein-3; IL's: Interleukins.*

Histological Examination

On day 21, after blood withdrawal, 3 mice from each group were sacrificed. Nasal mucosa, spleen and lung tissues were dissected and stored for 24 h in 10% formalin for histological examination. The specimens were dehydrated and placed in xylene for 1 h (3 times) and later in ethyl alcohol (70, 90 and 100%) for 2h each. The infiltration and impregnation were carried out by treating with paraffin wax twice, each time for one hour. For tissue slide preparation, specimens were

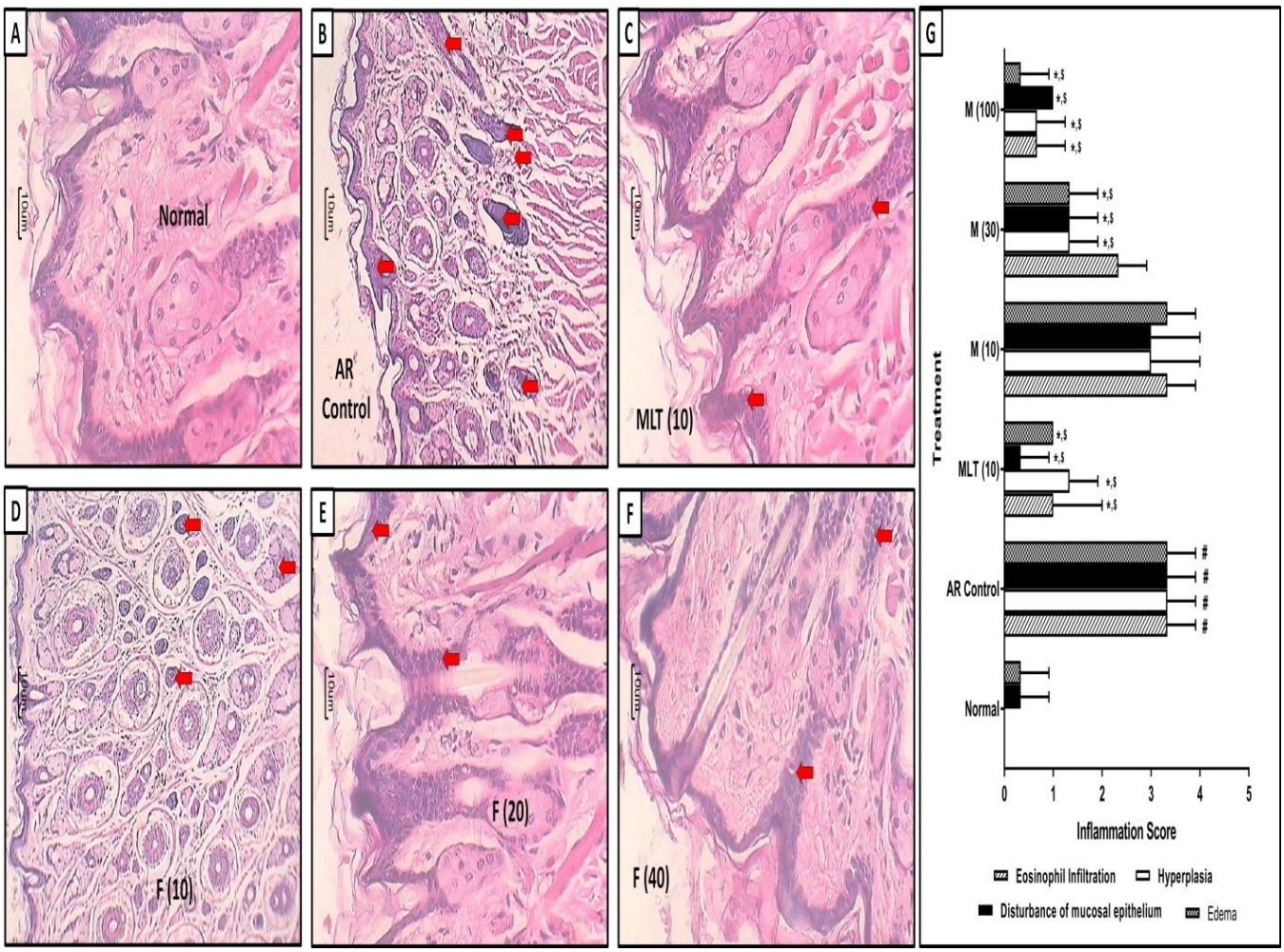

Fig. 2: ***Effect of fisetin treatment on OVA-induced alteration in nasal histopathology.*** *Photomicrograph of sections of nasal tissue from normal (A), AR control (B), Montelukast(10 mg/kg) treated (C), Fisetin(10 mg/kg) treated(D), Fisetin (20 mg/kg) treated (E) and Fisetin (40 mg/kg) treated (F) mice: Nasal H&E stain at 40X and 100X. The quantitative representation of histological score (G). Data are expressed as mean ± S.E.M. (n=3), and one-way ANOVA followed by Kruskal-Wallis test was applied for post hoc analysis. $^{\#}p$ <0.05 as compared with normal group, $^{*}p$ <0.05 as compared with AR control group and $^{\$}p$ <0.05 as compared with each other. AR: Allergic Rhinitis; HR: Hyperresponsiveness; OVA: Ovalbumin; MLT (10): Montelukast (10 mg/kg) treated; F (10): Fisetin (10 mg/kg) treated; F (20): Fisetin (20 mg/kg) treated; F (40): Fisetin (40 mg/kg) treated mice.*

cut into sections of 3-5 μm thickness and were stained with Hematoxylin and eosin (H&E). The specimens were then mounted on individual slides by use of Distrene Phthalate Xylene (DPX). Sections were examined under light microscope to obtain a general impression of the histopathology features of specimen and infiltration of cells in epithelium and sub-epithelium. The intensity of histological aberrations in the nasal, spleen and lung tissue was graded as Grade 0 (not present or very slight); Grade 1 (mild); Grade 2 (moderate); and Grade 3 (severe) as described in the literature.[38]

Statistical Analysis

Data were expressed as mean ± SEM (standard error of means), and analysis was performed using Graph Pad Prism 5.0 software (Graph Pad, San Diego, USA). Statistical comparisons were made between drug-treated groups and AR control animals. Data of biochemical parameters were analyzed using one-way ANOVA, Dunnett's multiple range test was applied for post hoc analysis. A score of nasal redness and nasal discharge was analyzed by nonparametric Kruskal–Wallis ANOVA. A value of $p < 0.05$ was considered to be statistically significant.

SUPPLEMENTARY FILE

Supplementary figure 1: Structure of fisetin (3, 3', 4', 7-tetrahydroxyflavone).

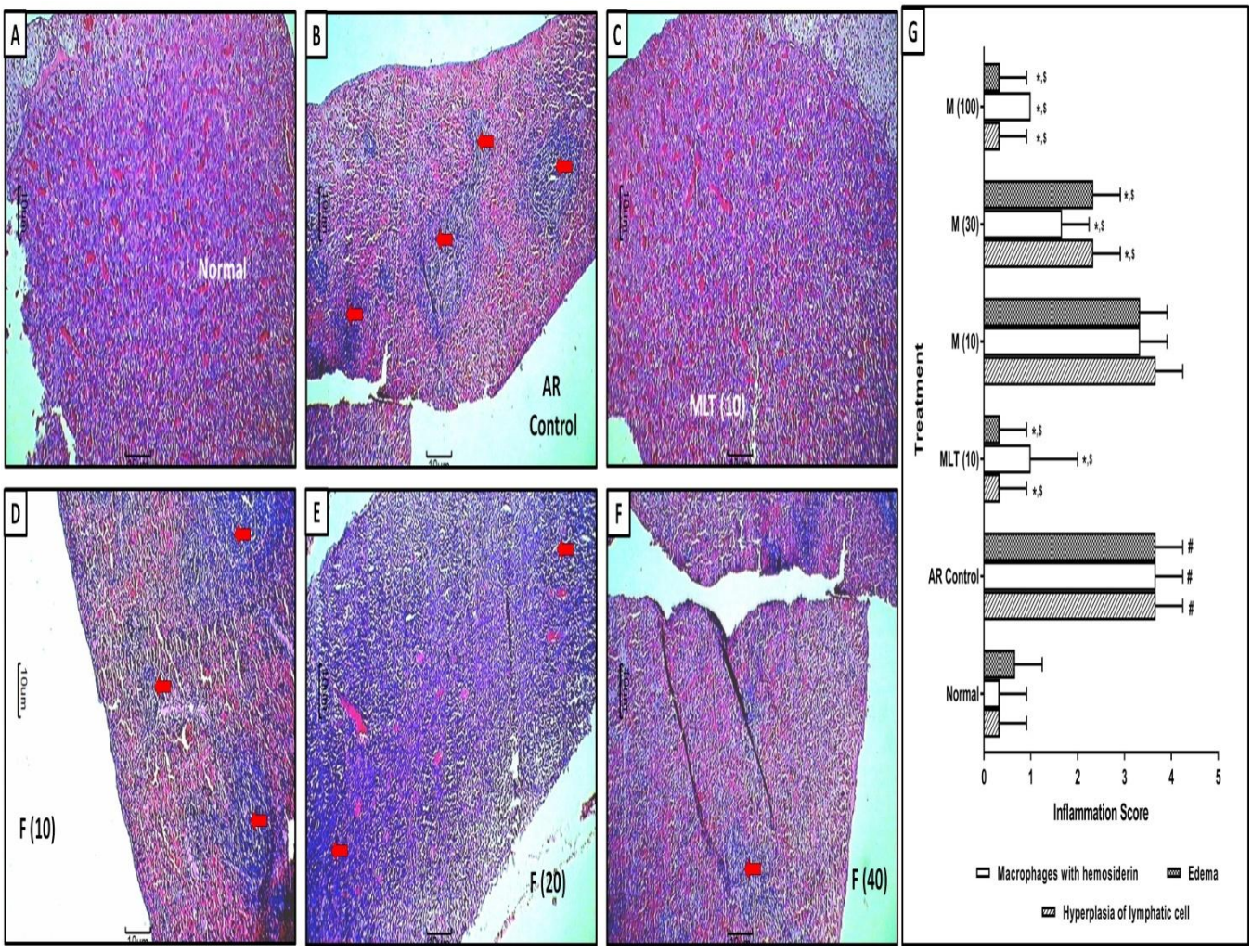

Fig. 3: ***Effect of fisetin treatment on OVA-induced alteration in spleen histopathology.*** *Photomicrograph of sections of spleen tissue from normal (A), AR control (B), Montelukast(10 mg/kg) treated (C),Fisetin(10 mg/kg) treated(D), Fisetin (20 mg/kg) treated (E) and Fisetin (40 mg/kg) treated (F) mice: Spleen H&E stain at 40X. The quantitative representation of histological score (G). Data are expressed as mean ± S.E.M. (n=3), and one-way ANOVA followed by Kruskal-Wallis test was applied for post hoc analysis. #p <0.05 as compared with normal group, *p <0.05 as compared with AR control group and $p <0.05 as compared with each other. AR: Allergic Rhinitis; HR: Hyperresponsiveness; OVA: Ovalbumin; MLT (10): Montelukast (10 mg/kg) treated; F (10): Fisetin (10 mg/kg) treated; F (20): Fisetin (20 mg/kg) treated; F (40): Fisetin (40 mg/kg) treated mice.*

RESULTS

Effect of Fisetin Treatment on OVA-Albumin Induced Alteration in Body Weight, Spleen Weight, and Lung Weight

OVA-Sensitization resulted in significant increase ($p < 0.05$) in relative spleen and lung weights in AR control mice as compared to normal mice. However, OVA-control mice did not show any significant alterations in body weight as compared to normal mice. Mice treated with fisetin (20 and 40 mg/kg) significantly inhibited ($p < 0.05$) OVA-induced increase in relative spleen and lung weights as compared to AR control animals. Montelukast (10 mg/kg) treatment also significantly decreased ($p < 0.05$) relative spleen and lung weights as compared to AR control mice. However, attenuation in OVA-induced increase in the relative spleen, and lung weights were more significant ($p < 0.05$) in montelukast (10 mg/kg) than fisetin treatment (**Table 2**).

Effect of Fisetin Treatment on OVA-Induced and Histamine-Induced Alterations in Nasal Symptoms

On day 21, the number of nasal rubs, sneezing, and discharge significantly increased ($p < 0.05$) after OVA challenge in OVA-sensitized mice as compared to normal mice. Treatment with fisetin (20 and 40 mg/kg) and montelukast significantly ($p < 0.05$) reduced OVA-induced nasal rubbing, sneezing and discharge as

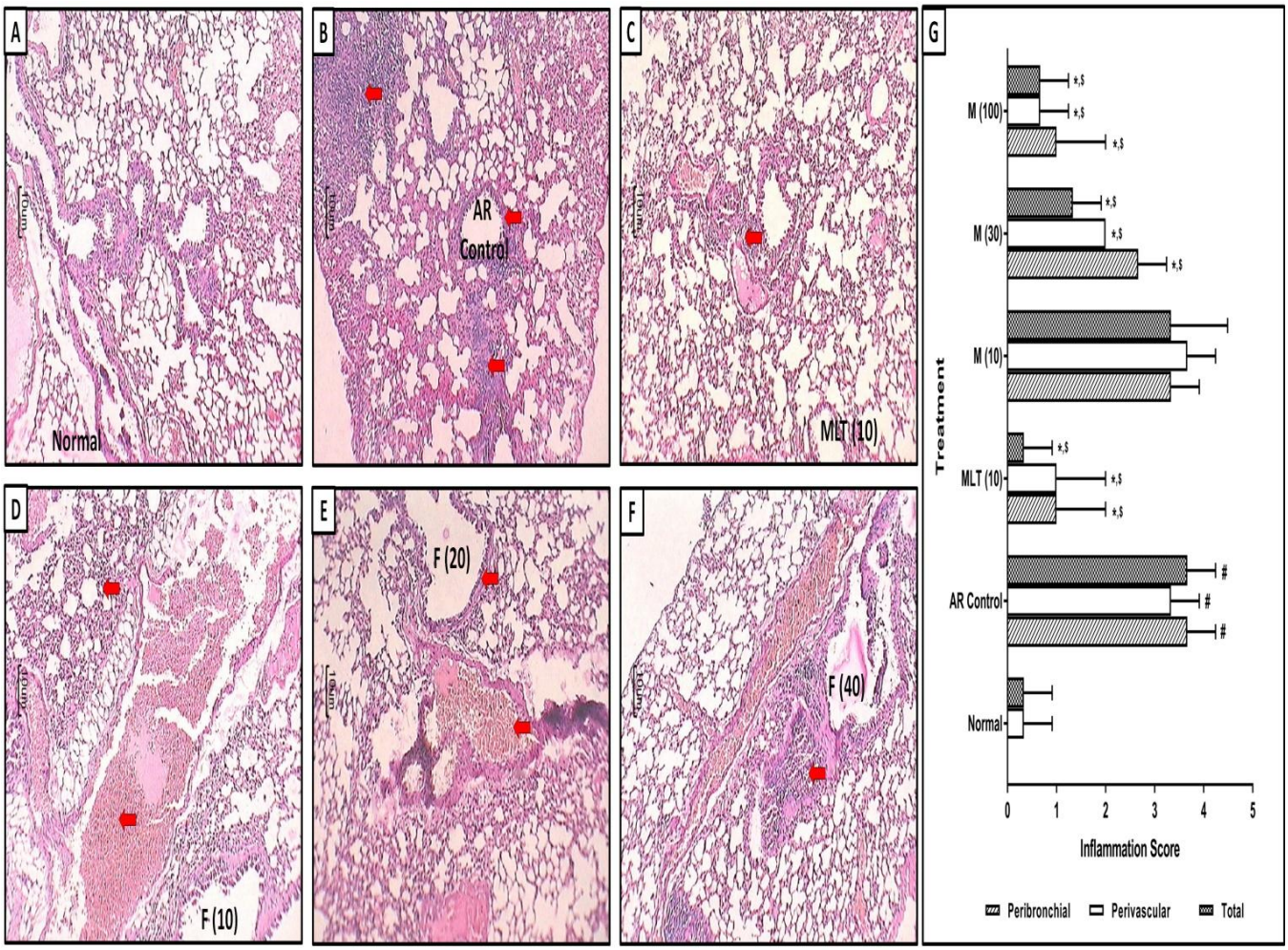

Fig. 4: ***Effect of fisetin treatment on OVA-induced alteration in lung histopathology.*** *Photomicrograph of sections of lung tissue from normal (A), AR control (B), Montelukast(10 mg/kg) treated (C), Fisetin (10 mg/kg) treated (D), Fisetin (20 mg/kg) treated (E) and Fisetin (40 mg/kg) treated (F) mice: Lung H&E stain at 40X. The quantitative representation of histological score (G). Data are expressed as mean ± S.E.M. (n=3), and one-way ANOVA followed by Kruskal-Wallis test was applied for post hoc analysis. #p <0.05 as compared with normal group, *p <0.05 as compared with AR control group and $p <0.05 as compared with each other. AR: Allergic Rhinitis; HR: Hyperresponsiveness; OVA: Ovalbumin; MLT (10): Montelukast (10 mg/kg) treated; F (10): Fisetin (10 mg/kg) treated; F (20): Fisetin (20 mg/kg) treated; F (40): Fisetin (40 mg/kg) treated mice.*

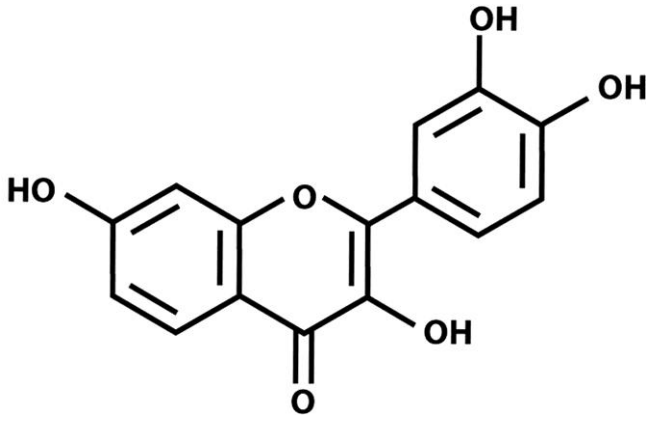

Fig. 5: *Structure of fisetin (3,3´,4´,7-tetrahydrofolate.*

compared to AR control mice. On day 24, histamine challenge in AR control mice caused significant increase ($p < 0.05$) in nasal rubbing and sneezing as compared to normal mice. Fisetin (20 and 40 mg/kg) treatment significantly inhibited ($p < 0.05$) histamine-induced nasal rubbing and sneezing as compared to AR control mice. Montelukast (10 mg/kg) treatment also showed significant attenuation ($p < 0.05$) in nasal rubbing and sneezing induced by histamine challenge as compared to AR control mice. Additionally, montelukast (10 mg/kg) showed more significant inhibition ($p < 0.05$) in OVA-induced as well as histamine-induced alterations in nasal symptoms as compared to fisetin treatment (**Table 2**).

Table 1: *Primer sequences for GATA3, IL-4, IL-5, IL-13, and β-actin.*

Sr. No.	*Gene*	*Primer Sequence*		*Base Pair (bp) Length*
		Forward Primer	*Reverse Primer*	
1.	GATA3	ACAGAAGGCAGGGAGTGTGT	GGTAGAGTCCGCAGGCATT	437
2.	IL-4	ACCTTGCTGTCACCCTGTTCTGC	GTTGTGAGCGTGGACTCATTCACG	352
3.	IL-5	GCTTCTGCATTTGAGTTTGCTAGCT	TGGCCGTCAATGTATTTCTTTATTAAG	276
4.	IL-13	TCTCGCTTGCCTTGGTGG	CATTCAATATCCTCTGGGTCCTGT	236
5.	β-actin	GTCACCCACACTGTGCCCATCT	ACAGAGTACTTGCGCTCAGGAG	764

Table 2: *Effect of fisetin treatment on OVA-induced alterations in body weight, relative spleen and lung weight, 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)*	*F (10)*	*F (20)*	*F (40)*
Body weight (gm)	30.07 ± 0.51	28.20 ± 0.61	29.55 ± 0.81	28.53 ± 0.78	29.60 ± 0.71	28.95 ± 0.62
Spleen wt / Body wt (mg/gm) ($X10^{-3}$)	3.58 ± 0.15	5.82 ± 0.25$^{\#}$	3.94 ± 0.26$^{*,\$}$	5.39 ± 0.32	4.80 ± 0.27$^{*,\$}$	4.32 ± 0.26$^{*,\$}$
Lung wt / Body wt (mg/gm) ($X10^{-3}$)	7.87 ± 1.15	17.13 ± 1.31$^{\#}$	9.24 ± 1.24$^{*,\$}$	14.12 ± 0.89	14.06 ± 0.90$^{*,\$}$	9.62 ± 0.49$^{*,\$}$
OVA challenge						
Rubbing (number)	17.67 ± 1.12	66.33 ± 1.41$^{\#}$	25.67 ± 0.56$^{*,\$}$	59.33 ± 1.71	47.33 ± 1.05$^{*,\$}$	32.83 ± 1.20$^{*,\$}$
Sneezing (number)	10.83 ± 0.48	41.17 ± 0.48$^{\#}$	15.50 ± 0.56$^{*,\$}$	37.50 ± 0.22	28.83 ± 0.70$^{*,\$}$	20.83 ± 0.31$^{*,\$}$
Discharge (score)	0.83 ± 0.17	2.67 ± 0.21$^{\#}$	1.33 ± 0.21$^{*,\$}$	2.50 ± 0.22	1.17 ± 0.17$^{*,\$}$	1.50 ± 0.22$^{*,\$}$
Histamine challenge						
Rubbing (number)	16.50 ± 0.99	72.83 ± 0.54$^{\#}$	33.17 ± 1.30$^{*,\$}$	66.50 ± 1.69	49.00 ± 1.29$^{*,\$}$	38.17 ± 1.42$^{*,\$}$
Sneezing (number)	9.17 ± 0.70	52.67 ± 1.02$^{\#}$	21.83 ± 0.65$^{*,\$}$	47.33 ± 1.05	38.33 ± 0.80$^{*,\$}$	23.50 ± 1.18$^{*,\$}$

Data are represented as Mean ± SEM (n = 6). Data of 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 tests. $^{\#}p$ <0.05 as compared with normal group, $^{*}p$ <0.05 as compared with AR control group and $^{\$}p$ <0.05 as compared with each other. Figures in parenthesis indicate oral dose in mg/kg. AR: Allergic Rhinitis; OVA: Ovalbumin; MLT (10): Montelukast (10 mg/kg) treated; F (10): Fisetin (10 mg/kg) treated; F (20): Fisetin (20 mg/kg) treated; F (40): Fisetin (40 mg/kg) treated.

Effect of Fisetin Treatment on OVA-Albumin Induced Alteration in Hematological Parameters

The intranasal challenge of OVA resulted in significant increase (p < 0.05) in total cell count, polymorphonuclear leukocytes, differential cell count and platelet count in AR control mice as compared to normal mice. However, when compared to AR control mice, there was significant decrease (p < 0.05) in elevated levels of total cell count, polymorphonuclear leukocytes, differential cell count (lymphocytes, eosinophils and monocytes) and platelet count in fisetin (20 and 40 mg/kg) and montelukast (10 mg/kg) treated group. However, mice treated with montelukast (10 mg/kg) showed more significant attenuation (p < 0.05) of increased hematological parameters as compared to fisetin treated mice (**Table 3**).

Table 3: *Effect of fisetin treatment on OVA-induced alterations in hematological parameters in AR mice.*

Parameter	*Treatment*					
	Normal	*AR Control*	*MLT (10)*	*F (10)*	*F (20)*	*F (40)*
TLC (X 10^3/mm^3)	2.31 ± 0.29	4.40 ± 0.16$^{\#}$	2.46 ± 0.18$^{*,\$}$	4.07 ± 0.24	3.19 ± 0.13$^{*,\$}$	2.75 ± 0.15$^{*,\$}$
PMN (%)	44.00 ± 1.18	58.33 ± 1.91$^{\#}$	44.50 ± 1.77$^{*,\$}$	56.00 ± 1.65	50.83 ± 1.78$^{*,\$}$	46.33 ± 1.67$^{*,\$}$
Lymphocytes (%)	40.00 ± 1.32	50.00 ± 1.88$^{\#}$	41.00 ± 1.71$^{*,\$}$	48.33 ± 1.69	46.50 ± 1.67$^{*,\$}$	42.17 ± 1.60$^{*,\$}$
Eosinophils (%)	2.50 ± 0.22	5.00 ± 0.00$^{\#}$	2.17 ± 0.17$^{*,\$}$	4.33 ± 0.33	2.83 ± 0.31$^{*,\$}$	2.33 ± 0.21$^{*,\$}$
Monocytes (%)	1.5 ± 0.22	3.33 ± 0.21$^{\#}$	1.33 ± 0.21$^{*,\$}$	3.33 ± 0.21	1.50 ± 0.22$^{*,\$}$	1.83 ± 0.17$^{*,\$}$
PLT (X 10^5/mm^3)	2.30 ± 0.18	4.34 ± 0.20$^{\#}$	2.40 ± 0.16$^{*,\$}$	4.20 ± 0.17	2.87 ± 0.22$^{*,\$}$	2.53 ± 0.15$^{*,\$}$

Data are represented as Mean ± SEM (n = 6) and was analysed by One-Way ANOVA followed by Tukey's multiple range test. $^{\#}p < 0.05$ as compared with normal group, $^{*}p < 0.05$ as compared with AR control group and $^{\$}p < 0.05$ as compared with each other. Figures in parenthesis indicate oral dose in mg/kg. AR: Allergic Rhinitis; HR: Hyperresponsiveness; MLT (10): Montelukast (10 mg/kg) treated; F (10): Fisetin (10 mg/kg) treated; F (20): Fisetin (20 mg/kg) treated; F (40): Fisetin (40 mg/kg) treated; TLC: Totalleukocyte count; PMN: Polymorphonuclear leukocytes; PLT: Platelet.

Table 4: *Effect of fisetin treatment on OVA-induced alterations in serum histamine, OVA-specific IgE, total IgE and IgG1, β-hexosaminidase, as well as NLF IL-4, IL-5, IL-13, IL-17, and IFN-γ levels in AR mice*

Parameters	*Treatment*					
	Normal	*AR Control*	*MLT (10)*	*F (10)*	*F (20)*	*F (40)*
Serum Histamine (µg/ml)	66.53 ± 3.05	360.80 ± 3.16$^{\#}$	96.51 ± 3.22$^{*,\$}$	320.30 ± 2.94	263.10 ± 2.72$^{*,\$}$	114.90 ± 1.52$^{*,\$}$
Serum OVA-specific IgE (ng/ml)	12.57 ± 1.46	59.11 ± 1.61$^{\#}$	23.37 ± 1.07$^{*,\$}$	52.47 ± 0.61	38.17 ± 1.11$^{*,\$}$	28.72 ± 1.34$^{*,\$}$
Serum Total IgE (ng/ml)	103.10 ± 4.65	494.20 ± 4.67$^{\#}$	133.90 ± 2.48$^{*,\$}$	396.90 ± 3.81	235.50 ± 4.28$^{*,\$}$	155.20 ± 2.73$^{*,\$}$
Serum Total IgG1 level (ng/ml)	0.23 ± 0.02	0.79 ± 0.02$^{\#}$	0.48 ± 0.02$^{*,\$}$	0.69 ± 0.02	0.50 ± 0.02$^{*,\$}$	0.47 ± 0.02$^{*,\$}$
Serum β-hexo-saminidase (ng/ml)	13.25 ± 0.47	47.89 ± 1.14$^{\#}$	17.63 ± 1.49$^{*,\$}$	39.25 ± 1.07	30.97 ± 1.45$^{*,\$}$	24.57 ± 1.71$^{*,\$}$
NLF IL-4 (pg/ml)	61.88 ± 3.18	141.20 ± 4.51$^{\#}$	76.27 ± 2.21$^{*,\$}$	142.20 ± 3.51	122.60 ± 3.59$^{*,\$}$	96.74 ± 4.03$^{*,\$}$
NLF IL-5 (pg/ml)	49.03 ± 2.85	111.50 ± 4.41$^{\#}$	56.54 ± 2.03$^{*,\$}$	107.90 ± 2.72	92.00 ± 2.92$^{*,\$}$	62.96 ± 4.11$^{*,\$}$
NLF IL-13 (pg/ml)	84.72 ± 3.40	201.90 ± 4.04$^{\#}$	116.80 ± 3.32$^{*,\$}$	182.00 ± 2.71	158.90 ± 2.96$^{*,\$}$	133.60 ± 3.90$^{*,\$}$
NLF IL-17 (pg/ml)	7.75 ± 1.91	42.02 ± 2.26$^{\#}$	15.05 ± 3.30$^{*,\$}$	39.64 ± 2.76	32.82 ± 1.55$^{*,\$}$	18.55 ± 2.87$^{*,\$}$
NLF IFN-γ (pg/ml)	61.58 ± 0.87	53.43 ± 2.97$^{\#}$	57.15 ± 3.19	53.38 ± 3.07	60.34 ± 1.92	62.85 ± 2.86
NLF IL-4/IFN-γ ratio	1.01 ± 0.05	2.67 ± 0.12$^{\#}$	1.36 ± 0.10$^{*,\$}$	2.69 ± 0.10	2.04 ± 0.08$^{*,\$}$	1.56 ± 0.10$^{*,\$}$

Data are represented as Mean ± SEM (n = 6) and was analysed by one-way ANOVA followed by Tukey's multiple range test. $^{\#}p < 0.05$ as compared with normal group, $^{*}p < 0.05$ as compared with AR control group and $^{\$}p < 0.05$ as compared with each other. Figures in parenthesis indicate oral dose in mg/kg. AR: Allergic Rhinitis; HR: Hyperresponsiveness; OVA: Ovalbumin; MLT (10): Montelukast (10 mg/kg) treated; F (10): Fisetin (10 mg/kg) treated; F (20): Fisetin (20 mg/kg) treated; F (40): Fisetin (40 mg/kg) treated; Ig: Immunoglobulin; IL's: Interleukins; IFN-γ: Interferon gamma; NLF: Nasal Lavage Fluid.

Effect of Fisetin Treatment on OVA-Albumin Induced Alteration in Serum Biochemical Parameters

Levels of serum histamine, OVA-specific IgE, total IgE and IgG1, β-hexosaminidase were significantly increased ($p < 0.05$) in AR control group as compared to normal group. Treatment with fisetin (20 and 40 mg/kg) significantly attenuated ($p < 0.05$) these elevated

levels of serum histamine, OVA-specific IgE, total IgE and IgG1, β-hexosaminidase when compared with AR control mice. Montelukast (10 mg/kg) treatment also significantly decreased ($p < 0.05$) these elevated levels of serum histamine, OVA-specific IgE, total IgE and IgG1, β-hexosaminidase as compared to AR control mice (**Table 4**).

Effect of Fisetin Treatment on OVA-Albumin Induced Alteration in Nasal Lavage Fluid IL-4, IL-5, IL-13, IL-17, and IFN-γ Levels

OVA-challenged mice showed a significant increase ($p < 0.05$) in nasal lavage fluid IL-4, IL-5, IL-13, IL-17, and IFN-γ levels as compared to normal mice. Treatment with fisetin (20 and 40 mg/kg) as well as montelukast (10 mg/kg) attenuated these elevated levels of IL-4, IL-5, IL-13, and IL-17 in nasal lavage fluid significantly ($p <0.05$) as compared to AR control mice. However, attenuation in these elevated levels of IL-4, IL-5, IL-13, and IL-17 in nasal lavage fluid was more significant ($p < 0.05$) in montelukast (10 mg/kg) treatment as compared to fisetin treatment. Treatment with fisetin (10, 20 and 40 mg/kg) as well as montelukast (10 mg/kg) did not show any significant inhibition in the elevated IFN-γ nasal lavage fluid levels as compared to AR control mice (**Table 4**).

Effect of Fisetin Treatment on OVA-Albumin Induced Alteration in Splenic GATA3, IL-4, IL-5, and IL-13 mRNA Expression Levels

There was significant ($p <0.05$) up-regulation in the splenic GATA3, IL-4, IL-5, and IL-13 mRNA expression in AR control mice after the intranasal challenge of OVA as compared to normal mice. Mice treated with fisetin (20 and 40 mg/kg) showed significant ($p < 0.05$) attenuation in these elevated splenic GATA3, IL-4, IL-5, and IL-13 mRNA expressions as compared to AR control mice. Treatment with montelukast (10 mg/kg) also showed significant ($p < 0.05$) down-regulation in splenic GATA3, IL-4, IL-5, and IL-13 mRNA expression when compared with AR control mice. However, treatment with montelukast (10 mg/kg) showed more significant ($p < 0.05$) down-regulation of splenic GATA3, IL-4, and IL-5 mRNA expression as compared to fisetin treatment.

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Effect of Fisetin Treatment on OVA-Albumin Induced Alteration in Nasal Mucosa Histopathology

Ovalbumin challenge produced marked disruption of mucosal epithelium in AR control mice. Histopathological evaluation of nasal mucosa after intranasal instillation of OVA showed significant ($p <0.05$) increase in eosinophil infiltration into the nasal mucosa in AR control mice (Fig. 2B) as compared to normal mice. There was no evidence of eosinophils infiltration in nasal mucosa of normal control mice. It showed normal architecture of mucosal epithelium with mild oedema (Fig. 2A). Mice treated with montelukast (10 mg/kg) significantly ($p <0.05$) decreased eosinophils infiltration and mucosal cell hyperplasia as compared to AR control mice (Fig. 2C). Fig. 2D depicts a predominant number of inflammatory cells in the nasal mucosa of mice treated with fisetin (10 mg/kg). Also, treatment with fisetin (20 and 40 mg/kg) significantly ($p <0.05$) reduced OVA-induced eosinophils infiltration into nasal mucosa as compared to AR control mice (Fig. 2E and Fig. 2F) (Fig. 2G).

Effect of Fisetin Treatment on OVA-Albumin Induced Alteration in Spleen Histopathology

Massive enlargement of the spleen reflected the induction of allergic rhinitis by intranasal challenge with OVA in mice. Spleen section from AR control mice showed significantly ($p < 0.05$) increased macrophages with hemosiderin and hyperplasic lymphoid cell as compared to normal mice (**Fig. 3**A). Staining of spleen with H&E showed the presence of a mixed population of pale-stained lymphoid cells. In normal mice, the number of macrophages with hemosiderin and hyperplasic lymphoid cells were lesser (**Fig. 3**B). Treatment with fisetin (10 mg/kg) did not produce any significant decrease in the number of mature lymphocytes and macrophages as compared to AR control mice (**Fig. 3**D). However, mice treated with fisetin (20 and 40 mg/kg) as well as montelukast (10 mg/kg) significantly ($p < 0.05$) decreased these elevated numbers of mature lymphocytes as compared to AR control mice (**Fig. 3**E, **Fig. 3**F, and **Fig. 3**C, respectively) (**Fig. 3**G).

Effect of Fisetin Treatment on OVA-Albumin Induced Alteration in Lung Histopathology

Lung tissue from the AR control mice demonstrated marked histopathologic abnormalities characterized by significant ($p < 0.05$) increase in peribronchial and perivascular inflammatory infiltration in alveoli and bronchial region (Fig. 4A) as compared to normal mice (Fig. 4B). Montelukast (10 mg/kg) treatment showed significant ($p < 0.05$) decrease in peribronchial and perivascular inflammatory infiltration as compared to AR control mice (Fig. 4C). However, mice treated with fisetin (10 mg/kg) failed to produce any attenuation in thenumber of inflammatory infiltration as compared to AR control mice (Fig. 4D). Treatment with Fisetin (20 and 40 mg/kg) showed significant ($p < 0.05$) attenuation in OVA-induced elevated inflammatory infiltration in alveoli and the bronchial region as compared to AR control mice (Fig. 4E and Fig. 4F, Fig. 4G).

DISCUSSION

Allergic rhinitis is an IgE mediated immune-inflammatory disease of nasal mucosa mainly characterized by sneezing, rhinorrhea, and lacrimation which are

exhibited due to the early phase response to an array of allergens. Amongst the various animal models, ovalbumin (OVA) induced AR is widely usedand well-established model which mimics all clinicopathological characteristics and mechanisms behind experimental and clinical immunomodulation.[13,14] Hence, in the present investigation, we have evaluated the potential of fisetin, a plant flavonoid, against OVA-induced AR. The findings of the present study showed that fisetin exerts its anti-allergic potential via modulation of the GATA3 pathway to inhibit the release of Th2 cytokines (IL-4, IL-5, IL-13) and IgE thus reducing OVA-induced nasal rubbing and sneezing during AR.

Accumulating reports have indicated that Th-lymphocytes play a vital role in initiation and progression of immune-inflammatory reactions (such as allergic rhinitis, asthma) via the release of various cytokines including IL-4, IL-5, and IL-13.[39] The release of these Th2 cytokines are associated with inflammatory infiltration including activation eosinophil, neutrophil, and leukocyte.[40,41] In the present study, we found that, compared to normal mice, OVA-challenged mice showed elevated levels of inflammatory cells (eosinophil, neutrophil, and leukocyte). Whereas, mice that received the fisetin treatment had significantly reduced levels of inflammatory cells and platelet count which may be attributed to its anti-allergic potential. The result of the present investigation is in accordance with findings of a previous investigator where fisetin significantly attenuated inflammatory infiltration which in turn decreased the levels of Th2 cytokines (IL-4, IL-5, IL-13).[23]

Numerous evidences have suggested that histamine released from activated mast cells causes infiltration of inflammatory cells into nasal mucosa.[14,42] This leads to increase in OVA-specific IgE levels in serum. Clinically, it has also been reported that in asthmatic patients, the released IgE are cross-linked with a FcεRI receptor on mast cell surface which resulted in its degranulation.[43] This mast cell degranulation was further associated with the release of pro-inflammatory cytokines including tumor necrosis factor-α (TNF-α), IL-4 and IL-6.[44-46] Furthermore, IgG1 was reported to form a complex with an inhaled allergen which further aggravated Th2 response.[47] Outcomes of our methodology coincides with these alterations after OVA challenge, indicating induction of allergic rhinitis in BALB/c mice. However, administration of fisetin caused significant inhibition in elevated levels of OVA-specific IgE, total IgE and IgG1 levels in serum reflecting its anti-allergic potential. The result of the present investigation is in accordance with findings of a previous investigator demonstrating its inhibitory potential against IgE and IgG1.[23]

It has been reported that elevated levels of Th2 cytokines are involved in the synthesis of IgE whereas Th1 cytokines (interferon gamma (IFN-γ)) are responsible for inhibition of IgE synthesis. Thus, an imbalance in Th1 and Th2 response is important in allergic conditions.[48] IL-4 is involved in the synthesis of OVA-specific IgE whereas IL-5 selectively associates with differentiation, activation, and survival of eosinophils. Thus, IL-5 is considered as a symptomatic characteristic of AR.[49] Furthermore, researchers have showed that IL-13 is another imperative cytokine which played a vital role during the late phase response. Thus, its inhibition is important for providing nasal blockage relief during allergic rhinitis.[50] In the present investigation, administration of fisetin significantly inhibited OVA-induced elevated expressions of IL-4, IL-5, and IL-13 in both nasal lavage fluid and spleen, which in turn decreased the OVA-induced nasal symptoms such as nasal rubbing, sneezing, and discharge. Additionally, this notion was further confirmed with histology of nasal mucosal tissue evaluated by H&E stain, where fisetin treated mice showed significantly decreased eosinophil infiltration due to its IL-5 inhibitory potential.

GATA-3 (GATA binding protein), a transcription factor, is a member of the GATA family particularly expressed in Th2 cells. Studies have reported that expression of GATA-3 was significantly up-regulated during the Th-cells differentiation whereas decreased during the Th1 pathway.[51,52] In the present study, OVA-challenge resulted in significantly up-regulated GATA-3mRNA expression in spleen tissue followed by elevated Th2 response reflected by increased IL-4, IL-5, and IL-13 mRNA expressions. Interestingly, fisetin treatment decreased the GATA-3 expression followed by down-regulation in Th2 cytokine response. Similar finding was reported by a previous investigator where fisetin reduced Th2 response via inhibition of predominant transcription factor GATA-3, thus exerting its anti-allergic effect.[53]

In the present study, AR was induced in BALB/c mice via intranasal instillation of antigen which is a simple, inexpensive and non-invasive method of inducing IgE-mediated allergic disease. Nakaya et al. (2006) has reported that, during the challenge of mice with intranasal instillation of OVA, some of the allergens was undoubtedly delivered to the respiratory tract resulting in lung lesions with chronic inflammation.[54] Infiltration by lymphocytes, plasma cells, eosinophils as well as mast cells in the bronchial mucosa along with epithelium hyperplasia in the bronchialregion were the characteristic features of this chronic inflammation of lungs.[55,56] Histological studies of lungs from AR control mice showed the presence of cells with inflammatory infiltration and epithelium hyperplasia in alveoli. However, treatment with fisetin attenuated these histological aberrations induced by OVA. The result of present investigation is in accordance with the findings of previous researchers where treatment with fisetin corrected histological abnormalities pro-

duced after OVA in asthmatic mice.[19,23,53]

It is ***concluded*** that the results obtained, demonstrated the anti-allergic potential of fisetin against ovalbumin-induced allergic rhinitis. Fisetin exerts its anti-allergic effect via inhibition of GATA3 pathway to modulate the Th2 response (IL-4, IL-5and IL-13) and decreases the IgE level which inhibits the OVA-induced nasal symptoms during AR. The present study strongly supports the protective role of fisetin in OVA-induced AR, suggesting that it can be considered as a potential therapeutic alternative for clinical development.

Disclosure of Interest

The authors report no conflict of interest.

Authors' Contribution

LZ: Concept and design, analysis, drafting and final approval of article. AK: Acquisition of data, drafting, critical revision, final approval of article. AM: Acquisition of data, analysis and interpretation. SB: Concept and design, analysis, interpretation, drafting, critical revision and final approval.

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