

RESEARCH PAPER

Modulative effect of a new hydrazide derivative on wheat-induced pulmonary inflammation in rats

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Abstract

We investigated the ameliorative anti-inflammatory effect of a previously synthesized hydrazide derivative (*N'*-(4-methoxybenzylidene)-6-(4-chlorophenyl)-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*b*]pyridine-4-carbohydrazide; MD) as an immunomodulator in a newly developed allergen-induced pulmonary inflammation (AIPI) rat model. Wheat and thresher dust were used as allergens to induce pulmonary inflammation while MD was used to reverse the inflammatory response. Blood and bronchoalveolar lavage fluid (BALF) were collected after killing the rats and inflammatory cells were counted. Histological analysis of lung airways was carried out by haematoxylin and eosin and periodic acid–Schiff staining while the level of total serum IgE, interleukin (IL)-4, IL-5 and cyclooxygenase (COX)-1 in BALF and *in vitro* T-cell proliferation in spleen were measured through enzyme-linked immunosorbent assay. mRNA expression level of IL-4, IL-5, IL-13, transforming growth factor β (TGF- β), interferon- γ , tumour necrosis factor α , COX-1 and COX-2 was evaluated by qRT-PCR. A liver and kidney function test was used to observe any toxic impact of MD. The results indicated that 2 mg of wheat and thresher dust led to higher levels of inflammatory cytokines in the blood, BALF and lung airways of rats. MD potentially down-regulated the inflammatory cell infiltration in BALF and pathological lesions in the lung airways of AIPI rats. MD significantly suppressed the elevated total serum IgE, along with IL-4, IL-5, IL-13, TGF- β , COX-1 and COX-2 mRNA expression and T-cell proliferation in spleen. In conclusion, MD at 10 mg kg⁻¹ exhibited a significant reduction in all the markers in both wheat- and thresher dust-induced pulmonary inflammation mainly by inhibiting pro-inflammatory cytokine production and T-cell proliferation. The data suggest that inhibition of the T-cell response may be responsible for the modulative effect of MD in an AIPI rat model.

KEYWORDS

Wheat, thresher dust, allergen, protein, pulmonary inflammation

1 | INTRODUCTION

Allergic diseases of the pulmonary airways such as allergic asthma and rhinitis are prevalent throughout the world, especially in western countries (Apter et al., 2008). Bronchial asthma is a chronic inflammatory disease of pulmonary airways characterized by inflammatory cell infiltration and increased mucus production of pulmonary airways (Subbarao, Mandhane, & Sears, 2009). These pathological changes lead to decreased lung function in bronchial asthma (Mallol et al., 2013). Bronchoconstriction is attributable

to hypertrophy of airway smooth muscle and airway hyper-responsiveness, which is the exaggerated response to various stimuli commonly observed in asthma and pulmonary inflammation (Temelkovski, Hogan, Shepherd, Foster, & Kumar, 1998; Wenzel, 2006). Pulmonary inflammation affects hundreds of millions of people and its growing incidence and complexity have attracted the attention of scientists researching at the molecular level (Pascual & Peters, 2005). Type 1 allergies, caused by characteristic immune responses to allergens, are primarily mediated by Th2 cells. Th2 cells synthesize high levels of interleukins (IL), i.e. IL-4, IL-5 and IL-13, which leads to

the production of IgE and the release of mediators from mast cells (Saito, Nakashima, Shima, & Ito, 2010). Accordingly, estimation of IgE production and the Th1/Th2 cytokine balance is an important tool in the evaluation of pulmonary inflammation (Bortolatto et al., 2008). Cyclooxygenase (COX) enzymes are responsible for the production of prostaglandin from arachidonic acid and the development of inflammation (Suke, Negi, Mediratta, Banerjee, & Sharma, 2013; Barnes, 2010).

Non-steroidal anti-inflammatory drugs (NSAIDs) are the drugs of choice for general inflammatory conditions but are not commonly used in asthma. NSAIDs are highly effective in inflammation but they have a number of deleterious side effects, such as gastrointestinal ulcers and even bleeding (Suleyman, Albayrak, Bilici, Cadirci, & Halici, 2010; Nepogodiev et al., 2014). NSAIDs exerted their anti-inflammatory and analgesic effects by inhibiting prostaglandin synthesis via COX (Vanegas & Schaible, 2001). Glucocorticoids are steroid hormones prescribed more frequently for asthma than NSAIDs, but many undesirable side effects are known for systemic glucocorticosteroids, such as adrenal suppression, decreased bone metabolism and decreased growth in children (Kelly, 2003; Allen, 2007). Therefore, investigators are still looking for new analgesic drugs with fewer side effects for the treatment of pulmonary inflammation in asthma.

Wheat (*Triticum aestivum*) is an important crop worldwide, being used for human food and livestock feed (Shewry, 2009). However, wheat products are also known to be responsible for a number of adverse reactions in humans, including intolerances and allergies (Ullrich, 2007). Wheat allergy is an unexpected and abrupt hypersensitivity response in the body resulting from the presence of various protein fractions in wheat flour. Complete blood count analysis and a skin prick test are used to diagnose and screen the allergic inflammation (Biesiekierski, 2017). The allergic reactions to wheat are triggered by albumin and globulin to a greater degree while gliadin and gluten cause allergy to a lesser degree (Barlow et al., 2015). These proteins act as aeroallergens because the baking of wheat flour makes them resistant to decay by proteases during the digestion process (Watson & Preedy, 2013; Breiteneder & Chapman, 2014). It was determined that there are five major allergens present in wheat flour responsible for the induction of asthma in bakery workers (Pahr et al., 2012). There is a direct and strong relationship between the extent and the intensity of exposure to the wheat allergens and the level of IgE (Baatjies & Jeebhay, 2013). Two main stages are involved in wheat processing: threshing and milling. Wheat threshing produces a major contributor of aeroallergen, thresher dust (Ahmad, Yousaf, & Asif, 2011), while wheat milling produces wheat flour, which is another source of aeroallergen involve in inducing allergic diseases. Animal models to study pulmonary inflammation responses of wheat and thresher dust are currently unavailable.

We developed a new allergen-induced pulmonary inflammation (AIPI) rat model based on wheat and thresher dust allergens and then investigated the modulatory effect of the hydrazide derivative *N'*-(4-methoxybenzylidene)-6-(4-chlorophenyl)-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*b*]pyridine-4-carbohydrazide (MD), which we previously synthesized. *In silico* and docking analysis showed MD to be a potent

New Findings

• What is the central question of this study?

What is the mechanism of wheat-induced pulmonary inflammation and how does a hydrazide derivative modulate it?

• What is the main finding and its importance?

A hydrazide derivative significantly reduced wheat-induced pulmonary inflammation in a rat model mainly by down-regulating inflammatory cell infiltration, pathological lesions in the lungs and the level of pro-inflammatory cytokines, COX-1, COX-2 and T-cell proliferation.

antiplatelet agent that is active against COX-1 and COX-2 enzymes, thus exhibiting good anti-inflammatory activity *in vitro* (Ramzan et al., 2017).

2 | MATERIALS AND METHODS

2.1 | Ethical approval

All the animal experiments were approved by institutional ethical review committee (approval no. D/024/2018). For the care of laboratory animals, international ethical guidelines were followed for maintaining the rats in an animal house. All experimental procedures were conducted according to the National Institutes of Health guidelines and the principles and regulations of *Experimental Physiology* as described by Grundy (2015). Sprague-Dawley (SD) rats 10–12 weeks old were obtained from the University of Health Sciences, Lahore, Pakistan. Male SD rats (150–250 g) were selected for experiments and maintained at controlled temperature ($25 \pm 5^\circ\text{C}$) and humidity ($50 \pm 10\%$) in the institutional animal house. Animals had free access to autoclaved tap water and pathogen-free feed for 24 h.

2.2 | Extraction of protein

Protein fractions were extracted from wheat and thresher dust samples by using phosphate-buffered saline (PBS) and phenylmethylsulfonyl fluoride (PMSF) (Chan & Phillips, 1994). Briefly, 2 g of each sample in 15 ml of 0.1 M PBS (pH 7.4) and 200 μl of 0.2 mM PMSF was stirred overnight. The resulting mixture was centrifuged at 22,132 g for 20 min. Supernatant was separated, lyophilized and stored at -20°C for future experimental analysis.

2.3 | Experimental design and animal model

Prior to investigating the ameliorative anti-inflammatory effect of the novel compound, the minimum concentration of wheat and thresher dust allergens required to induce pulmonary inflammation was determined by developing the first AIPI rat model. The AIPI rat

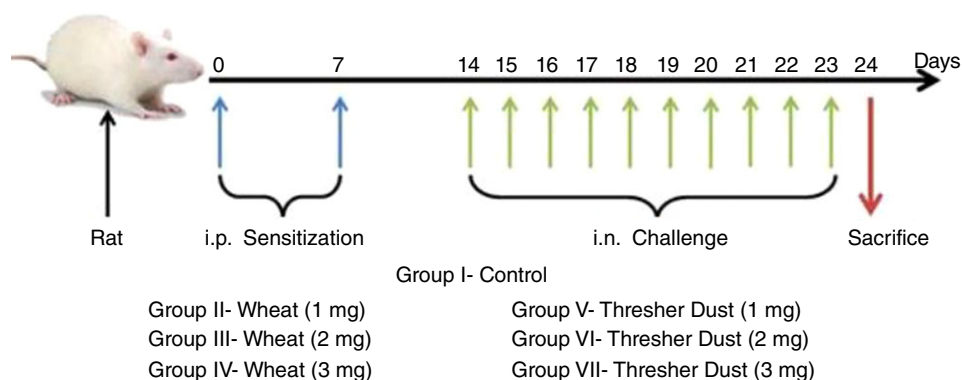


FIGURE 1 Diagrammatic representation of first AIPI rat model, developed by the i.n. exposure to wheat and thresher dust extract at three different doses. Number of rats in each group is 10

TABLE 1 Second AIPI rat model for the modulative effect of MD

Group	Description
Control	Rats were sensitized and challenged with PBS only
OVA	Rats were sensitized and challenged with 1 mg of OVA
OVA+MD1	Rats were sensitized and challenged with 1 mg of OVA and received 5 mg kg ⁻¹ of MD orally after 1 h of every challenge
OVA+MD2	Rats were sensitized and challenged with 1 mg of OVA and received 10 mg kg ⁻¹ of MD orally after 1 h of every challenge
OVA+MD3	Rats were sensitized and challenged with 1 mg of OVA and received 20 mg kg ⁻¹ of MD orally after 1 h of every challenge
OVA+Dex	Rats were sensitized and challenged with 1 mg of OVA and received 1 mg kg ⁻¹ of dexamethasone orally after 1 h of every challenge
Wheat	Rats were sensitized and challenged with 2 mg of wheat (2 mg optimized from first AIPI model)
Wheat+MD1	Rats were sensitized and challenged with 2 mg of wheat and received 5 mg kg ⁻¹ of MD orally after 1 h of every challenge
Wheat+MD2	Rats were sensitized and challenged with 2 mg of wheat and received 10 mg kg ⁻¹ of MD orally after 1 h of every challenge
Wheat+MD3	Rats were sensitized and challenged with 2 mg of wheat and received 20 mg kg ⁻¹ of MD orally after 1 h of every challenge
Wheat+Dex	Rats were sensitized and challenged with 2 mg of wheat and received 1 mg kg ⁻¹ of dexamethasone orally after 1 h of every challenge
Thresher dust	Rats were sensitized and challenged with 2 mg of thresher dust (2 mg optimized from first AIPI model)
Thresher dust+MD1	Rats were sensitized and challenged with 2 mg of thresher dust and received 5 mg kg ⁻¹ of MD orally after 1 h of every challenge
Thresher dust+MD2	Rats were sensitized and challenged with 2 mg of thresher dust and received 10 mg kg ⁻¹ of MD orally after 1 h of every challenge
Thresher dust+MD3	Rats were sensitized and challenged with 2 mg of thresher dust and received 20 mg kg ⁻¹ of MD orally after 1 h of every challenge
Thresher dust+Dex	Rats were sensitized and challenged with 2 mg of wheat and received 1 mg kg ⁻¹ of dexamethasone orally after 1 h of every challenge

model was developed by sensitizing the rats with intraperitoneal (i.p.) injection of allergen with alum at 0 and 7 days and the airway intranasal (i.n.) challenge was done 1 week after the sensitization through nasal inhalation of the allergen once daily for 10 consecutive days. Age-matched male SD rats were used for control.

Rats were divided into seven groups (10 rats/group). Group I served as control and rats were sensitized and challenged with PBS only. In Group II, III and IV, the rats were sensitized with 1, 2 and 3 mg of lyophilized wheat, respectively, emulsified in 50 mg of alum (Pierce Biotechnology, Rockford, IL, USA) in 1 ml PBS and challenged with lyophilized wheat extract (1, 2, 3 mg ml⁻¹ PBS, respectively). Likewise in Group V, VI and VII, the rats were sensitized and challenged with 1, 2 and 3 mg of lyophilized thresher dust, respectively. Rats were killed on day 24 after the last challenge (Figure 1).

Before the operation, the rats were anaesthetized by an intraperitoneal (i.p.) injection of pentobarbital (Nembutal®) (90 mg kg⁻¹). After some time the rats became silent/still and lost the feeling of pain, and were pinned on a cleaned board by placing the ventral side up. Abdominal skin was lifted with a forceps, and cut through with sterilized scissors. The rat was cut along the body midline, from the pubic region to the lower jaw. Immediately after cutting, blood was collected from the abdominal aorta carefully and whole lungs, liver, spleen and kidney were collected respectively.

After optimizing the concentration of wheat and thresher dust by the first AIPI rat model, a second AIPI rat model was developed to evaluate the ameliorative anti-inflammatory effect of MD at three different doses (Table 1). Ovalbumin (OVA; 1 mg) was used to induce pulmonary inflammation in asthmatic control rats (Raza Asim et al.,

2010; Yang et al., 2010) while dexamethasone (Dex; 1 mg kg⁻¹) was used as reference drug (Kumar et al., 2003) to treat the pulmonary inflammation. All the experimental rats were killed at day 24 and blood, bronchoalveolar lavage fluid (BALF), lungs, liver, kidney and spleen were collected for further evaluation of MD at the molecular level.

2.4 | Differential cell count analysis

For the determination of inflammatory cells in the blood of AIPI rats, rat blood was taken immediately after killing the rats and transferred to EDTA K3 (anticoagulant) tubes for blood cell count, kidney and liver function test and gel clot activator tube (ImuMed) for serum. Serum was then separated and stored at -20°C for the estimation of total IgE. Differential blood count including platelets, erythrocytes, leukocytes, haemoglobin and haematocrit was carried out on blood samples by haematological analyser (Abascus junior vet, Diatron, Vienna, Austria).

2.5 | Inflammatory cells in bronchoalveolar lavage fluid

BALF was obtained by infusing and removing three installations of chilled PBS buffer (2 ml) into the lung trachea and recovering it. BALF samples were centrifuged at 22,132 g for 20 min at 4°C, the supernatants were stored at -20°C for analysis of cytokine concentrations, and the pellet was resuspended in 300 µl of saline. Mixed BALF cells were then fixed with 95% ethanol, stained using Wright-Giemsa stain (Sun et al., 2011; Ma, Ma, Fu, & Ma, 2013) and differential cells were counted.

2.6 | Measurement of total serum IgE

The levels of serum total IgE were determined by enzyme linked immunosorbent assay (ELISA) according to protocol (Yang et al., 2010). Briefly, 96-well flat-bottom micro titre plates were coated overnight at 4°C with 2 µg ml⁻¹ mouse anti-rat IgE heavy chain (AbD Serotec, Munich, Germany) in 50 µl NaHCO₃ buffer (pH 9.6). The plates were washed 3 times with washing solution (0.1% Triton X-100 in PBS), and then blocked by incubation with 2% bovine serum albumin in washing solution for 1 h at 37°C. After washing, serum samples diluted 1000× were added and incubated for 1 h at 37°C. IgE was detected with horseradish peroxidase (HRP)-conjugated mouse anti-rat κ/λ chain (1 µg ml⁻¹) antibodies (AbD Serotec) by incubation for 30 min at 37°C. Then H₂O₂ and 3,3',5,5-tetramethylbenzidine were added as substrates and incubated at 37°C for 60 min. The reaction was terminated with 1 M H₂SO₄, and the plates were read at 450 nm with an ELISA reader (Bio-Rad, Hercules, CA, USA). The total IgE levels were expressed as µg ml⁻¹ after comparing the optical density (OD) values to rat IgE standards.

2.7 | Lung histological analysis

Lungs removed from killed rats were insufflated with 10% buffered formalin, fixed in formalin solution and processed. Sections were cut at 6 µm thickness and stained with hematoxylin and eosin (HE) to observe inflammation (McMillan et al., 2004) and periodic acid-Schiff

(PAS) to detect mucus producing goblet cells (Edwan, Perry, Talmadge, & Agrawal, 2004). Pathological alteration in lung tissues was evaluated including infiltration of eosinophils, lymphocytes and macrophages, goblet cell hyperplasia and alveolar thickening. The scoring of lung histology was performed in a blind fashion (Labomed Lx 400, Los Angeles, CA, USA). The pathological index was divided into four grades (0, 1, 2 and 3) for each index according to the severity of inflammation (Raza Asim et al., 2010).

2.8 | Measurement of pro-inflammatory cytokines in BALF

The levels of IL-4, IL-5 and COX-1 were measured in BALF by using a commercial BD Bioscience (San Diego, CA, USA) ELISA kit. All measurements were performed in duplicate. Briefly, the BALF samples were added in duplicate to 96-well plates with 100 µl per well. The appropriate biotin-conjugated antibodies were added to each well. The samples were incubated at room temperature for 2 h. The wells were then aspirated, and each well was washed five times. The substrate solutions were added to each well and were incubated for 30 min at room temperature in the dark. The OD of each well was determined using a microplate reader (Bio-Rad Model 680) that was set to 450 nm. The levels of cytokines were estimated from respective standard curve.

2.9 | mRNA expression of pro-inflammatory cytokines

To check the expression of RNA of pro-inflammatory cytokines by real time RT-PCR, total RNA was isolated from whole lung tissue using the Trizol method. The integrity of the RNA was confirmed by denaturing agarose gel electrophoresis and RNA concentration was quantified by measuring OD. Total cellular RNA was examined for IL-4, IL-5, IL-13, transforming growth factor β (TGF- β), tumour necrosis factor α (TNF- α), COX-1 and COX-2 mRNA by the RT-PCR method according to manufacturer's protocol (RevertAid, Fermentas Life Sciences, International Inc., Ontario, Canada). Briefly, RNA (1 µg) was reverse-transcribed into cDNA using oligo(dT) and heating the mixture at 70°C for 5 min. After that, 5× reaction buffer and RiboLock Inhibitor were added and incubated at 37°C for 5 min. Then, M-MuLV Reverse Transcriptase was added and incubated at 42°C for 60 min. The reaction was stopped by heating at 70°C for 10 min and cDNA was stored at -20°C until further use for PCR to measure the mRNA expression. Primer sequences for markers are given in Table 2.

2.10 | Measurement of T-cell proliferation

Spleen cells were isolated to obtain splenocytes from murine spleens (ProImmune Ltd, Oxford, UK; Shabbir, Shahzad, Ali, & Zia-Ur-Rehman, 2014). Briefly, whole spleens of normal rats were removed and washed with antibiotic (penicillin-streptomycin) containing PBS. Splenocytes were released by placing spleens in 70 µm cell strainers and meshed with the black rubber of a syringe plunger into the Petri dish. The cell suspension was centrifuged at 400 g for 10 min at room temperature and ammonium chloride lysing reagent (0.15 M NH₄Cl, 1 mM KHCO₃,

TABLE 2 Primer sequence of the markers used in real time RT-PCR

Marker	Primer sequence	FP/RP	Reference
β -Actin	5'-CTATCGGCAATGAGCGGTTCC-3'	FP	Asim et al. (2010)
	5'-TGTGTTGGCATAGAGGTCTTTACG-3'	RP	
IL-4	5'-ACAGGAGAAGGGACGCCAT-3'	FP	Lu et al. (2016)
	5'-GAAGCCCTACAGACGAGCTCA'-3	RP	
IL-5	5'-AGCACAGTGGTGAAAGAGACCTT-3'	FP	Lu et al. (2016)
	5'-TCCAATGCATAGCTGGTGATTT-3'	RP	
IL-13	5'-GGAGCTGAGCAACATCACACA-3'	FP	Lu et al. (2016)
	5'-GGTCTGTAGATGGCATTGCA-3'	RP	
TGF- β	5'-GGACTACTACGCCAAAGAAG-3	FP	Asim et al. (2010)
	5'-TCAAAAGACAGCCACTCAGG-3'	RP	
TNF- α	5'-CCTCTTCTATTCTGCTCGT-3'	FP	Shabbir et al. (2014)
	5'-TGAGATCCATGCCATTGGCC-3'	RP	
COX-1	5'-CTGCCCTCTGTACCCAAAGA-3'	FP	Shabbir et al. (2014)
	5'-GGACCCATCTTCCAGAGGT-3'	RP	
COX-2	5'-CCAGATGGCCAGAGGACTCA-3'	RP	Shabbir et al. (2014)
	5'-TGTGAGTCCCAGGGAATAGA-3'	RP	

COX, cyclooxygenase; FP, forward primer; IL, interleukin; RP, reverse primer; TGF- β , transforming growth factor- β ; TNF- α , tumor necrosis factor α .

0.1 mM EDTA) was added. Suspension was left for 3 min in the dark at room temperature and again centrifuged at 400 g for 5 min. The pellet was again suspended and washed with PBS. Suspension was again centrifuged at 400 g for 5 min, and pellet was resuspended in cell culture medium (RPMI-1640, L-glutamate, 10% fetal bovine serum, penicillin-streptomycin). A total of 2×10^4 cells per well were seeded with 100 μ l cell culture medium in 96-well cell culture flat bottom plate and incubated at 37°C for 24 h. Then, the cells were treated with 10 μ g of Con A (a proliferation stimulant), bromodeoxy uridine (BrdU) dissolved in cell culture medium (label), and 1 mg of MD and Dex. The plate was incubated for 24 h at 37°C. A BrdU-DNA assay was conducted according to the kit manufacturer's protocol (Calbiochem/Merck Millipore, Darmstadt, Germany). After incubation, contents of the 96-well plate was removed by inverting over a sink and gentle tapping over tissue paper; 200 μ l of denaturing solution was added to each well and incubated at 37°C for 30 min. The contents of the plate were again removed and anti-BrdU antibody was added to each well. The plate was incubated for 1 h at 37°C. After incubation each well was washed 3 times with wash buffer (1 $\times$) and peroxidase goat anti-mouse IgG HRP conjugate was added to each well. The plate was again incubated for 30 min at 37°C followed by washing 3 times with wash buffer to remove unbound antibody. Whole plate was flooded with dH₂O and the contents were removed by inverting over a sink. Substrate solution was then added and incubated in the dark for 15 min. Stop solution was added to each well in the same order as the substrate solution was added. Absorbance was measured at 450 nm wavelength within 15 min of adding stop solution by using an ELISA reader (Bio-Rad Model 680).

2.11 | Measurement of liver and kidney function

Liver and Kidney functions were determined in blood of AIPI rats, to evaluate any injurious implication of MD on these parameters. A

fully automatic chemical analyser (Olympus, AU-480; Tokyo, Japan) was used for measuring the levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN) and creatinine in blood (Shabbir et al., 2014).

2.12 | Statistical analysis

Statistical analysis was performed with Prism version 7.01 (GraphPad Software Inc., La Jolla, CA, USA) and using one way ANOVA followed by Tukey's multiple comparison test. In cases of severity score non-parametric analysis, the Kruskal-Wallis test was performed to determine the significance of difference among the groups. Whole data are expressed as means $\pm$ SD. The values were considered statistically significant at $P < 0.05$.

3 | RESULTS

3.1 | Differential cell count analysis

Haematological parameters including haemoglobin, inflammatory cells and blood cells were measured in the blood of the first AIPI rat model. Analysis showed that only inflammatory cells including lymphocytes, neutrophils, monocytes and eosinophils were elevated significantly as compared to control in the wheat 1, wheat 2, wheat 3, thresher dust 1, thresher dust 2 and thresher dust 3 groups. No statistical difference was found in the case of lymphocytes in the blood of wheat 1 and thresher dust 1 rats. All other parameters showed no prominent difference as compared to control (Tables 3 and 4).

The haematological parameters were also assessed for the second AIPI rat model to check the efficacy of MD as an immune-modulatory anti-inflammatory agent *in vivo*. The percentage of differential

TABLE 3 Haematological parameters in AIPI rats induced by wheat

Haematological parameters	Control	Wheat 1	Wheat 2	Wheat 3
Haemoglobin (g l ⁻¹)	131 ± 10.6	127 ± 11.9	123 ± 10.6	124 ± 11.02
Lymphocytes (%)	24 ± 3.0	30 ± 4.6*	34 ± 9.9	52 ± 2.4**
Neutrophils (%)	14 ± 4.9	19 ± 2.4**	32 ± 11.9*	53 ± 2.6**
Monocytes (%)	15 ± 2.6	19 ± 3.3**	25 ± 2.0*	36 ± 3.7**
Eosinophils (%)	7.0 ± 1.6	16 ± 3.4**	31 ± 2.9*	41 ± 4.4**
Platelets (10 ³ µl ⁻¹)	335 ± 4.6	321 ± 65	318 ± 91	321 ± 39
Total RBCs (10 ⁶ µl ⁻¹)	8.0 ± 0.11	8.5 ± 0.12	8.5 ± 0.21	8.8 ± 0.15
MCHC (%)	27 ± 3.5	27 ± 6.9	29 ± 7.3	29 ± 5.8
MCV (fl)	54 ± 1.8	53 ± 9.8	48 ± 4.0	44 ± 3.6
MCH (pg)	10 ± 2.1	10 ± 2.3	9.0 ± 2.9	8.0 ± 2.6
HCT (%)	54 ± 1.8	52 ± 3.8	50 ± 4.0	50 ± 3.6

Data are presented as mean ± SD; *n* = 10 for each group. HCT, haematocrit; MCH, mean corpuscular haemoglobin; MCHC, mean cell haemoglobin concentration; MVC, mean corpuscular volume; RBC, red blood cell. **P* < 0.05 and ***P* < 0.01 show statistical difference between two groups.

TABLE 4 Haematological parameters in AIPI rats induced by thresher dust

Haematological parameters	Control	Thresher dust 1	Thresher dust 2	Thresher dust 3
Haemoglobin (g l ⁻¹)	131 ± 12.6	137 ± 14.9	135 ± 14.3	129.16 ± 18.8
Lymphocytes (%)	24 ± 3.0	27 ± 9.4	41 ± 8.6**	48 ± 8.4**
Neutrophils (%)	14 ± 4.9	23 ± 2.6**	29 ± 6.4**	53 ± 11.6***
Monocytes (%)	15 ± 6.6	16 ± 6.0	28 ± 2.3**	38 ± 4.7***
Eosinophils (%)	7.0 ± 1.6	9.0 ± 6.0	21 ± 3.4**	25 ± 2.4**
Platelets (10 ³ µl ⁻¹)	335 ± 4.6	337 ± 63	321 ± 65	299 ± 39
Total RBCs (10 ⁶ µl ⁻¹)	8.0 ± 0.11	9.1 ± 0.39	9.6 ± 0.12	7.8 ± 0.15
MCHC (%)	27 ± 3.5	29 ± 4.8	27 ± 6.9	29 ± 5.8
MCV (fl)	54 ± 1.8	56 ± 3.0	53 ± 4.8	44 ± 9.6
MCH (pg)	10 ± 2.1	13 ± 1.4	10 ± 2.3	9.0 ± 2.6
HCT (%)	54 ± 1.8	61 ± 9.0	52 ± 9.8	50 ± 3.6

Data are presented as mean ± SD; *n* = 10 for each group. HCT, haematocrit; MCH, mean corpuscular haemoglobin; MCHC, mean cell haemoglobin concentration; MVC, mean corpuscular volume; RBC, red blood cell. ***P* < 0.01 and ****P* < 0.001 show statistical difference between two groups.

TABLE 5 Modulative effect of MD on haematological parameters in OVA AIPI rats

Haematological parameters	Control	OVA	OVA+MD1	OVA+MD2	OVA+MD3	OVA+Dex
Haemoglobin (g l ⁻¹)	131 ± 7.6	110 ± 9.6	118 ± 8.6	125 ± 4.9	123 ± 8.9	125 ± 5.9
Lymphocytes (%)	22 ± 3.0	57 ± 2.4***	47 ± 2.9	33 ± 7.6*	29 ± 3.4*	30 ± 6.4*
Neutrophils (%)	14 ± 4.9	65 ± 11.6**	42 ± 12.9	31 ± 12.4*	27 ± 1.6**	20 ± 5.6**
Monocytes (%)	15 ± 6.6	46 ± 4.7**	35 ± 4.0*	21 ± 6.3**	22 ± 4.0**	21 ± 4.0**
Eosinophils (%)	7.0 ± 1.6	49 ± 4.4***	31 ± 8.9*	13 ± 3.4**	11 ± 6.0***	14 ± 6.0**
Platelets (10 ³ µl ⁻¹)	335 ± 4.6	329 ± 39	318 ± 91	319 ± 65	327 ± 72	322 ± 72
Total RBCs (10 ⁶ µl ⁻¹)	8.0 ± 2.11	7.8 ± 1.15	7.5 ± 2.21	7.5 ± 1.12	6.8 ± 2.9	8.2 ± 2.39
MCHC (%)	27 ± 3.5	25 ± 5.8	26 ± 7.3	30 ± 6.9	39 ± 4.8	29 ± 4.8
MCV (fl)	54 ± 3.8	49 ± 3.6	49 ± 4.0	52 ± 3.8	56 ± 3.0	54 ± 3.1
MCH (pg)	10 ± 2.1	07 ± 2.6	09 ± 2.9	11 ± 2.3	14 ± 1.5	13 ± 1.4
HCT (%)	54 ± 1.8	47 ± 3.6	49 ± 4.0	54 ± 3.8	60 ± 7.0	56 ± 4.2

Data are presented as mean ± SD; *n* = 10 for each group. HCT, haematocrit; MCH, mean corpuscular haemoglobin; MCHC, mean cell haemoglobin concentration; MVC, mean corpuscular volume; RBC, red blood cell. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 show statistical difference between two groups.

TABLE 6 Modulative effect of MD on haematological parameters in wheat AIPI rats

Haematological parameters	Control	Wheat	Wheat+MD1	Wheat+MD2	Wheat+MD3	Wheat+Dex
Haemoglobin (g l ⁻¹)	131 ± 10.6	123 ± 11.02	122 ± 10.6	125 ± 11.9	128 ± 12.9	122 ± 9.9
Lymphocytes (%)	24 ± 3.0	51 ± 2.4**	45 ± 9.9	31 ± 4.6*	27 ± 3.4**	29 ± 5.4*
Neutrophils (%)	14 ± 4.9	53 ± 2.6**	32 ± 11.9*	21 ± 2.4**	17 ± 2.6**	18 ± 3.6**
Monocytes (%)	15 ± 2.6	36 ± 3.7**	25 ± 2.0*	19 ± 3.3**	17 ± 4.1**	16 ± 3.1**
Eosinophils (%)	7.0 ± 1.6	41 ± 9.4**	31 ± 2.9*	17 ± 3.4**	9.0 ± 3.0**	14 ± 6.0**
Platelets (10 ³ µl ⁻¹)	335 ± 4.6	321 ± 39	318 ± 91	321 ± 65	337 ± 72	328 ± 72
Total RBCs (10 ⁶ µl ⁻¹)	8.0 ± 0.11	8.8 ± 0.15	8.5 ± 0.21	8.6 ± 0.12	9.1 ± 0.39	8.4 ± 0.39
MCHC (%)	27 ± 3.5	29 ± 5.8	29 ± 7.3	27 ± 6.9	29 ± 4.8	25 ± 4.8
MCV (fl)	54 ± 1.8	44 ± 3.6	48 ± 4.0	53 ± 9.8	57 ± 3.0	55 ± 3.0
MCH (pg)	10 ± 2.1	8.0 ± 2.6	9.0 ± 2.9	10 ± 2.3	13 ± 1.4	12 ± 1.4
HCT (%)	54 ± 1.8	50 ± 3.6	49 ± 4.0	52 ± 3.8	60 ± 3.0	53 ± 3.0

Data are presented as mean ± SD; *n* = 10 for each group. HCT, haematocrit; MCH, mean corpuscular haemoglobin; MCHC, mean cell haemoglobin concentration; MVC, mean corpuscular volume; RBC, red blood cell. **P* < 0.05 and ***P* < 0.01 show statistical difference between two groups.

TABLE 7 Modulative effect of MD on haematological parameters in thresher dust AIPI rats

Haematological parameters	Control	Thresher dust	Thresher dust+MD1	Thresher dust+MD2	Thresher dust+MD3	Thresher dust+Dex
Haemoglobin (g l ⁻¹)	131 ± 12.6	123.16	122 ± 11.6	135 ± 14.3	138 ± 14.9	128 ± 15.9
Lymphocytes (%)	24 ± 3.0	51 ± 8.4**	45 ± 8.9	31 ± 8.6**	27 ± 9.4**	29 ± 9.4**
Neutrophils (%)	14 ± 4.9	53 ± 6.6**	32 ± 8.9*	26 ± 2.4*	17 ± 3.6**	21 ± 2.6**
Monocytes (%)	15 ± 6.6	36 ± 4.7**	25 ± 4.1*	19 ± 5.3**	17 ± 6.0**	16 ± 4.3**
Eosinophils (%)	7.0 ± 1.6	41 ± 10.4**	35 ± 2.9	17 ± 3.4**	9.0 ± 6.0**	14 ± 6.0**
Platelets (10 ³ µl ⁻¹)	335 ± 4.6	299 ± 39	318 ± 91	321 ± 65	327 ± 63	328 ± 71
Total RBCs (10 ⁶ µl ⁻¹)	8.0 ± 0.11	7.8 ± 0.15	8.5 ± 0.21	9.6 ± 0.12	9.1 ± 0.39	8.4 ± 0.39
MCHC (%)	27 ± 3.5	29 ± 5.8	29 ± 7.3	27 ± 6.9	29 ± 4.8	25 ± 4.8
MCV (fl)	54 ± 1.8	44 ± 3.6	48 ± 4.0	53 ± 3.8	57 ± 3.0	55 ± 3.0
MCH (pg)	10 ± 2.1	8.0 ± 2.6	9.0 ± 2.9	10 ± 2.3	13 ± 1.4	12 ± 1.4
HCT (%)	54 ± 1.8	50 ± 3.6	49 ± 4.0	52 ± 3.8	60 ± 3.0	53 ± 3.0

Data are presented as mean ± SD; *n* = 10 for each group. HCT, haematocrit; MCH, mean corpuscular haemoglobin; MCHC, mean cell haemoglobin concentration; MVC, mean corpuscular volume; RBC, red blood cell. **P* < 0.05 and ***P* < 0.01 show statistical difference between two groups.

inflammatory cells increased in OVA-, wheat- and thresher dust-induced rats and was suppressed significantly by MD at the dose of 10 and 20 mg kg⁻¹ while MD at a dose of 5 mg kg⁻¹ in AIPI rats did not suppress the differential inflammatory cells significantly. Furthermore, no change was observed in other haematological parameters, i.e. haemoglobin, platelets, mean cell haemoglobin concentration, mean corpuscular volume, mean corpuscular haemoglobin and haematocrit (Tables 5–7).

3.2 | Inflammatory cells in bronchoalveolar lavage fluid

Inflammatory cells including totals cells, lymphocytes, macrophages, neutrophils and eosinophils were stained and counted in the BALF of both AIPI rat models. The results for the first AIPI model showed that total cells in BALF of rats immunized and challenged with wheat (*P* < 0.05) and thresher dust (*P* < 0.05) were increased significantly compared with rats in control group at both concentrations (2 and

3 mg ml⁻¹). No significant difference was found in the case of eosinophils, lymphocytes, macrophages and neutrophils in BALF of both groups, i.e. wheat 1 and thresher dust 1. In BALF the inflammatory cell infiltration increased significantly (*P* < 0.01) in wheat 2, wheat 3, thresher dust 2 and thresher dust 3 groups compared with control except for lymphocytes in thresher dust 3 rats where the difference was found to be increased (*P* < 0.001) (Figures 2, 3).

In the second AIPI rat model the total inflammatory cells in the BALF induced by OVA, wheat and thresher dust were increased significantly (*P* < 0.001) compared with control rats (Figures 4a, 5a, 6a). Eosinophils, lymphocytes, macrophages, neutrophils and total cells in the BALF of rats induced by wheat were also increased significantly compared with rats in control group. In the BALF of AIPI rats exposed to OVA, the differential inflammatory cells and total cells were decreased significantly when treated with MD in the OVA+MD2 (10 mg kg⁻¹) and OVA+MD3 (20 mg kg⁻¹) groups (Figure 4b–e). Inflammatory cells including eosinophils, lymphocytes, macrophages and neutrophils and total cells were diminished significantly in the

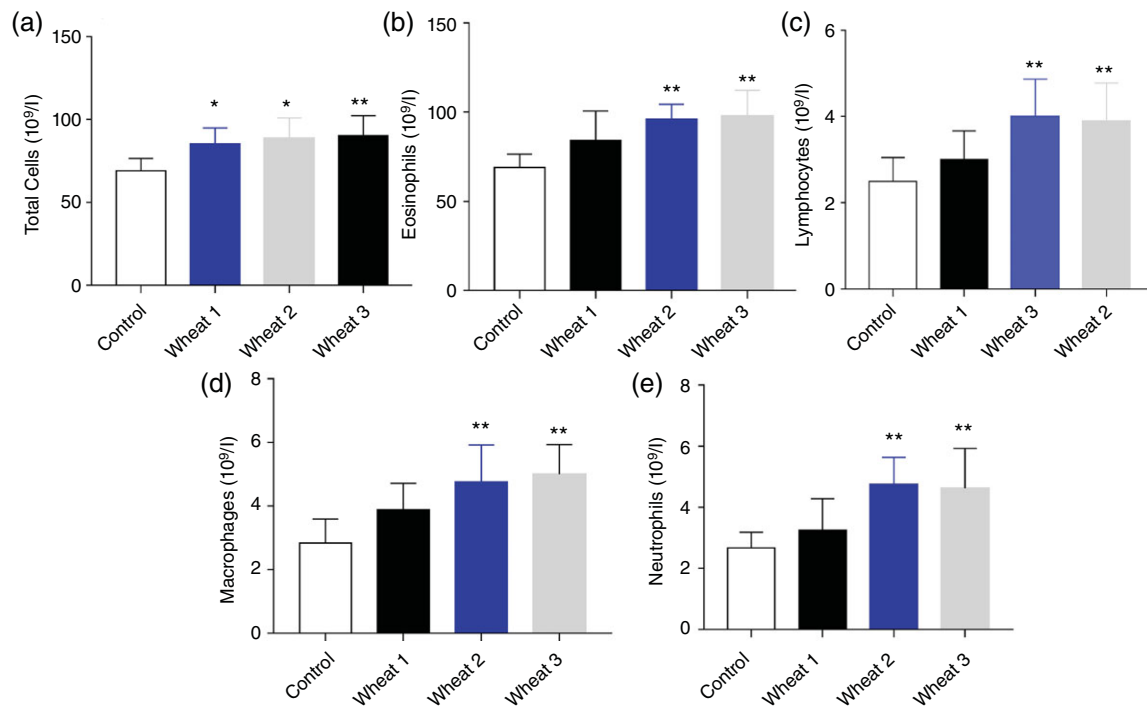


FIGURE 2 Inflammatory cells in BALF of wheat AIPI rat group. AIPI rats challenged with three doses of wheat: wheat 1 (1 mg), wheat 2 (2 mg) and wheat 3 (3 mg). (a) Total inflammatory cells, (b) eosinophils, (c) lymphocytes, (d) macrophages, and (e) neutrophils. Data are mean $\pm$ SD. * P < 0.05, ** P < 0.01 show statistical difference between two groups

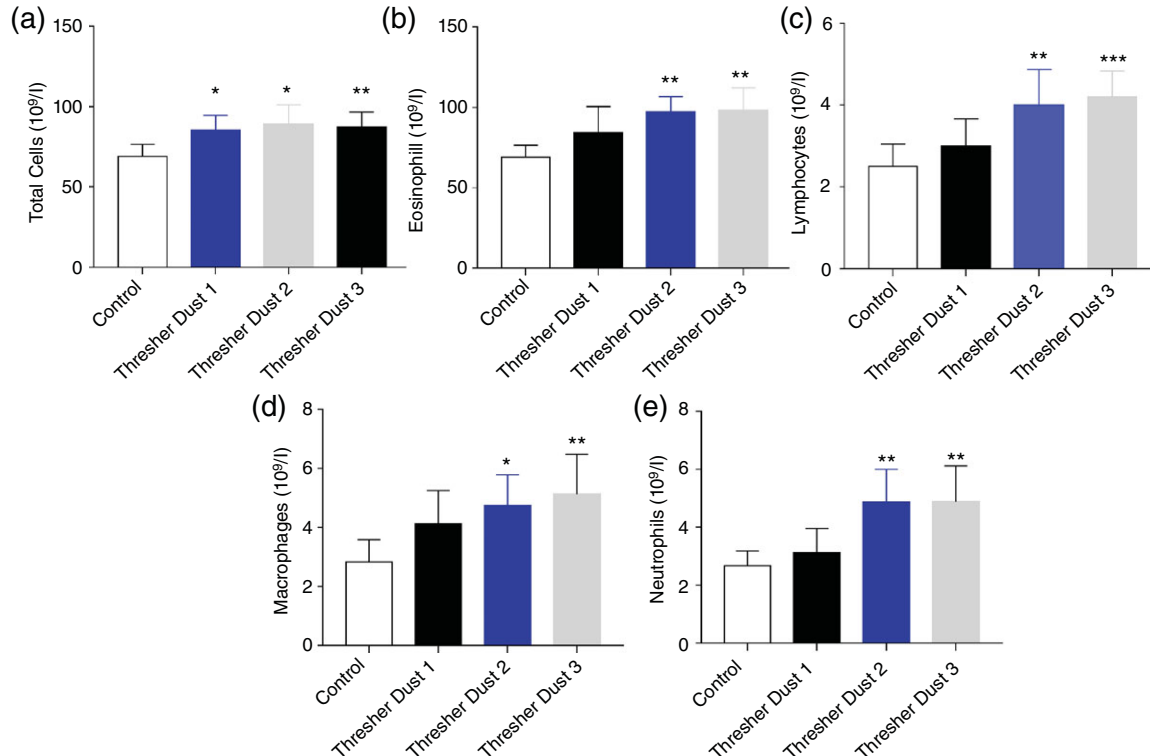


FIGURE 3 Inflammatory cells in BALF of thresher dust AIPI rat group. AIPI rats challenged with three doses of thresher dust: thresher dust 1 (1 mg), thresher dust 2 (2 mg) and thresher dust 3 (3 mg). (a) Total inflammatory cells, (b) eosinophils, (c) lymphocytes, (d) macrophages, and (e) neutrophils. Data are mean $\pm$ SD. * P < 0.05, ** P < 0.01, *** P < 0.001 show statistical difference between two groups

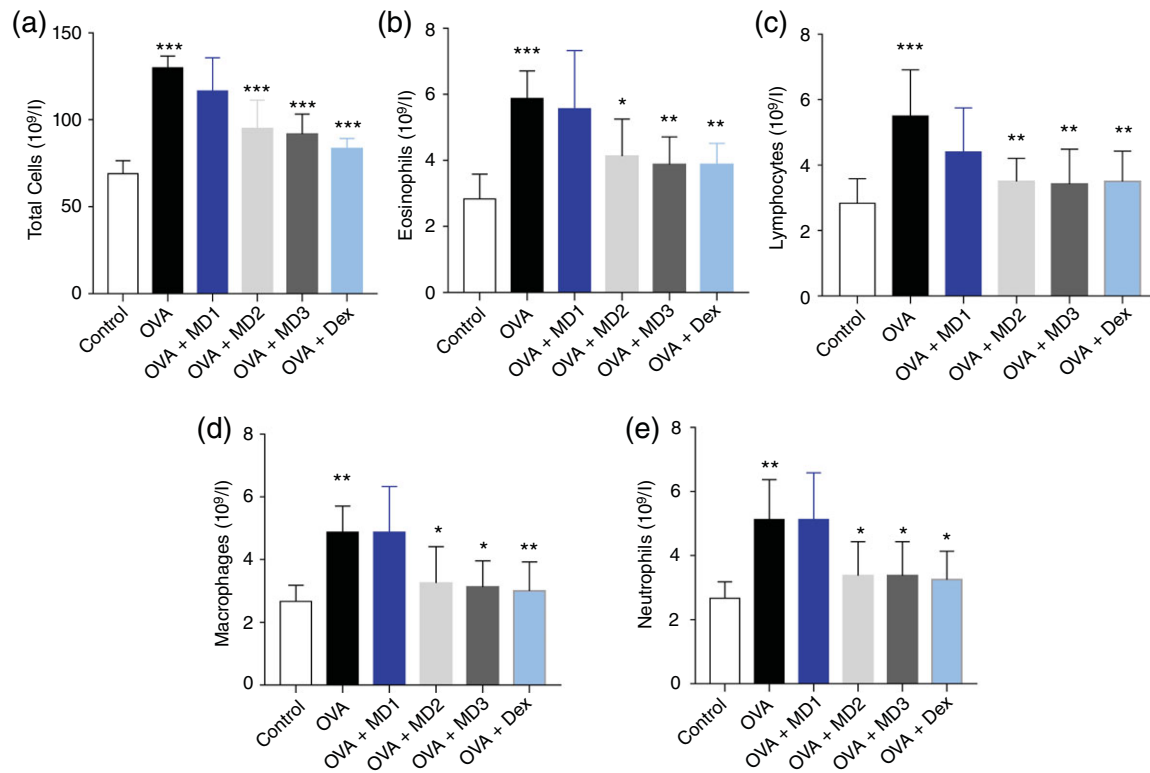


FIGURE 4 Inflammatory cell count in BALF of OVA AIPI rat group. Rats were divided into 6 groups: control, OVA sensitized, OVA sensitized and received MD at doses MD1 (5 mg kg^{-1}), MD2 (10 mg kg^{-1}) and MD3 (20 mg kg^{-1}), and dexamethasone (Dex; 1 mg kg^{-1}). (a) Total cells, (b) eosinophils, (c) lymphocytes, (d) macrophages, and (e) neutrophils. Data are mean $\pm$ SD. $n = 10$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ show statistical difference between two groups

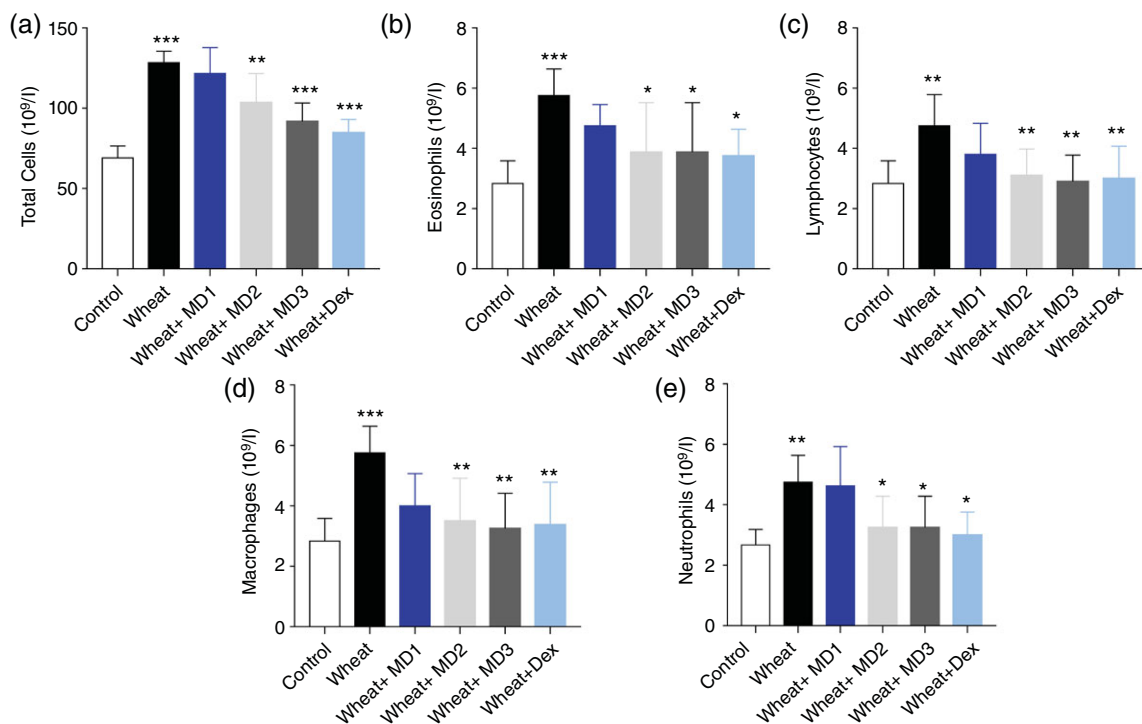


FIGURE 5 Inflammatory cell count in BALF of wheat AIPI rat group. Rats were divided into 6 groups: control, wheat sensitized, wheat sensitized and received MD at doses MD1 (5 mg kg^{-1}), MD2 (10 mg kg^{-1}) and MD3 (20 mg kg^{-1}), and dexamethasone (Dex; 1 mg kg^{-1}). (a) Total cells, (b) eosinophils, (c) lymphocytes, (d) macrophages, and (e) neutrophils. Data shown as mean $\pm$ SD. $n = 10$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ show statistical difference between two groups

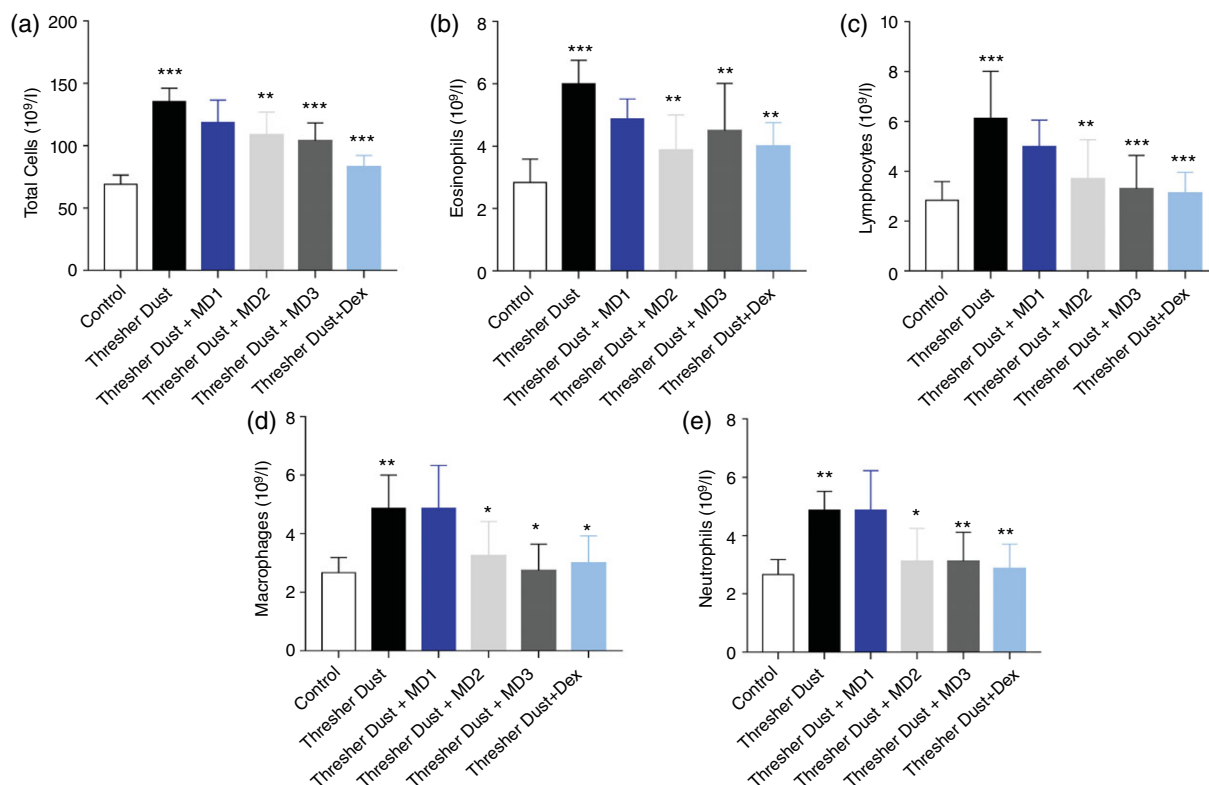


FIGURE 6 Inflammatory cell count in BALF of thresher dust AIPI rat group. Rats were divided into 6 groups: control, thresher dust sensitized and received MD at doses MD1 (5 mg kg⁻¹), MD2 (10 mg kg⁻¹) and MD3 (20 mg kg⁻¹), and dexamethasone (Dex; 1 mg kg⁻¹). (a) Total cells, (b) eosinophils, (c) lymphocytes, (d) macrophages, and (e) neutrophils. Data shown as mean $\pm$ SD. $n = 10$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ show statistical difference between two groups

BALF of AIPI rats induced by wheat and treated with MD in the wheat+MD2 and wheat+MD3 groups (Figure 5b–e). Moreover, MD also suppressed significantly the differential inflammatory cells in the BALF of rats exposed to thresher dust when used at a dose of 10 and 20 mg kg⁻¹ (Figure 6b–e).

3.3 | ELISA for total serum IgE

Total serum IgE was determined by ELISA in the serum of AIPI rats. Results for the first AIPI rat model showed that the level of serum IgE increased significantly in AIPI rats sensitized and challenged with 2 and 3 mg of wheat ($P < 0.01$) compared with rats exposed to PBS. No significant increased level of IgE was found in wheat 1 rats compared with rats in control group. Almost similar results were also observed in the case of thresher dust AIPI rats except for the rats exposed to thresher dust 3 where we found significant difference ($P < 0.001$; Figure 7).

In the second AIPI rat model, ELISA was used to estimate the suppressive effect of the novel synthesized compound MD on the IgE level in the serum. IgE level was increased in the serum of rats induced by OVA ($P < 0.001$), wheat flour ($P < 0.001$) and thresher dust ($P < 0.001$) compared with rats in control group sensitized and challenged with PBS. MD inhibited the airway inflammation by decreasing significantly IgE level in the serum of AIPI rats belonging to the OVA+MD2 ($P < 0.01$), OVA+MD3 ($P < 0.01$), wheat+MD2 ($P < 0.05$), wheat+MD3 ($P < 0.01$), thresher dust+MD2 ($P < 0.05$) and

thresher dust+MD3 ($P < 0.05$) groups when compared with the IgE level measured in the serum of rats in control group. No statistical difference was observed between the AIPI rats treated with 5 mg kg⁻¹ of MD (Figure 8a–c).

3.4 | Lung histological analysis

Pathological alterations were determined in the lung of the AIPI model induced by wheat and thresher dust by H&E staining. The results of the first model showed massive inflammation in the rats of the wheat 2, wheat 3, thresher dust 2 and thresher dust 3 groups. Very light pathological changes were seen the lungs of rats in the wheat 1 and thresher dust 1 groups (Figure 9).

Results of the second AIPI rat model showed that wheat and thresher dust extracts induced prominent and significant airway allergy in rats. The results of HE staining showed massive recruitment of inflammatory cells around the airway, alveolar spaces and perivascular areas in the rats' lungs immunized and challenged with OVA, wheat and thresher dust as compared with control rats, while there were no inflammatory abnormalities seen in the lungs of the control group rats exposed to PBS. AIPI rats induced by OVA, wheat and thresher dust with MD significantly reduced the airway inflammation by decreasing the inflammatory cell infiltration in the airways, alveoli and perivascular areas when MD was used at dose of 10 and 20 mg kg⁻¹ (Figures 10–12).

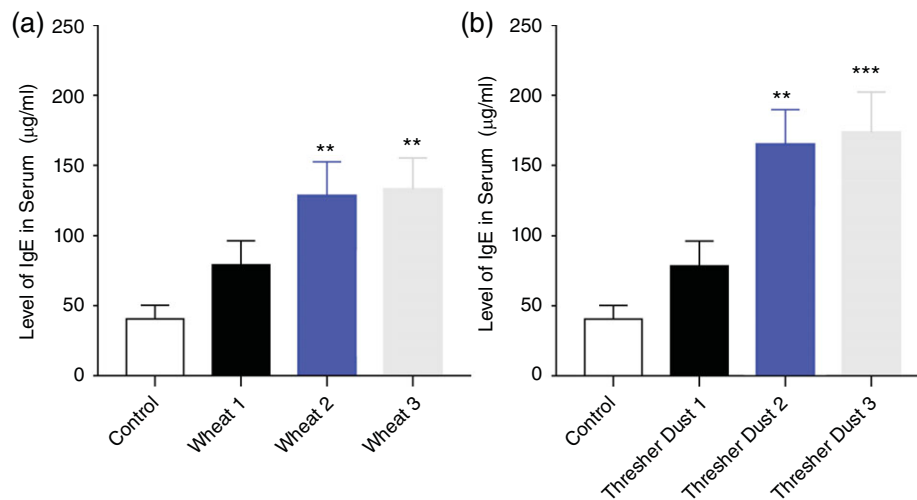


FIGURE 7 Total serum IgE level. (a) Total serum IgE level of wheat AIPI rats. (b) IgE level in serum of thresher dust AIPI rats. Data shown as mean $\pm$ SD. $n = 10$. ** $P < 0.01$, *** $P < 0.001$ show statistical difference between two groups

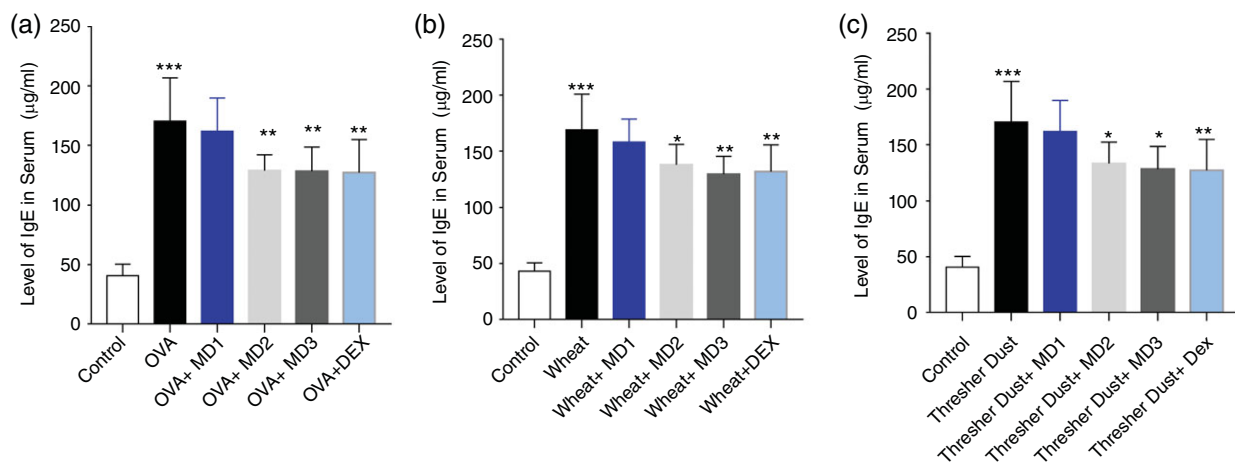


FIGURE 8 Total IgE level in Serum of AIPI rats. Effects of different concentrations of MD, MD1 (5 mg kg⁻¹), MD2 (10 mg kg⁻¹), MD3 (20 mg kg⁻¹), and dexamethasone (Dex; 1 mg kg⁻¹) on level of serum IgE in AIPI. (a) OVA, (b) wheat flour, and (c) thresher dust. Data shown as mean $\pm$ SD. $n = 10$ in each group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ show statistical difference between two groups

Dose-dependent suppression was seen in the histological score for inflammatory cell infiltration in the lungs of allergen-exposed rats treated with MD while the severity score was not significantly changed in all AIPI rats at a dose of 5 mg kg⁻¹ of MD (Figure 13).

The successfulness of our AIPI rat model was further assessed by PAS staining of goblet cells and mucus in the airways. From histological images it was observed that there were qualitatively more goblet cells in the airways of allergen-induced rats as compared to control rats. Histological images showed decrease goblet cell hyperplasia prominently with 10 and 20 mg kg⁻¹ of MD when compared with AIPI rats (Figures 14–16).

3.5 | ELISA and qRT-PCR for pro-inflammatory cytokines in BALF

ELISA and real time RT-PCR were carried out to evaluate the level of pro-inflammatory cytokines in BALF and their mRNA expression in

the lungs of AIPI rats. In the first AIPI rat model, the results showed a significantly increased level of IL-4 cytokine in wheat 2 ($P < 0.01$), wheat 3 ($P < 0.001$), thresher dust 2 ($P < 0.01$) and thresher dust 3 ($P < 0.01$) groups when compared with control. No significant differences were seen between the wheat 1 and thresher dust 1 group when compared with control. Furthermore, mRNA expression of IL-4 increased in lungs of AIPI rats with increasing concentration of allergen ($P < 0.05$). No significant increase was found between the control and thresher dust 1 group (Figure 17).

In the second AIPI rat model, the levels of IL-4, IL-5 and COX-1 were evaluated through ELISA in BALF of AIPI rats with three doses of MD. Level of IL-4, IL-5 and COX-1 were found to be increased in AIPI rats exposed to OVA, wheat and thresher dust while MD at dose of 10 and 20 mg kg⁻¹ reversed the amplified levels of IL-4, IL-5 and COX-1 (Figures 18–20).

The molecular basis of the effects of AIPI rats given compound MD was revealed by mRNA expression of T-helper cell cytokines

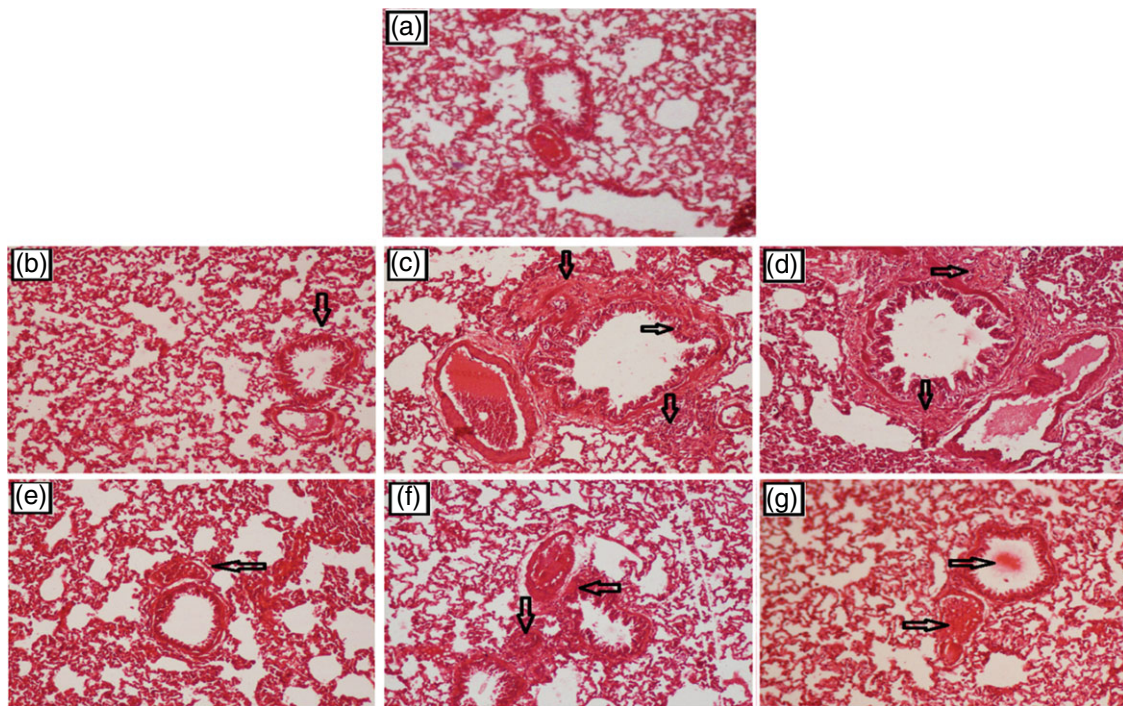


FIGURE 9 Pathological changes in lung of AIPI rats. Three doses of wheat and thresher dust allergens were used to evaluate the airway inflammation in rats; lung tissues were stained with H&E. (a) Control group rats, (b) wheat 1 rat group, (c) wheat 2 rat group, (d) wheat 3 rat group, (e) thresher dust 1 rat group, (f) thresher dust 2 rat group, and (g) thresher dust 3 rat group. $n = 10$ for each group. Arrow shows inflammatory cell infiltration in the airways

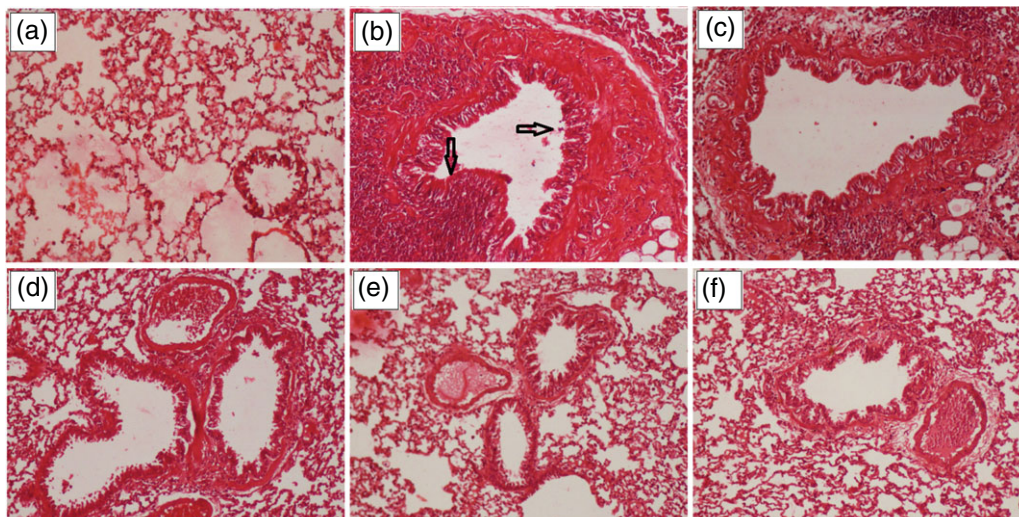


FIGURE 10 HE staining of lung sections for OVA AIPI rat group. The AIPI rats were immunized and challenged with OVA and received MD1 (5 mg kg^{-1}), MD2 (10 mg kg^{-1}), MD3 (20 mg kg^{-1}) and dexamethasone (Dex; 1 mg kg^{-1}). (a) Control, (b) OVA sensitized, (c) OVA sensitized and received MD1, (d) OVA sensitized and received MD2, (e) OVA sensitized and received MD3, (f) OVA sensitized and received Dex. Arrow shows inflammatory cell infiltration in the airways

by real time RT-PCR. The relative mRNA expression of IL-4, IL-5, IL-13 and TGF- β was significantly enhanced in the rats sensitized by OVA, wheat and thresher dust as compared to control rats. MD successfully reversed the amplified mRNA gene expression of IL-4, IL-5, IL-13 and TGF- β markedly in the lungs of AIPI rats at doses of 10 and 20 mg kg^{-1} . No effect of MD at a dose of 5 mg kg^{-1}

was seen on these cytokines in the lungs of all groups of AIPI rats (Figures 21–24).

No effect of allergens was observed on the mRNA expression of IFN- γ and TNF- α in the lung of AIPI rats (Figures 25, 26). Furthermore, COX mRNA expression was also checked in the AIPI rats. Results showed that COX-1 and COX-2 increased significantly

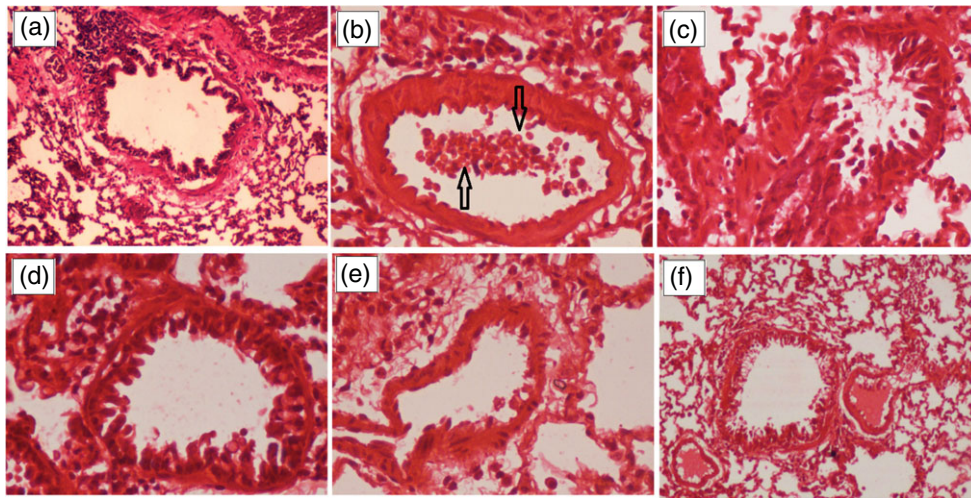


FIGURE 11 HE staining of lung sections for wheat AIPI rat group. AIPI rats were immunized and challenged with wheat and received MD1 (5 mg kg^{-1}), MD2 (10 mg kg^{-1}), MD3 (20 mg kg^{-1}) and dexamethasone (Dex; 1 mg kg^{-1}). (a) Control, (b) wheat sensitized, (c) wheat sensitized and received MD1, (d) wheat sensitized and received MD2, (e) wheat sensitized and received MD3, (f) wheat sensitized and received Dex. Arrow shows inflammatory cell infiltration in the airways

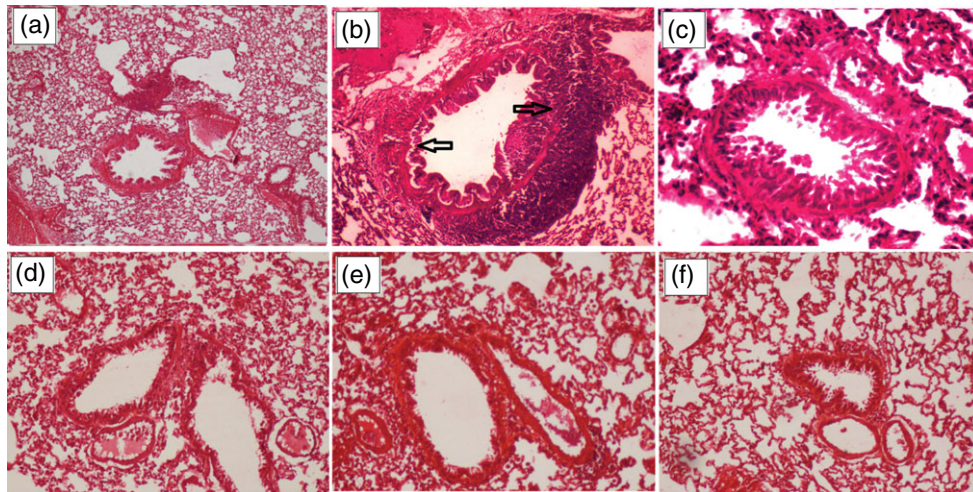


FIGURE 12 HE staining of lung sections for thrasher dust AIPI rat group. AIPI rats were immunized and challenged with thrasher dust and received MD1 (5 mg kg^{-1}), MD2 (10 mg kg^{-1}), MD3 (20 mg kg^{-1}) and dexamethasone (Dex; 1 mg kg^{-1}). (a) Control, (b) thrasher dust sensitized, (c) thrasher dust sensitized and received MD1, (d) thrasher dust sensitized and received MD2, (e) thrasher dust sensitized and received MD3, (f) thrasher dust sensitized and received Dex. Arrow shows inflammatory cell infiltration in the airways

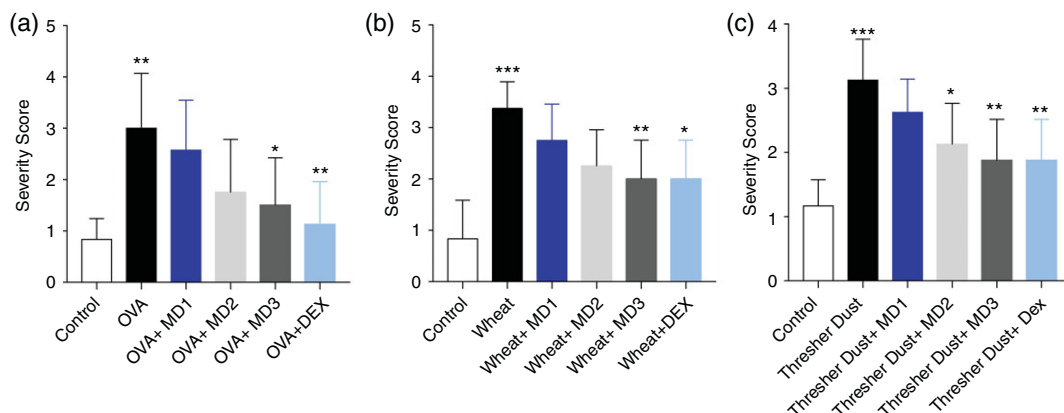


FIGURE 13 Severity score of inflammatory cell infiltrations in the airways and alveoli of AIPI rats. (a) OVA AIPI rat group, (b) wheat AIPI rat group, and (c) thrasher dust AIPI rat group. Data shown as mean $\pm$ SD. $n = 10$ rats/group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ show statistical difference between two groups

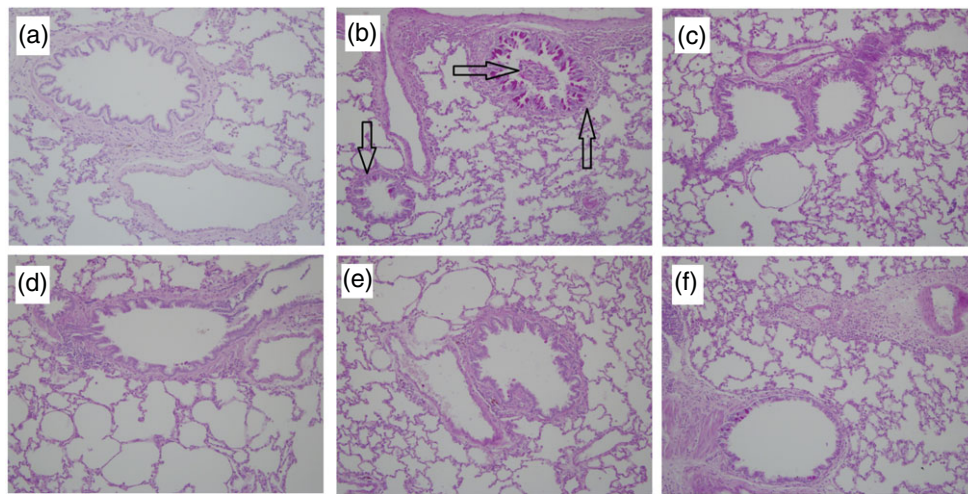


FIGURE 14 PAS staining of lung sections for goblet cells in OVA AIPI rat group. AIPI rats were sensitized with OVA and received MD1 (5 mg kg^{-1}), MD2 (10 mg kg^{-1}), MD3 (20 mg kg^{-1}) and dexamethasone (Dex; 1 mg kg^{-1}). (a) Control, (b) OVA sensitized, (c) OVA sensitized and received MD1, (d) OVA sensitized and received MD2, (e) OVA sensitized and received MD3, (f) OVA sensitized and received Dex. Arrows show goblet cells in airways

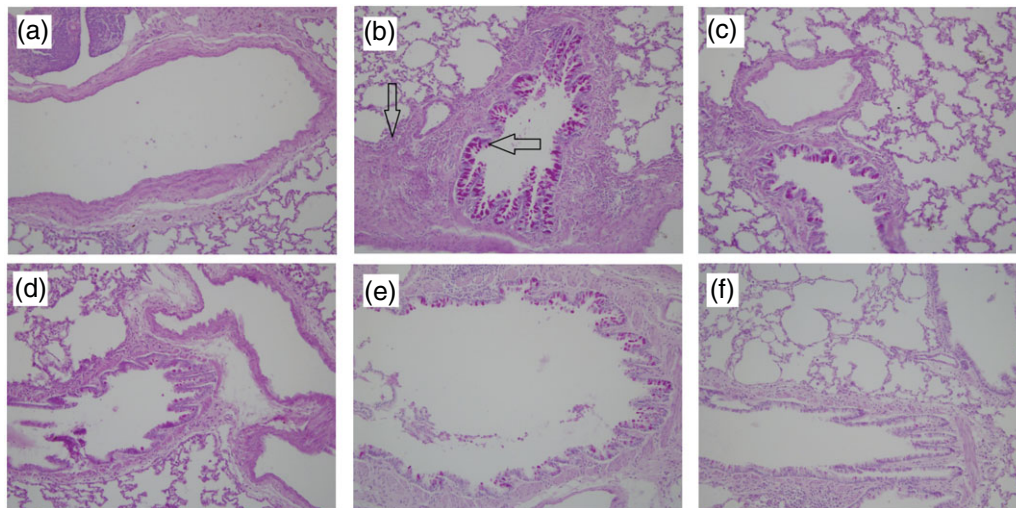


FIGURE 15 PAS staining of lung sections for goblet cells in wheat AIPI rat group. AIPI rats were sensitized with wheat and received MD1 (5 mg kg^{-1}), MD2 (10 mg kg^{-1}), MD3 (20 mg kg^{-1}), and dexamethasone (Dex; 1 mg kg^{-1}). (a) Control, (b) wheat sensitized, (c) wheat sensitized and received MD1, (d) wheat sensitized and received MD2, (e) wheat sensitized and received MD3, (f) wheat sensitized and received Dex. Arrows show goblet cells in airways

in the rats exposed to OVA, wheat and thresher dust and this increase in the mRNA expression was suppressed significantly when treated with MD at the doses of 10 and 20 mg kg^{-1} . Minimum suppression ($P < 0.05$) was observed by MD at both doses characterized by in AIPI rats while MD at dose 5 mg kg^{-1} showed no significant suppressive effects on COX-1 and COX-2 gene expression (Figures 27, 28).

3.6 | Liver and kidney function evaluation

AST, ALT, BUN and creatinine levels were estimated in blood of AIPI rats. ASL, ALT and creatinine were increased in the OVA+MD3 rat group but no change was observed in the case of BUN (Table 8). In the case of the wheat+MD3 and thresher dust+MD3 rat groups, ASL and

ALT were increased markedly in AIPI rats when compared with control rats (Tables 9, 10). Furthermore, no change was observed in either liver or kidney function tests in the AIPI rats exposed to OVA, wheat and thresher dust and given Dex, as reference drug.

3.7 | Effect on T-cell proliferation

Spleen of normal rats was used to determine the effect of OVA, wheat and thresher dust allergens on T-cell proliferation. The results of BrdU for the T-cells isolated from spleen showed that these allergens increased significantly the T-cell proliferation ($P < 0.01$) after their exposure. MD (10 mg kg^{-1}) and Dex (1 mg kg^{-1}) suppressed the T-cell proliferation significantly when compared to control (Figure 29).

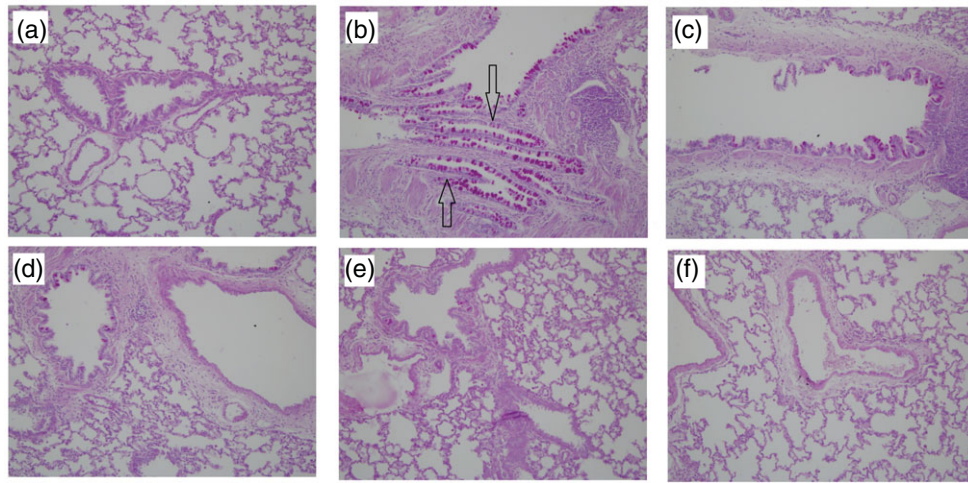


FIGURE 16 PAS staining of lung sections for goblet cells in thresher dust AIPI rat group. AIPI rats were sensitized with thresher dust and received MD1 (5 mg kg^{-1}), MD2 (10 mg kg^{-1}), MD3 (20 mg kg^{-1}), and dexamethasone (Dex; 1 mg kg^{-1}). (a) Control, (b) thresher dust sensitized, (c) thresher dust sensitized and received MD1, (d) thresher dust sensitized and received MD2, (e) thresher dust sensitized and received MD3, (f) thresher dust sensitized and received Dex. Arrows show goblet cells in airways

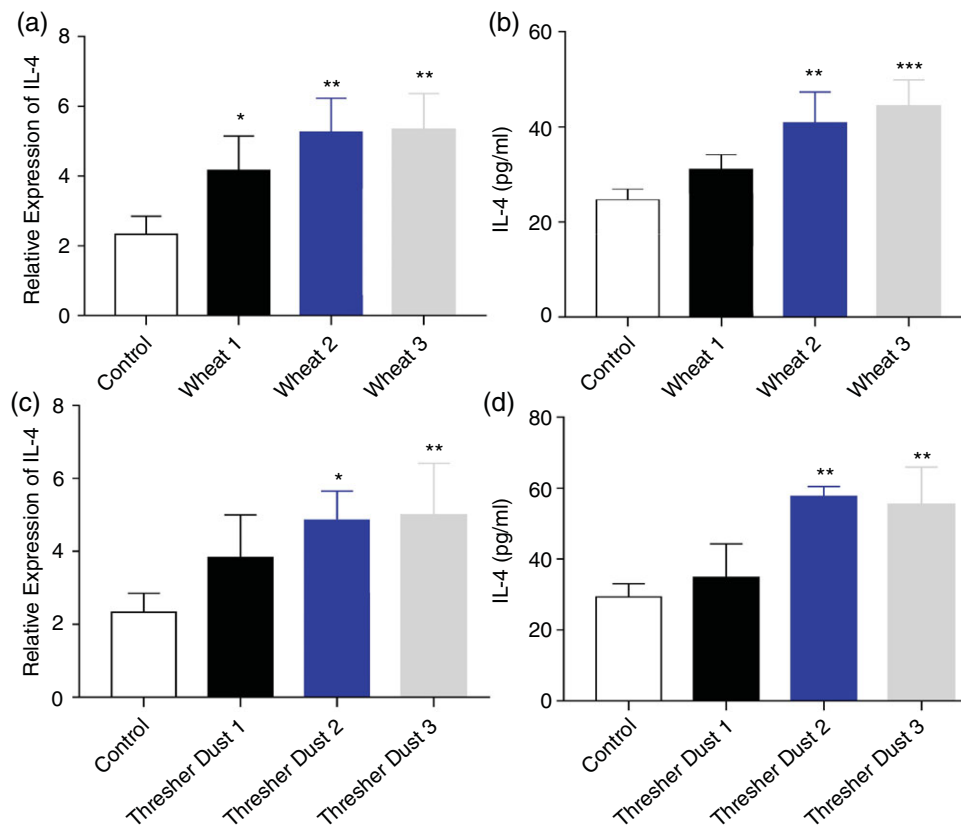


FIGURE 17 Relative expression and serum level of IL-4 cytokine. Total level of IL-4 cytokine was measured by ELISA, and mRNA level of IL-4 was estimated by RT-PCR. (a) Relative mRNA expression of IL-4 gene in wheat group rats, (b) total IL-4 protein level in wheat group rats, (c) relative mRNA expression of IL-4 gene in thresher dust group rats, and (d) Total IL-4 protein level in thresher dust group rats. Data shown as mean $\pm$ SD. $n = 10$ in each group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ show statistical difference between two groups

4 | DISCUSSION

Pulmonary airway inflammation is inflammatory cell infiltration in the lung airways, hyper-mucus secretion and enhanced IgE level

(Woodruff et al., 2009). To investigate the molecular basis of allergenic effects of wheat and thresher dust proteins, we have developed an AIPI rat model sensitized and challenged with wheat and thresher dust extracts. PBS was used to extract the protein from wheat and

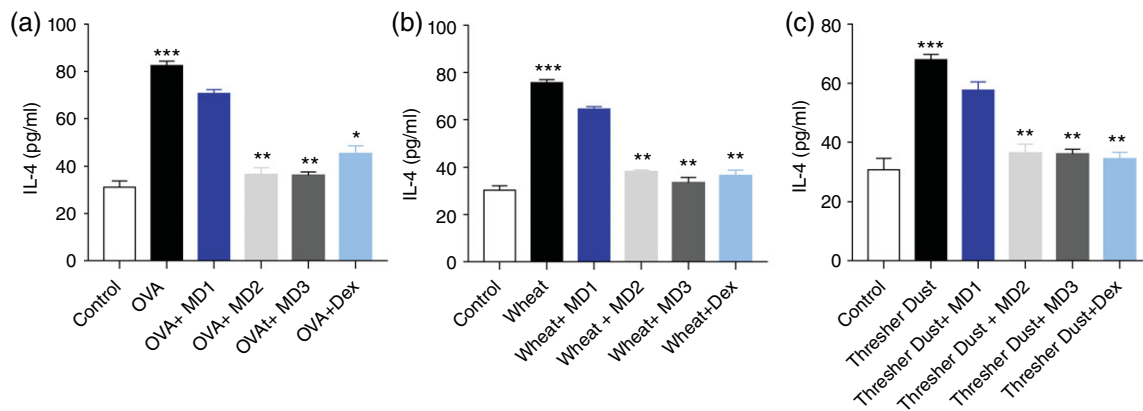


FIGURE 18 IL-4 level in AIPI rats. Anti-inflammatory effects of MD1 (5 mg kg^{-1}), MD2 (10 mg kg^{-1}) and MD3 (20 mg kg^{-1}) were determined in AIPI rats while dexamethasone (Dex; 1 mg kg^{-1}) was used as reference. (a) OVA AIPI rat group, (b) wheat AIPI rat group, and (c) thresher dust AIPI rat group. Data shown as mean $\pm$ SD. $n = 10$ in each group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ show significant difference between two groups

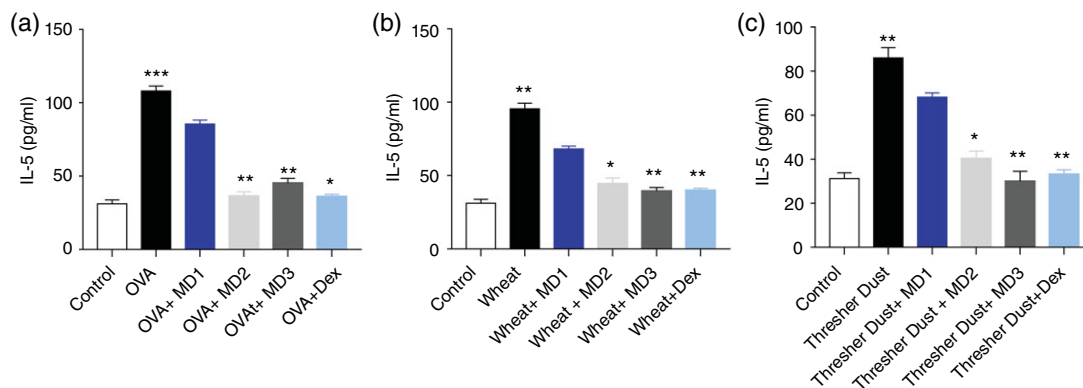


FIGURE 19 IL-5 level in AIPI rats. Anti-inflammatory effects of MD1 (5 mg kg^{-1}), MD2 (10 mg kg^{-1}) and MD3 (20 mg kg^{-1}) were determined in the AIPI rats while dexamethasone (Dex; 1 mg kg^{-1}) was used as reference. (a) OVA AIPI rat group, (b) wheat AIPI rat group, and (c) thresher dust AIPI rat group. Data shown as mean $\pm$ SD. $n = 10$ in each group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ show significant difference between two groups

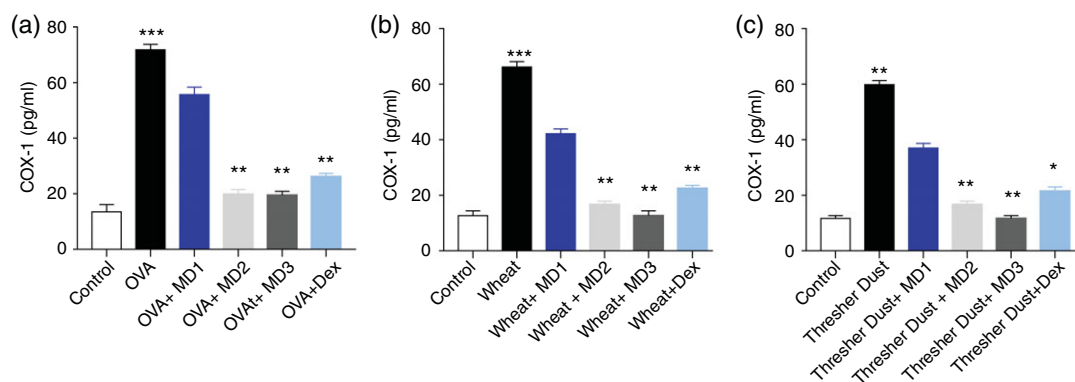


FIGURE 20 COX-1 level in AIPI rats. Anti-inflammatory effects of MD1 (5 mg kg^{-1}), MD2 (10 mg kg^{-1}) and MD3 (20 mg kg^{-1}) were determined in the AIPI rats while dexamethasone (Dex; 1 mg kg^{-1}) was used as reference. (a) OVA AIPI rat group, (b) wheat AIPI rat group, and (c) thresher dust AIPI rat group. Data shown as mean $\pm$ SD. $n = 10$ in each group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ show significant difference between two groups

thresher dust samples, which was estimated using Lowry's method (Lowry, Rosebrough, Farr, & Randall, 1951). It was estimated that there was 994.7 and 980.5 $\mu\text{g } \mu\text{l}^{-1}$ protein found in samples of wheat and thresher dust, respectively. We previously found that the protein fraction in wheat and thresher dust responsible for

allergy in asthmatic patient falls between 10 and 15 kDa. In this region, α -amylase/trypsin or thioredoxin H protein fractions are present (Battais, Richard, Jacquenet, Denery-Papini, & Moneret-Vautrin, 2008) and these fractions might be involved in AIPI in rats. A previously established method for the AIPI model was used with

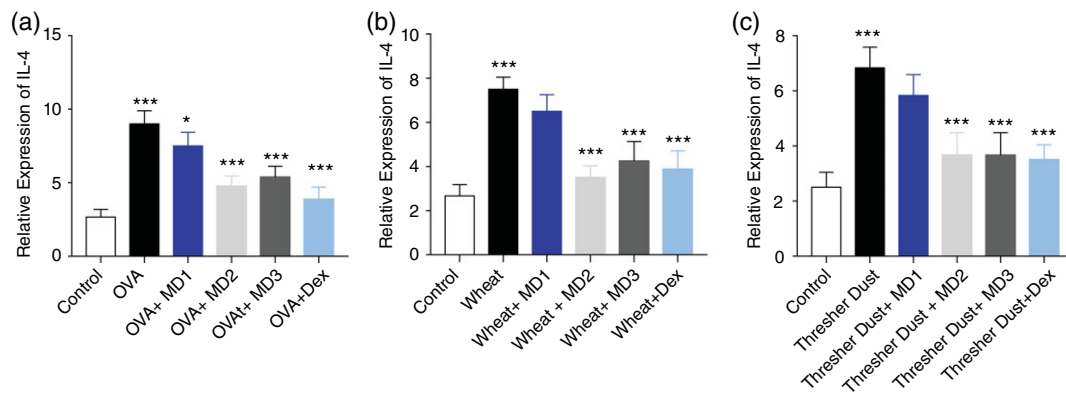


FIGURE 21 mRNA expression of IL-4 cytokine. Quantitative real time RT-PCR was used to determine the IL-4 expression in lung of AIPI rat that received MD at doses MD1 (5 mg kg⁻¹), MD2 (10 mg kg⁻¹) and MD3 (20 mg kg⁻¹). β -Actin was used to find the relative expression. (a) OVA AIPI rat group, (b) wheat AIPI rat group, and (c) thresher dust AIPI rat group. Data shown as mean $\pm$ SD. $n = 10$ in each group. * $P < 0.05$, *** $P < 0.001$ show the statistical difference between two groups

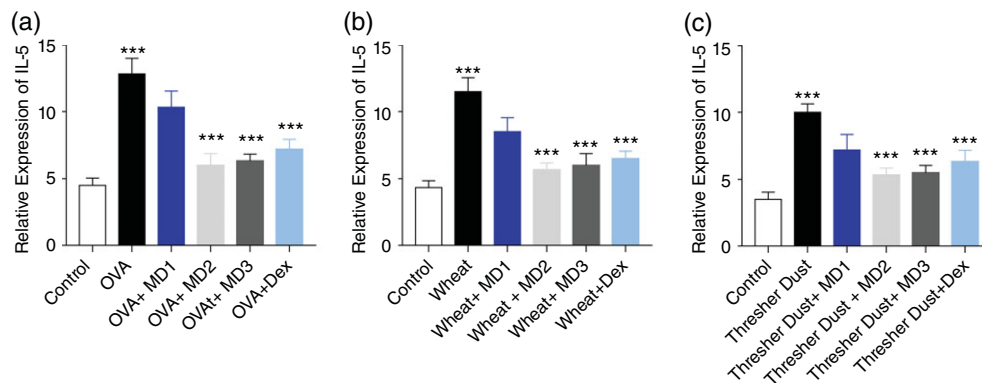


FIGURE 22 mRNA expression of IL-5 cytokine. Quantitative real time RT-PCR was used to determine the IL-5 expression in lung of AIPI rat that received MD at doses MD1 (5 mg kg⁻¹), MD2 (10 mg kg⁻¹) and MD3 (20 mg kg⁻¹). β -Actin was used to find the relative expression. (a) OVA AIPI rat group, (b) wheat AIPI rat group, and (c) thresher dust AIPI rat group. Data shown as mean $\pm$ SD. $n = 10$ in each group. *** $P < 0.001$ shows the statistical difference between two groups

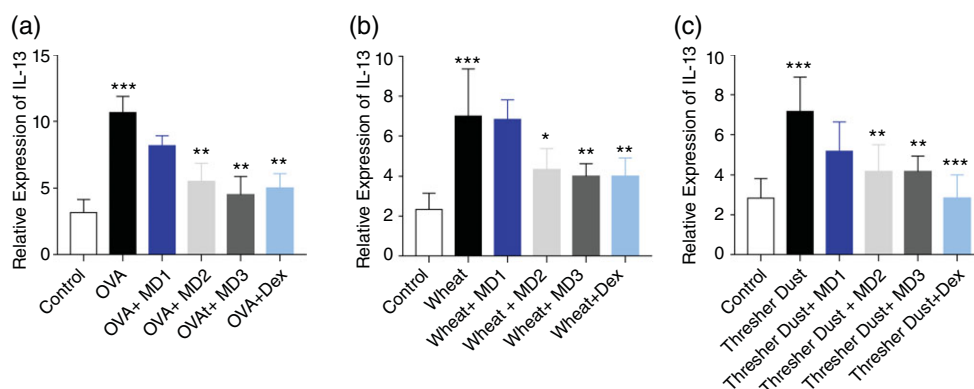


FIGURE 23 mRNA expression of IL-13 cytokine. Quantitative real time RT-PCR was used to determine the IL-13 expression in lung of AIPI rat that received MD at doses MD1 (5 mg kg⁻¹), MD2 (10 mg kg⁻¹) and MD3 (20 mg kg⁻¹). β -Actin was used to find the relative expression. (a) OVA AIPI rat group, (b) wheat AIPI rat group, and (c) thresher dust AIPI rat group. Data shown as mean $\pm$ SD. $n = 10$ in each group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ show the statistical difference between two groups

some alterations, in which a mixture of OVA and alum was used on the first day to sensitized (i.p.) the rats and then after 1 week of sensitization, rats were challenged (i.n.) with OVA for 7 consecutive days (Raza Asim et al., 2010). In the AIPI rat model, the rats were

sensitized (i.p.) with wheat and thresher dust along with alum twice a week and after 1 week of sensitization (i.p.) the rats were challenged with the respective allergen for 10 consecutive days (Figure 1). Allergen-dependent asthma or airway inflammation is characterized

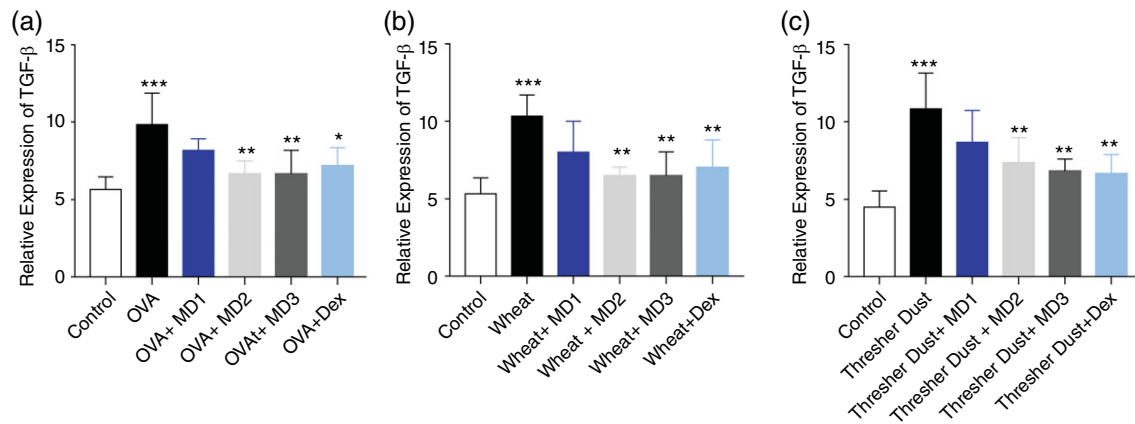


FIGURE 24 mRNA expression of TGF- β cytokine. Quantitative real time RT-PCR was used to determine the TGF- β expression in lung of AIPI rat that received MD at doses MD1 (5 mg kg⁻¹), MD2 (10 mg kg⁻¹) and MD3 (20 mg kg⁻¹). β -Actin was used to find the relative expression. (a) OVA AIPI rat group, (b) wheat AIPI rat group, and (c) thresher dust AIPI rat group. Data shown as mean $\pm$ SD. $n = 10$ in each group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ show the statistical difference between two groups

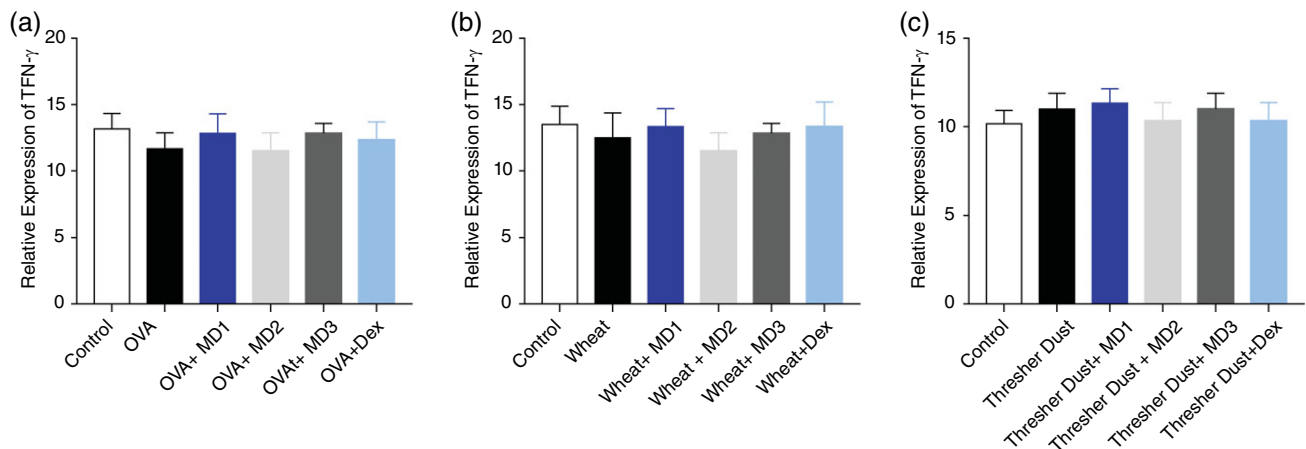


FIGURE 25 mRNA expression of IFN- γ cytokine. Quantitative real time RT-PCR was used to determine the IFN- γ expression in lung of AIPI rat that received MD at doses MD1 (5 mg kg⁻¹), MD2 (10 mg kg⁻¹) and MD3 (20 mg kg⁻¹). β -Actin was used to find the relative expression. (a) OVA AIPI rat group, (b) wheat AIPI rat group, and (c) thresher dust AIPI rat group. Data shown as mean $\pm$ SD. $n = 10$ for each group

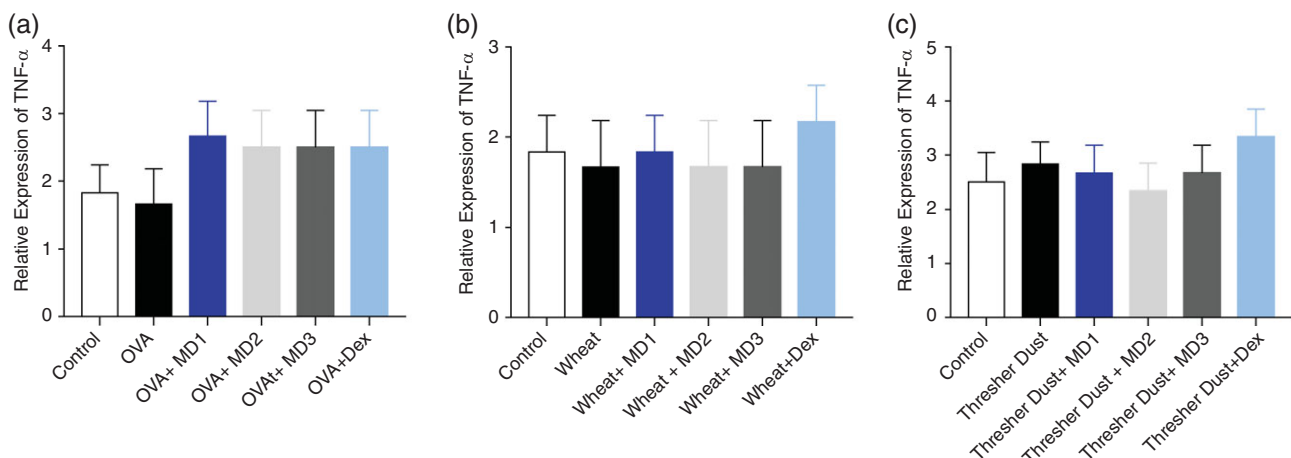


FIGURE 26 mRNA expression of TNF- α cytokine. Quantitative real time RT-PCR was used to determine the TNF- α expression in lung of AIPI rat and received MD at doses; MD1 (5 mg kg⁻¹), MD2 (10 mg kg⁻¹), MD3 (20 mg kg⁻¹). β -actin was used to find the relative expression. (a) OVA AIPI rat group (b) wheat AIPI rat group and (c) thresher dust AIPI rat group. Data shown as mean $\pm$ SD. $n = 10$ for each group

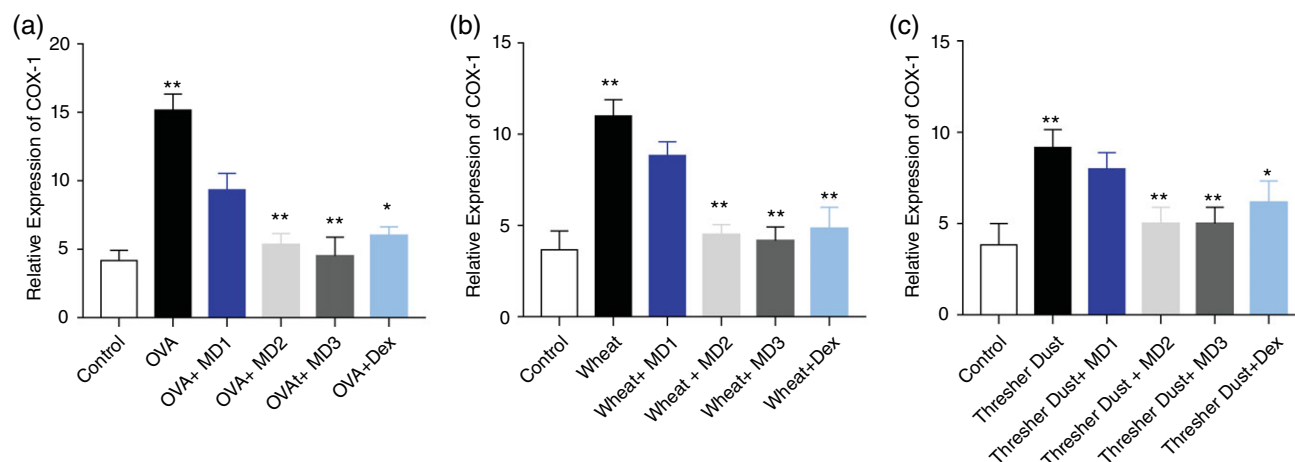


FIGURE 27 mRNA expression of COX-1. Quantitative real time RT-PCR was used to determine the COX-1 expression in lung of AIPI rat that received MD at doses MD1 (5 mg kg⁻¹), MD2 (10 mg kg⁻¹) and MD3 (20 mg kg⁻¹). β -Actin was used to find the relative expression. (a) COX-1 expression of OVA group rats, (b) COX-1 expression of wheat group rats, and (c) COX-1 expression of thresher dust group rats. Data shown as mean $\pm$ SD. $n = 10$ in each group. * $P < 0.05$, ** $P < 0.01$ show the statistical difference between two groups

TABLE 8 Liver and kidney function test of OVA AIPI rats

Group	AST (U l ⁻¹)	ALT (U l ⁻¹)	Creatinine (mg dl ⁻¹)	BUN (mg dl ⁻¹)
Control	66 $\pm$ 8.7	24 $\pm$ 2.5	0.58 $\pm$ 0.02	17 $\pm$ 1.4
OVA	71 $\pm$ 9.1	27 $\pm$ 3.7	0.72 $\pm$ 0.02	18 $\pm$ 2.1
OVA+MD1	70 $\pm$ 6.6	26 $\pm$ 3.5	0.51 $\pm$ 0.03	17 $\pm$ 1.8
OVA+MD2	65 $\pm$ 4.7	27 $\pm$ 3.4	0.69 $\pm$ 0.01	18 $\pm$ 2.3
OVA+MD3	89 $\pm$ 6.1*	47 $\pm$ 4.6*	0.91 $\pm$ 0.01*	16 $\pm$ 2.2
OVA+Dex	71 $\pm$ 7.8	25 $\pm$ 6	0.51 $\pm$ 0.02	21 $\pm$ 1.6

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen. Data presented as mean $\pm$ SD. $n = 10$ for each group.

TABLE 9 Liver and kidney function test of wheat AIPI rats

Group	AST (U l ⁻¹)	ALT (U l ⁻¹)	Creatinine (mg dl ⁻¹)	BUN (mg dl ⁻¹)
Control	61 $\pm$ 6.1	24 $\pm$ 3.6	0.4 $\pm$ 0.05	18 $\pm$ 3.3
Wheat	63 $\pm$ 5.9	25 $\pm$ 4.7	0.6 $\pm$ 0.02	20 $\pm$ 3.2
Wheat+MD1	62 $\pm$ 6.6	25 $\pm$ 3.5	0.5 $\pm$ 0.02	21 $\pm$ 4
Wheat+MD2	61 $\pm$ 4.8	22 $\pm$ 3.4	0.5 $\pm$ 0.01	19 $\pm$ 2.5
Wheat+MD3	101 $\pm$ 11.1*	42 $\pm$ 11.6*	0.9 $\pm$ 0.08	21 $\pm$ 3.2
Wheat+Dex	70 $\pm$ 6.2	25 $\pm$ 3.8	0.4 $\pm$ 0.02	21 $\pm$ 3.1

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen. Data presented as mean $\pm$ SD. $n = 10$ for each group.

TABLE 10 Liver and kidney function test of thresher dust AIPI rats

Group	AST (U l ⁻¹)	ALT (U l ⁻¹)	Creatinine (mg dl ⁻¹)	BUN (mg dl ⁻¹)
Control	60 $\pm$ 10	24 $\pm$ 6	0.4 $\pm$ 0.02	17 $\pm$ 3.2
Thresher dust	61 $\pm$ 18	23 $\pm$ 8.7	0.5 $\pm$ 0.02	18 $\pm$ 3.1
Thresher dust+MD1	62 $\pm$ 6.6	24 $\pm$ 3.5	0.6 $\pm$ 0.03	20 $\pm$ 4.2
Thresher dust+MD2	59 $\pm$ 4.7	22 $\pm$ 3.4	0.6 $\pm$ 0.01	17 $\pm$ 2.3
Thresher dust+MD3	99 $\pm$ 6.1*	41 $\pm$ 5.6*	0.9 $\pm$ 0.2	18 $\pm$ 3.1
Thresher dust+Dex	69 $\pm$ 10	26 $\pm$ 6	0.5 $\pm$ 0.02	19 $\pm$ 4.1

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen. Data presented as mean $\pm$ SD. $n = 10$ for each group.

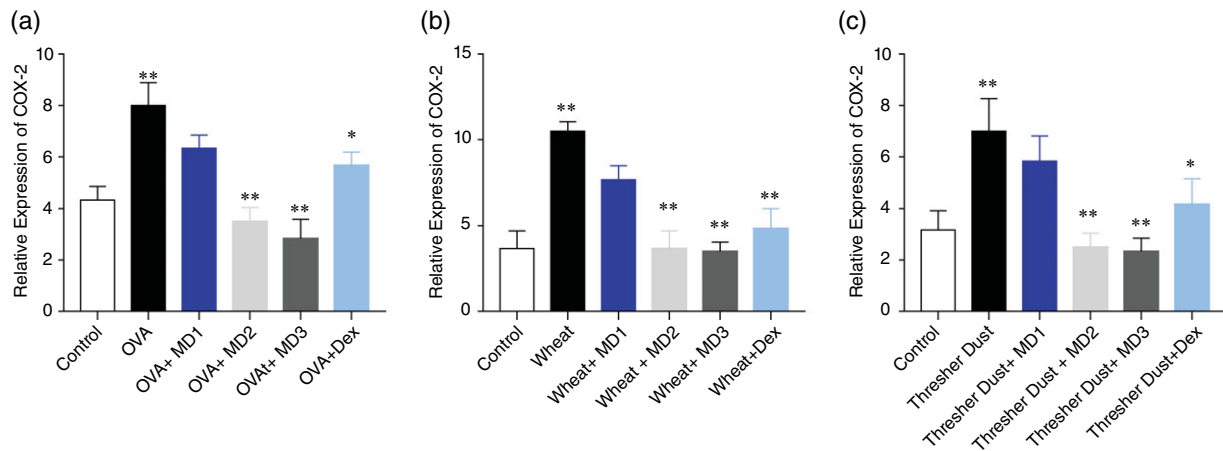


FIGURE 28 mRNA expression of COX-2. Quantitative real time RT-PCR was used to determine the COX-2 expression in lung of AIPI rat that received MD at doses MD1 (5 mg kg⁻¹), MD2 (10 mg kg⁻¹) and MD3 (20 mg kg⁻¹). β -Actin was used to find the relative expression. (a) COX-2 expression of OVA group rats, (b) COX-2 expression of wheat group rats, and (c) COX-2 expression of thresher dust group rats. Data shown as mean $\pm$ SD. $n = 10$ in each group. * $P < 0.05$, ** $P < 0.01$ show the statistical difference between two groups

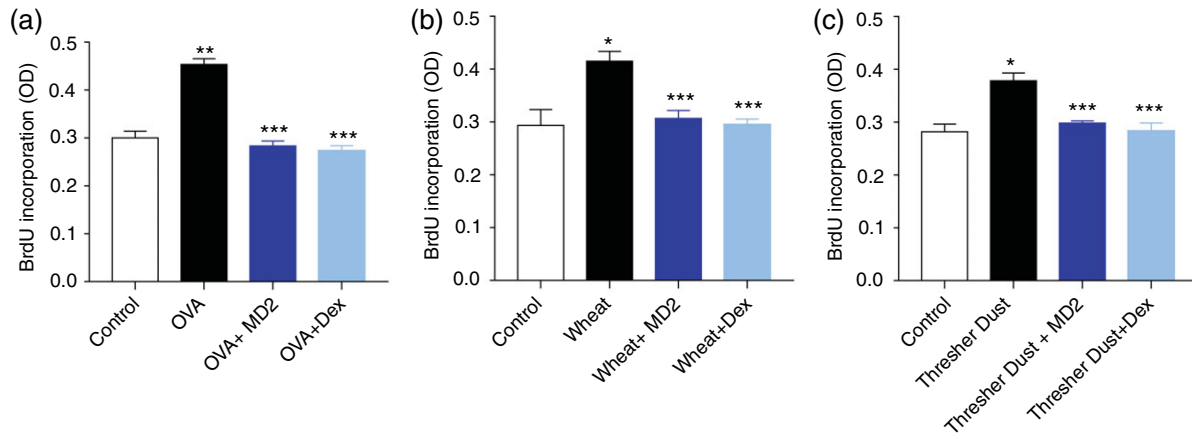


FIGURE 29 Effect of MD on T-cell proliferation. Spleen of normal rats was used to determine the effects of allergen on T-cell proliferation. Furthermore, an inhibitory effect of MD2 at 10 mg ml⁻¹ was also evaluated on T-cell proliferation. (a) OVA used to stimulate T-cell proliferation, (b) wheat extract used to stimulate T-cell proliferation, and (c) thresher dust used to stimulate T-cell proliferation. Data shown as mean $\pm$ SD. $n = 10$ in each group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ show significant difference between two groups

by a massive increase of inflammatory cells especially of IgE-related Th2 cells. These T cells express IL-4, IL-5 and IL-13, Th2 signalling cytokines that regulate many stages of inflammation in a coordinated manner (Chen et al., 2004; Cohn, Elias, & Chupp, 2004). Inflammatory cells including eosinophils, macrophages, neutrophils and lymphocytes were increased along with IgE in serum. The intraperitoneal route to sensitize the rats is considered to be an effective way to elicit high levels of IgE and production of Th2 cytokines in spleen cells as well as airway inflammation (Hoymann, 2007; Careau, 2006). Alum was used as an adjuvant that also might be involved in promoting the development of a Th2 phenotype by the immune system when it is exposed to an antigen (Bungener et al., 2008). The present results showed that the mRNA expression and level of IL-4 in lungs of AIPI rats exposed to 2 and 3 mg of wheat and thresher dust were increased significantly when compared with control rats. The infiltration of eosinophils in an asthmatic attack can cause tissue lesion and more severe clinical symptoms (Blacqui re et al., 2010).

Pathological changes including qualitatively enhanced goblet cells and infiltration of inflammatory cells in the airways of the AIPI model were significant when exposed to wheat and thresher dust allergens. Therefore, our results combined implied that the thresher dust and wheat allergen-induced pulmonary model of rat can mimic the inflammatory character of allergic asthma. IL-4 stimulated the isotonic pattern of transformation and IgE secretion by B cells. In addition, IL-4 inhibits apoptosis of eosinophils and T lymphocytes, chemical induction of eosinophils and initiation by promoting the expression of eotaxin, thus promoting iodine inflammation (Pappas, Papaioannou, Kostikas, & Tzanakis, 2013; Chung, 2015). For this reason mRNA expression of IL-4 was increased in the lung of our novel AIPI rats by both wheat and thresher dust allergen at concentrations of 2 and 3 mg ml⁻¹.

It was also reported that NSAIDs showed inhibitory effects against COX enzymes and inhibit prostaglandin 2 (Crofford, 2013). Improving the side effects of existing drugs and synthesis of safe and selective

drugs are going to be of major interest for pharmaceutical chemists. MD, a novel hydrazine recently synthesized by our research group, showed good anti-inflammatory and anti-platelet activity *in vitro*. Molecular docking of MD against COX (1 and 2) showed an impressive binding interaction with the naproxen pocket and selected residues (Ramzan et al., 2017). According to quantitative structure–activity relationship studies, polar surface area (PSA) of the drug is less than 60–70 Å². It was reported previously that PSA should not exceed 120 Å² for an orally active drug that is transported by a transcellular route and PSA should be < 100 Å² or < 60–70 Å² for brain penetration (Van De Waterbeemd, Camenisch, Folkers, Chretien, & Raevsky, 1998; Tronde et al., 2003; Remko, Boháč, & Kováčiková, 2011).

The anti-inflammatory effect of MD was determined at the molecular level on the AIPI rats by exposing them to 2 mg ml⁻¹ of both allergens, i.e. wheat and thresher dust. Three different oral doses of 5, 10 and 20 mg kg⁻¹ of MD were used against AIPI rats to check the ameliorative effects. Histopathology, i.e. H.E and PAS staining, suggested a reduction in wheat- and thresher dust-induced inflammatory infiltration in lungs when the AIPI rats were treated with MD. The anti-inflammatory effect of MD was dose dependent in manner. The present study also confirmed that medium (10 mg kg⁻¹) and high (20 mg kg⁻¹) doses of MD significantly suppressed the level of total serum IgE and inflammatory cells in both blood and BALF of AIPI rats exposed to OVA, wheat and thresher dust. Th2-dependent inflammatory cell accumulation and enhanced IgE were the hallmarks of airway inflammation when E3 rats were exposed to OVA (Sun et al., 2011). Histological and biochemical evaluation of the AIPI rats showed similarities to human allergic asthma. IL-4 is one of the major markers for the Th2 immune response (Fallon et al., 2002) and IL-5 is crucial for inflammation by eosinophils, because they are involved in the differentiation of precursor cells from bone marrow into eosinophils, and also helps to prolong the survival of the eosinophils (Fort et al., 2001). It was also reported that IL-4 in the Th2 response initiated allergen-specific IgE production while IL-5 involved in the production of eosinophils and their proliferation in lung tissues as well as in blood (Palmqvist, Wardlaw, & Bradding, 2007). TGF- β is a multifunctional cytokine that causes the proliferation of fibroblasts and smooth muscle cells and epithelial airway repair (Li, Wan, Sanjabi, Robertson, & Flavell, 2006). It also has an immunoregulatory effect, which results in suppression of Th1 and Th2 cells (Konkel et al., 2017). A higher expression of TGF- β 1 was reported as well as - β 2 in asthma bronchitis, which is associated with subepithelial fibrosis (Choy et al., 2011; Smith et al., 2016). Th2 cytokines, i.e. IL-4, IL-5, IL-13 and TGF- β , were up-regulated in all groups of AIPI rats except control due to wheat and thresher dust allergens, and oral administration of MD down-regulated the level of IL-4, IL-5, IL-13 and TGF- β in a dose-related fashion. Thus, the present AIPI rats showed a Th2 allergic response and decline in level of Th2 cytokine, suggesting that MD alters the Th2 pathway, thus reducing the airway allergy. Relative expression of INF- γ and TNF- α remains unaffected in the present AIPI model. Pathological changes including airway thickening and qualitatively enhanced goblet cells were suppressed by MD in AIPI rats.

COX is an enzyme responsible for the production of prostaglandins from arachidonic acid, and NSAIDs exhibit an anti-inflammatory

property through inhibition of COX activity (Crofford, 2008). Our data showed that mRNA levels of both COX-1 and COX-2 were enhanced in lung of AIPI rats. MD significantly suppressed both COX-1 and COX-2 enzymes and this inhibitory effect of MD was found to be dose dependent. COX-1 and COX-2 are the two leading isoforms in mammals (Morita, 2002). COX-1 is generally recognized as a 'housekeeping' enzyme and is involved in physiological functioning of prostaglandins (Alhouayek & Muccioli, 2014). In mammals, COX-2 enzyme is involved in various inflammatory diseases including arthritis and airway allergy (Choi, Aid, & Bosetti, 2009). In the present work, no gastropathy/ulcers were observed in the experimental rats after using MD as a COX-1 and COX-2 inhibitor. According to clinical trial evidence, the use of COX-2 inhibitors appears to provide a safe and effective means of anti-inflammatory and analgesic treatment in asthmatic patients with true aspirin-exacerbated respiratory disease (Morales et al., 2014).

Our results revealed no massive effects on the haematological parameters except inflammatory cells in AIPI rats. Furthermore, in the present AIPI rat model AST, ALT, BUN and creatinine levels remained almost the same as in control rats exposed to PBS and no significant increase was found. But liver function parameter, ASL and ALT, were enhanced in AIPI rats treated with a high dose (20 mg kg⁻¹) of MD. These results suggested that MD does not have any hepatotoxic and nephrotoxic effects while MD showed toxicity at a dose of 20 mg kg⁻¹. It is well known that spleen is one of the major sites for the proliferation of T-cells in rats immunized with alum along with allergen (Raza Asim et al., 2010; Ather et al., 2011). *In vitro* evaluation showed that MD (10 mg kg⁻¹) significantly suppressed the wheat- and thresher dust-induced splenocyte proliferation and thus down-regulated the production of T-cells.

5 | CONCLUSION

Here we report the first ever AIPI rat model of lyophilized wheat and thresher dust extracts and conclude that 2 mg of lyophilized wheat and thresher dust was the amount required minimally to induce pulmonary airway inflammation in rats. Increased level of total IgE, cytokines (IL-4, IL-5, TGF- β and IL-13) and COX expression revealed that wheat and thresher dust allergens are involved in initiation of the Th2 immune response. The study also concluded that a hydrazide derivative (MD) at a dose of 10 mg kg⁻¹ significantly modulated the mRNA expression of pro-inflammatory cytokines by inhibiting splenocyte proliferation in OVA-, wheat- and thresher dust-exposed rats. *In vivo* results showed the immune-modulatory and anti-inflammatory potential of MD, which also supports the *in silico* findings. In future, clinical investigation is required to establish the anti-inflammatory response of MD.

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AUTHOR CONTRIBUTIONS

M.M.A. and M.A.R.B. were responsible for directing, interpreting and writing the manuscript. S.S. and M.M.S. were involved in designing the protocols, performing the *in vivo* experiments, revising the manuscript and analysing the data. M.N. was involved in interpreting the histochemistry results. M.S. assisted in handling the animal model and data analysis. A.R. provided expertise to synthesized the hydrazide derivative *N'*-(4-Methoxybenzylidene)-6-(4-chlorophenyl)-3-methyl-1-phenyl-1*H*-pyrazolo [3,4-*b*]pyridine-4-carbohydrazide. All authors have read and approved the final version of this manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

COMPETING INTERESTS

The authors declare no conflict of interests.

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