Original research paper

Punicalagin attenuated allergic airway inflammation *via* regulating IL4/ IL-4Rα/ STAT6 and Notch- GATA3 pathways

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ABSTRACT

Allergic asthma is an inflammatory disease of the airways which has a complex etiology. Punicalagin, a major polyphenol present in pomegranates, is reported to possess various biological properties including antioxidant and antiproliferative effects. The current research aimed to evaluate the antiasthmatic effects of punicalagin in an ovalbumin (OVA)-induced experimental model of asthma in female BALB/c mice. Treatment group animals received punicalagin (12.5, 25 or 50 mg kg^{-1} body mass) per day for 21 days from day 1 of OVA injection. Dexamethasone (DEX) was administered to a separate group of mice, as the standard drug control. Inflammatory cell infiltration into the bronchoalveolar lavage fluid (BALF) was substantially decreased in punicalagin-treated mice. Punicalagin reduced Th2-derived cytokines and OVA-specific IgE levels. The IL-4/STAT6 and Notch/GATA3 signalling pathways were regulated on punicalagin administration. The data obtained illustrate the potency of punicalagin as an anti-asthmatic drug. Conclusively, the study's observations suggest the potential therapeutic efficiency of punicalagin in allergic asthma.

Keywords: asthma, GATA3, inflammation, interleukins, Notch signal, punicalagin

Allergic asthma is a major global health concern with increasing incidence rates (1). Genetic predisposition and environmental factors are considered to be the major influencers of asthma (2). The symptoms of asthma include chronic inflammation, hyperresponsiveness, cellular infiltration of the airways, hypersecretion of mucus and airway remodelling which clinically manifest as wheezing, chest tightness, variable expiratory airflow and shortness of breath (3, 4). Currently, corticosteroids are a very effective class of drugs against asthma (5). Nevertheless, prolonged administration of inhaled steroids is very often associated with adverse side effects, particularly with higher doses (6). Therefore, the identification of new therapeutic compounds with better safety is inevitable.

Understanding the underlying molecular mechanisms in asthma pathogenesis helps with the development and identification of novel therapeutic agents. Several studies in recent years have been found to focus on the use of phytochemicals as alternate therapeutic

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regimens in the management of several clinical conditions (7). Punicalagin, a 2,3-hexa-hydroxydiphenoylgallagyl-D-glucose), is widely present in pomegranates (8). Punicalagin is found to promote general wellbeing and also exhibits potential antioxidant, antiproliferative and anti-inflammatory effects (8, 9). The current work aims to assess the possible protective role of punicalagin in the asthma-induced rodent model.

T helper 2 cells (Th2) cells are well documented as major players in the pathogenesis of inflammation in allergic asthma. Th2 cells secrete cytokines as interleukins- IL-4, IL-5 and IL-13. The cytokines- IL-4 and IL-13 stimulate the secretion of an allergen-associated immunoglobulin E (IgE) response (10). IL-5 is essential in eosinophils' survival, proliferation, differentiation, and tissue infiltration (11). Th2-derived cytokines also stimulate the production and release of inflammatory mediators from the mast cells and are implicated in eosinophils recruitment, excessive secretion of mucus, airway hypersensitivity, and airway remodelling (12).

Inhibition of Th2 cell-derived effector cytokines would be an effective strategy in asthma treatment (13). GATA-binding protein-3 (GATA-3), one of the major transcription factors plays a key role in regulating Th2 differentiation and expressions of Th2-derived cytokines (14). Th2-interleukins- IL-4 and IL-13 that possess IL-4Rα subunit in their receptors induce activation of Signal Transducer and Activator of Transcription 6 (STAT6) (15). STAT6 signalling is a critical pathway in airway inflammation and mucus production (16). Activated STAT6 enters the nucleus from the cytosol and induces transcription of GATA3 and subsequently resulting in Th2 cell differentiation. Further, STAT6 is the major regulator of IL-4- and IL-13-mediated production of Th2 chemokines (14).

The Notch signalling pathway induces differentiation of Th2 cells *via* regulating GATA3 and IL4 transcription. The Notch ligands- Jagged1 and 2, are implicated in Th2-
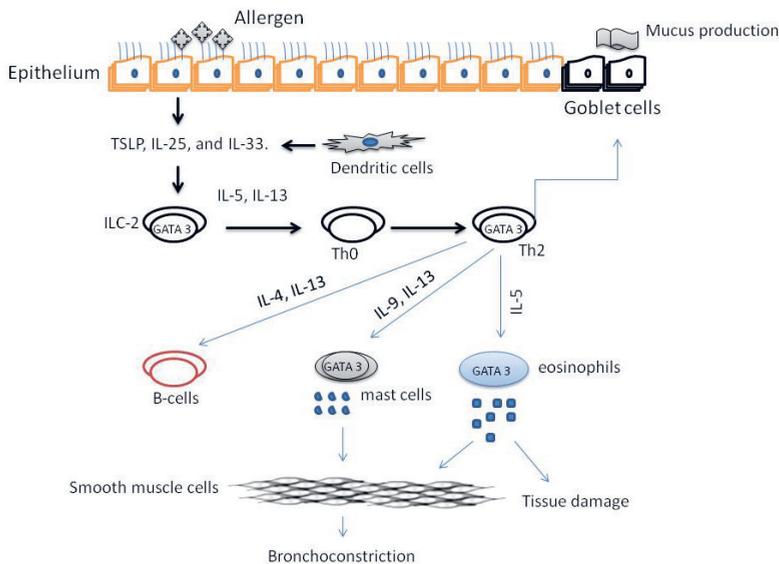

Fig. 1. Signalling pathway associated with allergic asthma with respect to GATA-3 and Th2 cells. Where TSLP is thymic stromal lymphopoietin ILC2: innate lymphocyte type 2, Th2: T helper cell type 2.

mediated responses (17). Thus, the Notch/GATA3 signal is crucial in asthma pathogenesis. Here we evaluated the effects of punicalagin on Th2-mediated immune responses and the Notch/GATA3 signalling in OVA-induced experimental asthma (Fig. 1).

<div align="center">EXPERIMENTAL</div>

Chemicals and antibodies

Antibodies against GATA3, STAT6, p-STAT6, JAK1, p-JAK1 (Cell signalling Technology, USA), IL-4Rα, Jagged1, Jagged2, Notch1 intracellular domain (NICD1), Notch 2 intracellular domain (NICD2), Notch1, Notch 2 and GAPDH were purchased from Santa Cruz Biotechnology, USA. Methacholine, ovalbumin (OVA), punicalagin (98 %), and other reagents were acquired from Sigma-Aldrich (USA).

Experimental study design and grouping of animals

BALB/c mice (female; n = 90; 6 weeks old; 25–35 g) were obtained from the Institute Laboratory Animal Centre. The mice were housed in sterile acrylic cages (n = 3/cage) under 12 h/12 h day/night cycle and controlled laboratory environment (temperature 22 ± 3 °C, relative humidity 55 ± 5 %). The mice were allowed to have unlimited access to a standard pellet diet and drinking water throughout the investigation period.

The animals were adapted to the laboratory environment for five days before the initiation of the study. The protocols and study design were permitted by the Institutional Ethical Committee. The mice were induced allergic asthma *via* sensitization to OVA through inhalation. Equal volumes of OVA (500 μg mL^{-1} PBS) and aqueous alum (10 %) were mixed thoroughly and pH adjusted to 6.5. Following incubation for an hour at room temperature, the OVA-alum mix was centrifuged for five min at 750 *g*. The OVA- alum pellet obtained was then re-suspended in double distilled water to its initial volume.

On day one of the study, the mice received OVA at 100 μg (0.2 mL of 500 μg mL^{-1}) *via* intraperitoneal injection (*i.p.*). Subsequently, on day eight, the mice received 250 μg of OVA (2.5 mg OVA mL^{-1}; 100 μL; *i.p.*) and were challenged with intranasal administration of OVA at 125 μg (50 μL of 2.5 mg mL^{-1} solution) on days 15, 18, and 21. Mice were given *i.p.* ketamine-xylazine anesthesia (0.2 mL ketamine at 0.44 mg mL^{-1} xylazine at 6.3 mg mL^{-1} in normal saline) prior to the OVA challenge. Punicalagin at doses of 12.5, 25, 50 mg kg^{-1} b.m. was administered *via* oral gavage for 21 days to the treatment group of mice, from day 1 to day 21 of OVA injection. Control mice were not subjected to OVA induction of allergic asthma but received equal volumes of normal saline for punicalagin. On days of OVA administration, punicalagin was given 60 minutes prior to OVA injection. Mice administered dexamethasone (Dex; 2 mg kg^{-1} b.m. *i.p.*) an hour before OVA injection were grouped as standard drug control.

Determination of airway hyperresponsiveness

24 h post the last OVA challenge; any functional changes in the airways were evaluated using aerosolized methacholine. Whole-body plethysmography was employed to measure pulmonary function (18). Changes in lung compliance (Cdyn) and airway resistance (RI) were recorded to measure pulmonary function. Cdyn and RI in response to

increasing doses of methacholine (3.125, 6.25, 12.5, or 25 mg mL^{-1}) were noted. Briefly, mice were tracheotomised, cannulated and laid in the Buxco whole-body plethysmograph chamber, connected to the ventilator. The mice received methacholine by nebulization. The RI and Cdyn were presented as percentages (%) of the corresponding basal values.

Bronchoalveolar lavage fluid collection

The mice were sacrificed after exposure to pentobarbital overdose (50 mg kg^{-1} b.m.) at 24 h after the last OVA challenge (19). Ice-cold PBS (0.5 mL) was infused into one of the lungs and bronchoalveolar lavage fluid (BALF) was collected by tracheal cannulation. The procedure was done three times, and BALF was collected (total volume of 1.5 mL). The BALF was pooled and centrifuged (250 g) at 4 °C for 5 min. The supernatant was used for the measurement of cytokines and OVA-specific IgE levels. The cell pellets were suspended in PBS and treated with Wright-Giemsa stain (Sigma-Aldrich) to determine inflammatory cell counts in the BALF.

Determination of cytokines in BALF

Cytokines IL-4, IL-5 and IL-13, in BALF were determined by enzyme-linked immunosorbent assay (ELISA) method using a kit obtained from Sigma-Aldrich (20). The analysis was performed according to the manufacturer's instructions.

OVA-specific IgE determination

Serum obtained from centrifugation of whole blood was used for analysis. OVA-specific IgE levels in serum and BALF were determined by ELISA using BD OptEIA™ Set Mouse OVA-specific IgE kit (BD Biosciences, USA) following the manufacturer's instructions.

RT-PCR analysis

Total RNA of the excised lung tissues was isolated with TRIzol® (Invitrogen, USA). cDNA was synthesized from the extracted RNA using Omniscript Reverse Transcriptase (Qiagen Inc., USA). Samples were then subjected to RT-PCR using the iQ SYBR Green supermix (Bio-Rad, USA). The samples were amplified (denaturation at 95 °C for 10 s; annealing and extension at 55 °C for 30 s) and detected using an S1000 Thermal cycler real-time PCR system (Bio-Rad). The following primers were employed for analysis:

GATA3 Forward – 5'-AGGCAACCACGTCCCGTCCT 3'
    Reverse- 5'-TTTGCCGCCATCCAGCCAGG3'
Notch1 Forward- 5'-CCGTGGCTCCATTGTCTACCT-3'
    Reverse- 5'-CATCGGTGGCACTCTGGAA-3'
Notch2 Forward- 5'-CCAAGCGGAAGCAAGCAT-3'
    Reverse- 5'-GGCGCTTGTGATTGCTAGAGT-3'

Immunoblotting

The lung tissues were homogenized and then lysed using a protein lysis buffer containing protease inhibitors (Sigma Aldrich). The protein content of each lysate was subjected to protein quantification with a BCA assay. Protein samples were run on 10 % SDS-

-PAGE gel and finally transferred to the PVDF membrane. The PVDF membrane was then blocked using 5 % non-fat dry milk at room temperature for 3 h. It was followed by overnight incubation with primary antibodies against the target proteins at 4 °C. After that, membranes were washed using TBST buffer and incubated with secondary antibodies for 60 minutes. A western blot image-forming system (Tanon 5200, China) was used to visualize the protein bands through enhanced chemiluminescence.

Statistical analysis

IBM SPSS Statistics for Windows, version 22.0 (IBM Corp., USA) was used to perform data analyses in this study. The experiment results were shown as mean ± SD ($n = 6$). Statistical significance was evaluated by ANOVA and Duncan's Multiple Range Test (DMRT). p-values < 0.05 were marked as significant.

RESULTS AND DISCUSSION

Punicalagin reduced airway hyperresponsiveness

Hyper-sensitivity of the airway is one of the characteristic features of asthma (21). It is well documented that contractile agonists such as OVA induce airway hypersensitivity responses. In the study, airway resistance (RI) and lung compliance (Cdyn) were noted in response to methacholine administered at increasing doses (3.125, 6.25, 12.5, or 25 mg mL^{-1})
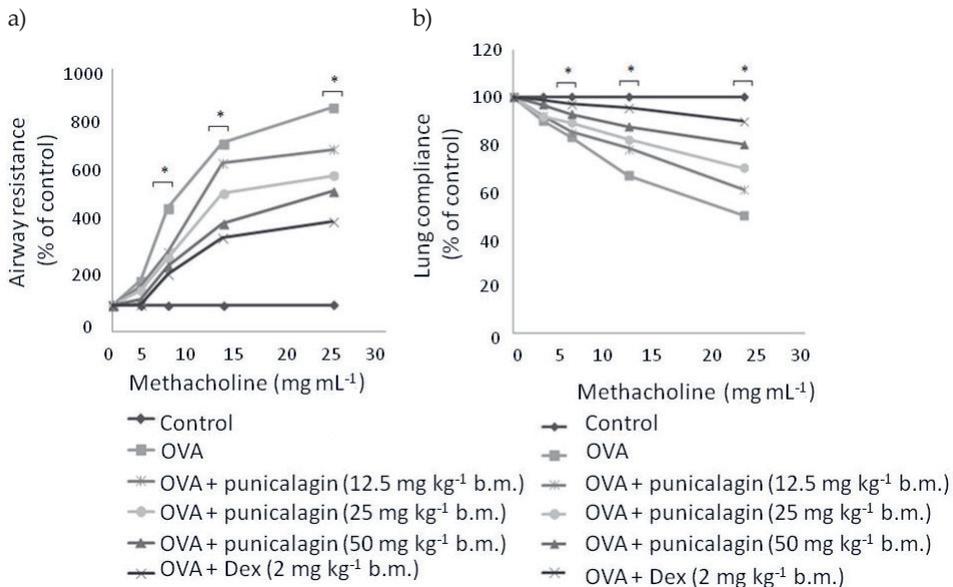

Fig. 2. The effect of punicalagin on airway resistance and lung compliance. Punicalagin (12.5, 25 and 50 mg) treated OVA-challenged mice exhibited: a) significantly reduced airway resistance and b) improved lung compliance. * represents $p < 0.05$ *vs.* control.

to mechanically ventilated mice. The OVA-challenged animals exhibited considerably ($p <$ 0.05) amplified airway resistance and caused a substantial decrease in lung compliance in comparison to the normal control (Fig. 2). Punicalagin (12.5, 25 and 50 mg) treated OVA-challenged mice exhibited significantly ($p < 0.05$) improved lung compliance (Fig. 1b) with reduced airway resistance (Fig. 1a). Positive control mice administered with Dex displayed RI and Cdyn values statistically similar to the group treated with 50 mg punicalagin.

Punicalagin inhibited cellular infiltration

Infiltration and accumulation of inflammatory cells into the airways following a trigger by an allergen is considered a significant event leading to AHR and inflammation. Increased counts of eosinophils and lymphocytes in BALF under asthmatic conditions have been reported (22) and are considered hallmark features of asthma. The effects of systemic administration of punicalagin on inflammatory cell infiltration into the BALF of OVA-induced asthmatic mice were assessed. Following OVA sensitisation and challenge, the asthmatic mice revealed severe infiltration of eosinophils, lymphocytes and other inflammatory cells into the BALF compared to the normal control mice (Fig. 3). Punicalagin supplementation significantly ($p < 0.05$) repressed OVA-induced eosinophilia and reduced cellular infiltration. Punicalagin (50 mg) was found to exhibit suppressive effects statistically similar to the effects of the standard drug, dexamethasone.

Punicalagin decreased OVA-specific IgE levels

Elevated allergen-specific IgE levels upon exposure to an allergic agent are considered one of the key events of the early inflammatory responses in asthma. Higher levels of IgE
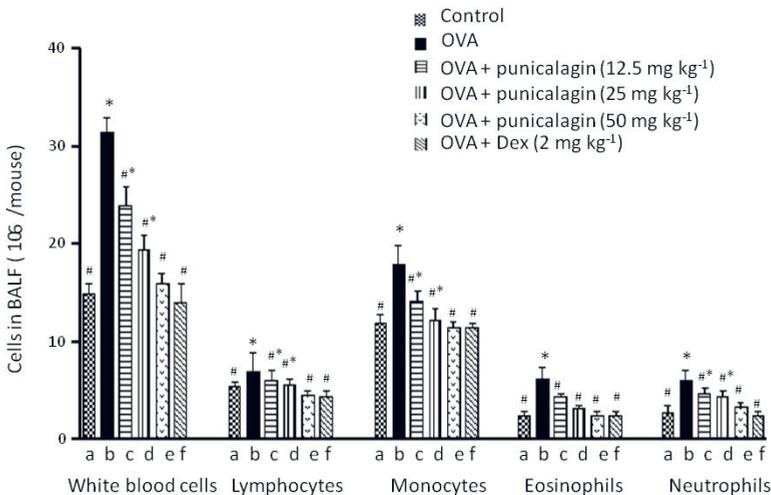

Fig. 3. Effect of punicalagin on cell accumulation in BALF. Punicalagin supplementation significantly repressed OVA-induced eosinophilia and also reduced cellular infiltration.* represents $p < 0.05$ compared to control and [#] represents $p < 0.05$ compared to OVA alone group.
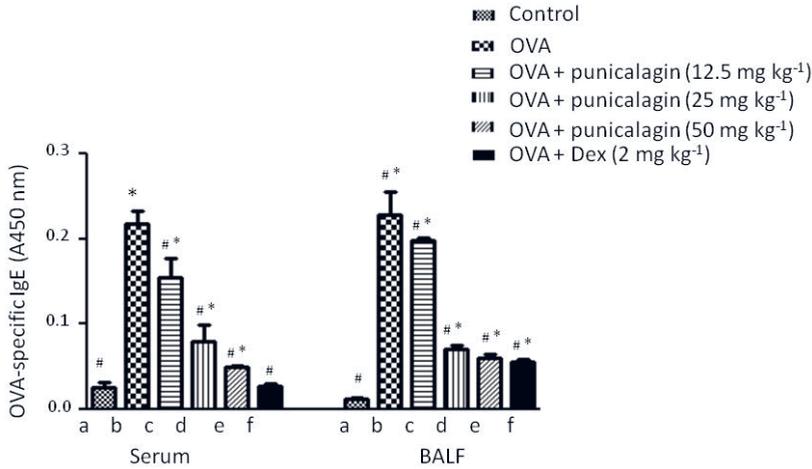
Fig. 4. Punicalagin reduces OVA-specific IgE levels in serum and BALF. Punicalagin treatment to OVA-induced mice produced a significant drop in the levels of OVA-specific IgE. * represents $p < 0.05$ compared to control and # represents $p < 0.05$ compared to OVA alone group.

observed in asthmatic conditions are the main contributor to mast cell degranulation and bronchial constriction. While the early inflammatory responses in asthma are IgE-dependent allergic responses, the later phase in asthma is presented with the recruitment of inflammatory cells, predominantly lymphocytes and eosinophils in the bronchial tissues. In the study, OVA-specific IgE levels were measured in the serum and BALF 24 h after the final OVA injection. We noticed a multi-fold increase ($p < 0.05$) in OVA-specific IgE levels in asthmatic mice (Fig. 4). Interestingly, punicalagin treatment to OVA-induced mice for a period of 3 weeks produced a significant ($p < 0.05$) drop in the levels of OVA-specific IgE. The punicalagin dose of 50 mg caused more pronounced effects compared to lower doses of 12.5 mg and 25 mg.

Punicalagin reduced Th2 cytokine levels

Inflammatory cytokines are major players in asthma pathogenesis (23). The Th2--derived cytokines (IL-4, IL-5, and IL-13) are demonstrated as important mediators of the inflammatory cascade. Cytokines IL-4 and IL-13 induce allergen specific-IgE production in the mast cells and stimulate the production of various other chemokines by the mast cells. Further, IL-4 and IL-13 are found to promote structural remodelling of the airways (15). IL-5 is one of the main cytokines involved in the recruitment of eosinophils at the inflammatory sites (24). Th2 cytokine levels (IL-4, IL-5, and IL-13) in the BALF were determined following OVA induction. In comparison to the normal non-asthmatic mice, we observed a multi-fold ($p < 0.05$) increase in levels of cytokines in OVA-induced mice. IL-4 levels were increased to 314 pg mL^{-1}, IL-5 to 436 pg mL^{-1} and IL-14 to 465 pg mL^{-1} following sensitization and challenge to OVA (Fig. 5). Systemic administration of punicalagin to OVA-induced mice brought a significant ($p < 0.05$) fall in cytokine levels. Furthermore, the decrease was
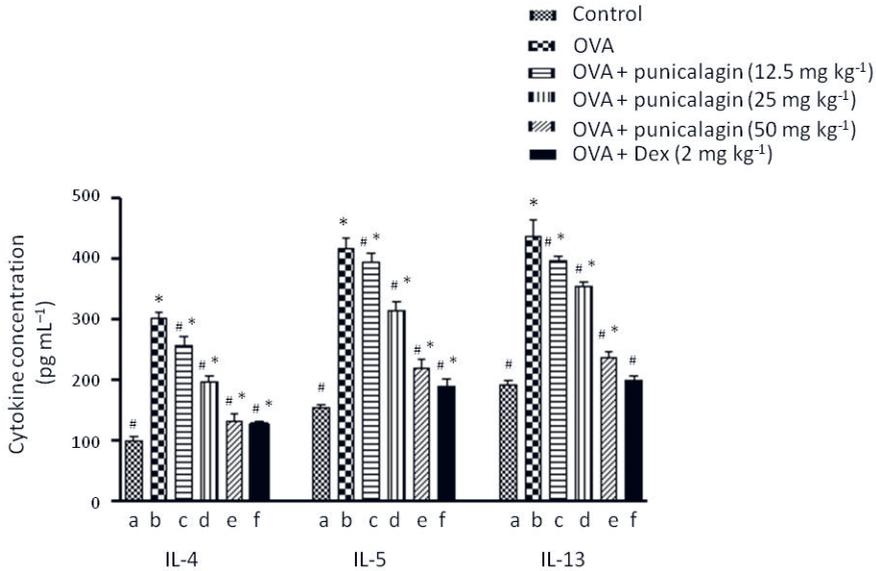
Fig. 5. Effect of punicalagin on cytokines levels in BALF. Administration of punicalagin to OVA-induced mice brought a significant fall in cytokine levels compared to control. * represents $p < 0.05$ compared to control and $^{\#}$ represents $p < 0.05$ compared to OVA alone group.

dose-dependent, where 50 mg showed maximal effects. The levels of IL-4, IL-5, and IL-13 were reduced to 144, 235 and 247 pg mL^{-1}, respectively in treatment with punicalagin at 50 mg kg^{-1}.

Punicalagin regulates the STAT6 signalling pathway

Th2 cell-mediated immune responses are known to be tightly regulated by various pathways, including the IL-4, STAT6 and the Jagged1/Jagged2-Notch1/Notch2 signalling pathway thought to play the main role in asthma. The pathways are crucial in allergic responses and hypersecretion of mucus (17). STAT6 signalling is well documented in bronchial inflammation and mucus secretion. The activation of STAT6 after OVA injections was evaluated by measuring the phosphorylation levels of STAT6 protein. A significantly ($p < 0.05$) raised protein expressions of IL-4Rα and JAK1 were noticed along with raised ($p < 0.05$) phosphorylation of STAT6, illustrating amplified activation of STAT6 in asthmatic conditions (Fig. 6). While the IL-4Rα increased by almost 2.2 fold, phosphorylation levels of STAT6 increased 1.8 fold against total STAT levels in OVA control mice when compared to normal control. Supplementation of punicalagin to OVA-induced mice significantly decreased IL-4Rα, JAK1 expressions and STAT6 activation. p-STAT6 /STAT6 levels dropped to 1.1 fold in mice given 50 mg punicalagin *vs.* 1.8 fold in asthma control. Phosphorylation of JAK was also found to be remarkably reduced on punicalagin supplementation. The pJAK/JAK decreased to 1.23 fold with 50 mg punicalagin *vs.* 2.0 fold in OVA-induced asthma control mice.
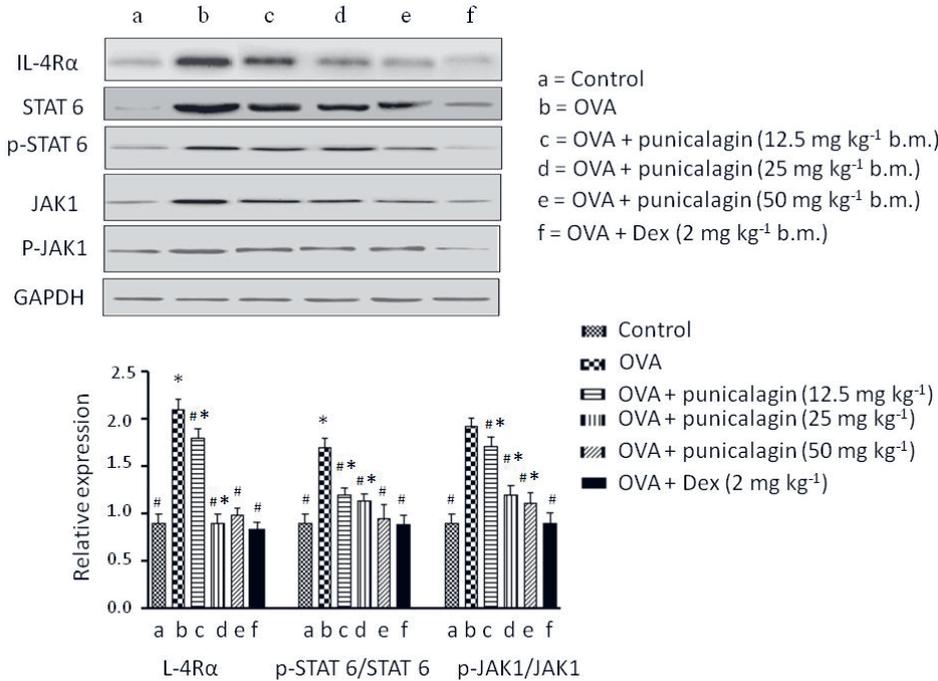
Fig. 6. Punicalagin regulates STAT6 activation. Punicalagin treatment to OVA-induced mice significantly decreased IL-4Rα, JAK1expressions and STAT6 activation. * represents $p < 0.05$ compared to control and $^{\#}$ represents $p < 0.05$ compared to OVA alone group.

Punicalagin modulated GATA3 expressions and Notch signalling following OVA induction

The expression of GATA3, one of the chief regulators of Th2 cell differentiation, was observed to be upregulated after OVA induction (25). The protein IL-4 induces the STAT6 signalling that subsequently induces GATA3 expression (26). Further, signalling pathways, such as T cell receptor (TCR), Notch, and WNT have been demonstrated to induce GATA3 expression (27). GATA3, Notch1, and Notch 2 expressions were assessed in the lung tissues of experimental animals. Significantly up-regulated ($p < 0.05$) expressions of GATA3, Notch 1, and Notch 2 mRNA levels in the OVA control group (Fig. 7) were observed. Protein expressions of Jagged1 and Jagged2 were upregulated 1.83 fold and 1.96 fold respectively, compared to the normal control mice, while NICD1 and NICD2 levels were increased by 2.33 and 2.60 fold, indicating activation of Notch signal subsequent to OVA-sensitization and challenge. Punicalagin-treated OVA-induced animals exhibited significantly ($p < 0.05$) down-regulated protein expressions compared to OVA control mice. Punicalagin at all the three tested doses also caused significant inhibition of GATA3, Notch 1, and Notch2 mRNA levels. Interestingly, we noted the standard drug also caused similar inhibition. Punicalagin (50 mg) treatment was observed to down-regulate the expression levels statistically close to normal levels.
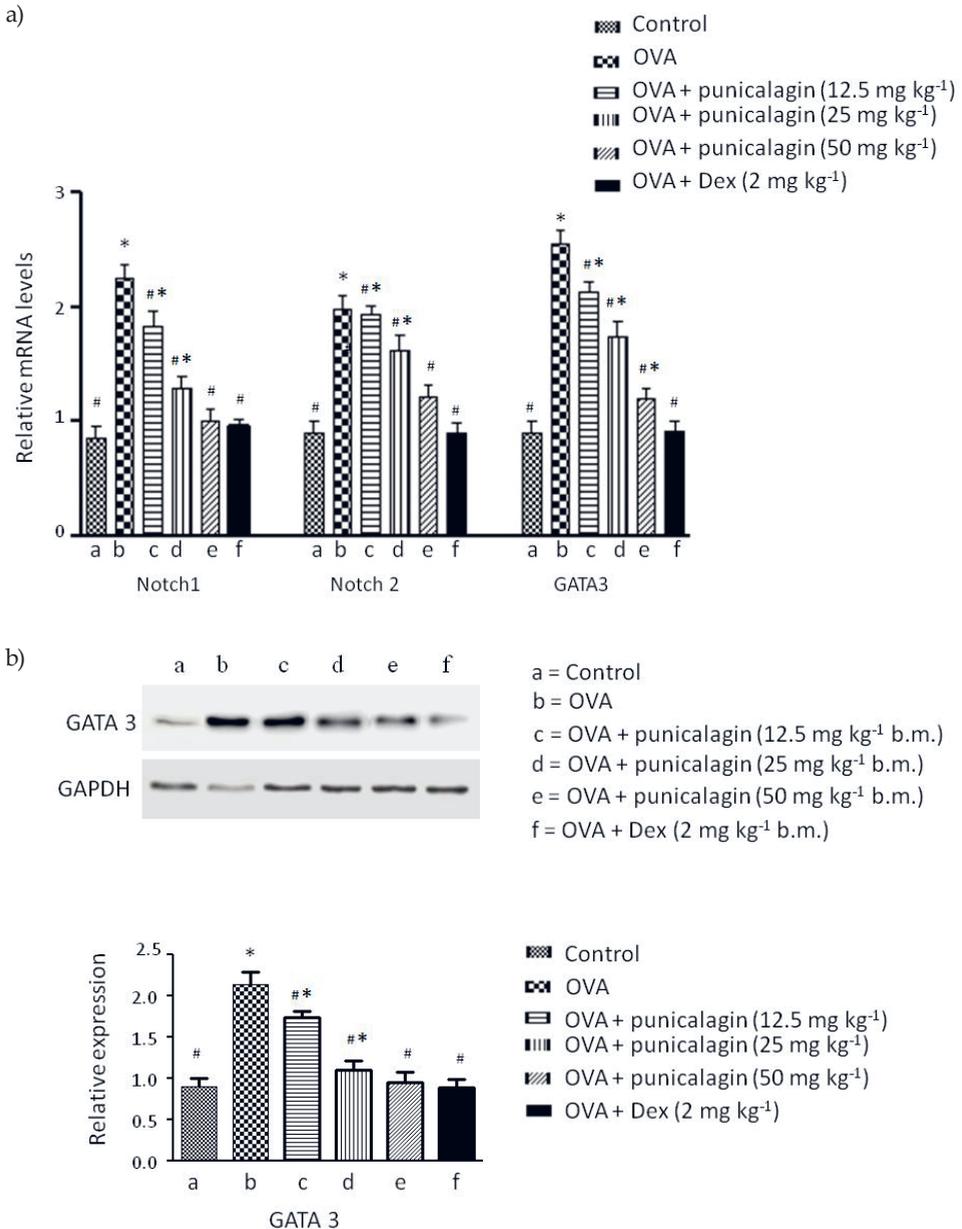
a)

b)

Fig. 7. a) Effect of punicalagin on GATA3, Notch 1 and Notch2 mRNA and b) protein expression of GATA3 in BALB/c mice lung tissue (*p < 0.05). c) Effect of different concentrations of punicalagin on the protein levels of NICD 1, NICD 2, Jagged1 and Jagged2. d) Effect of different concentrations of punicalagin on the protein levels of Notch 1 and Notch 2. * represents p < 0.05 compared to control, while as [#] represents p < 0.05 compared to OVA alone group.

c)

a = Control
b = OVA
c = OVA + punicalagin (12.5 mg kg⁻¹ b.m.)
d = OVA + punicalagin (25 mg kg⁻¹ b.m.)
e = OVA + punicalagin (50 mg kg⁻¹ b.m.)
f = OVA + Dex (2 mg kg⁻¹ b.m.)

Control
OVA
OVA + punicalagin (12.5 mg kg⁻¹)
OVA + punicalagin (25 mg kg⁻¹)
OVA + punicalagin (50 mg kg⁻¹)
OVA + Dex (2 mg kg⁻¹)

d)

a = Control
b = OVA
c = OVA + punicalagin (12.5 mg kg⁻¹ b.m.)
d = OVA + punicalagin (25 mg kg⁻¹ b.m.)
e = OVA + punicalagin (50 mg kg⁻¹ b.m.)
f = OVA + Dex (2 mg kg⁻¹ b.m.)

Control
OVA
OVA + punicalagin (12.5 mg kg⁻¹)
OVA + punicalagin (25 mg kg⁻¹)
OVA + punicalagin (50 mg kg⁻¹)
OVA + Dex (2 mg kg⁻¹)

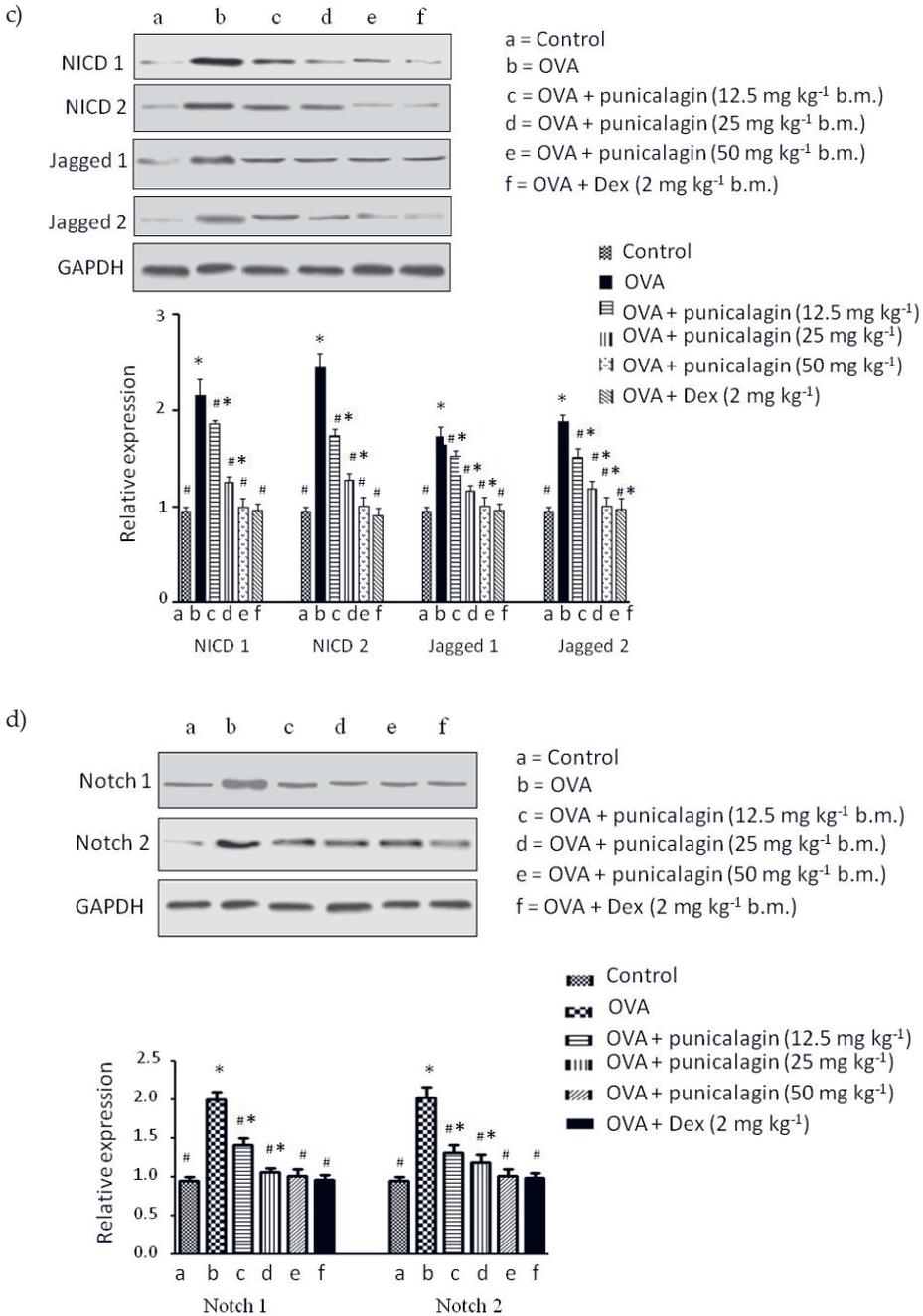Fig. 7. Continued.

CONCLUSIONS

The observations of the current study demonstrate the efficacy of punicalagin as a therapeutic compound in the management of allergic asthma and other inflammatory conditions. Punicalagin effectively regulated the major pathways involved in inflammatory responses. Additionally, the present study could be extended to comprehend the molecular events underlying the beneficial effects of punicalagin against inflammatory disorders. Nevertheless, extended studies are vital to assess the pharmacokinetics and arrive at an effective dosage.

Authors contributions. – Conceptualization, methodology and investigation, L.Y.; original draft preparation, review and editing, J.L. Both the authors have read and agreed to the published version of the manuscript.

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