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Vitexin restores lung homeostasis by targeting vicious loop between inflammatory aggravation and autophagy mediated via multiple redox cascade and myeloid cells alteration in experimental allergic asthma

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ABSTRACT

Background: Allergic asthma is one of the leading respiratory diseases with complex pathology. Attributes of vitexin, a trihydroxyflavone, has been studied to alleviate Th2 cytokines response in allergic asthma. However, its efficacy and underlying mechanism in mitigating allergic asthma particularly mediated by oxi-inflammatory stress, autophagy and apoptosis, yet to be delineated.

Purpose: Present study aimed to decipher efficacy and governing molecular mechanism of vitexin in mitigating allergic asthma particularly mediated by vicious loop of oxi-inflammatory stress, autophagy and apoptosis.

Methods: To ascertain this, OVA-LPS induced mice model was used and protective attributes of vitexin for different mediators, pathological facets and sensing pathways of allergic asthma were evaluated.

Results: Vitexin treatment remarkably inhibited OVA-LPS induced inflammatory cell infiltration, mast cell activation, alveolar collapse, congestion, fibrosis in lung architecture. These results were accompanied by suppression of immune cells hyperactivation, mucus secretion, goblet cell proliferation, persistent inflammation which were affirmed by alleviation in levels of IgE, Th1/Th2/Th17, IL-4/IFN-γ, chemokines, endopeptidases (MMP-1, MMP-13), oxidative effectors with concomitant increase in IL-15, IL-10, MMP-9 and MMP-3. Additionally, noticeable decline in p-connexin 43, p-c-Fos, TGF-β, Smad2/3/4, Caspase9/3, LC3A/B expression and upregulation in beclin-1, p62 co-localization and Bcl2/Bax indicate reversal of lung vascular permeability, mast cell degranulation, fibrosis, apoptosis, autophagosome impairment. Subsequent allergic inflammatory cascades analysis revealed p-NF-κB, p-PI3K, p-Akt, p-p38, p-Stat3, GATA3 upregulation and p-PTEN downregulation in sensitized mice, which were decisively counteracted by vitexin. *In silico* studies signified target specificity of vitexin with these proteins. Suppression in myeloid cells activation and enhancements of Tregs demonstrated immunomodulatory potential of vitexin in allergic airways.

Conclusion: Collectively, to our knowledge, this is the first report that confers vitexin meditated multi-faceted protective attribute in mitigation of allergic asthma that could be linked to its suppressive effects on vicious cycle of pathological process particularly regulated via oxi-inflammation, autophagy and apoptosis. Thus, signify vitexin as safe therapeutic strategy.

Abbreviation: BSA, Bovine serum albumin; BALF, Bronchoalveolar lavage fluid; CD, Cluster of differentiation; DAPI, 4′,6-Diamidino-2-Phenylindole, Dihydrochloride; FMO, Fluorescence minus one; H & E, Hematoxylin & Eosin; IgE, ImmunoglobulinE; IHC, Immunohistochemistry; IL, Interleukin; iNOS, Inducible nitric oxide synthase; MDC, Monodansylcadaverine; MMPs, Matrix metalloproteinases; OVA-LPS, Ovalbumin-Lipopolysaccharide; PBS, Phosphate buffer saline; PI3K, Phosphoinositide 3-kinases; PIC, Protease inhibitor cocktail; PTEN, Phosphatase and tensin homolog; Stat-3, Signal transducer and activator of transcription-3; TGF-β, Transforming growth factor.

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Introduction

Asthma is a multifactorial, heterogenous chronic allergic pulmonary inflammatory disease mainly characterized by influx of inflammatory cells and remodeling (Tian et al., 2010). Amongst several types of asthma, allergic inflammatory asthma is an incurable, burgeoning pulmonary disease that affect millions of people worldwide and has become major public health concern (Soriano et al., 2020). The precise etiology of allergic asthma is still unstipulated, but it is evident that allergic asthma appears to begin with recurrent exposure to allergens, leading to lung injury and inflammation which ultimately culminates into disturbed lung homeostasis (Porter et al., 2011). Over the past 30 years, allergic asthma was believed to be mainly driven by disruption in the regulation of Th2 cell mediated immune response. However, with evolving genome-wide studies this notion has changed, findings have suggested rather than this being a single factor mediated pathology, simultaneous perturbation in several signaling cascade and kinases involved in regulation of inflammation, Th1/Th2/Th17 cytokines milieu, tissue homeostasis, autophagy and airway remodeling such as PTEN/PI3K/Akt/NF-κB, AMPK/Beclin1-LC3/p62, TGF-β-Smad-Caspase, MAPKs/GATA3-Stat3 are annotated as drivers of disease (Lambrecht et al., 2019). Intriguingly, dampening the activity of these redox signaling cascade and kinases can be considered for development of new strategies to tame allergic asthma.

Several synthetic therapies have already been used for asthma, but only few of them are effective and these are also associated with side effects (Mochimaru et al., 2017). Taking pathogenic complexity into account, it is reasonable to opine that rather than fixing a single pathological defect, simultaneous modulation of several molecular flaws with one entity could be more beneficial. In this context, flavonoids and other anti-oxidant bioactive molecules have gained much attention for prevention of inflammation associate pulmonary pathologies. Vitexin is a trihydroxyflavone that exist mainly in different traditional medicinal plant species including *Crataegus* spp., *Vitex* spp., and *Passiflora* spp. (Venturini et al., 2018). It was reported to be safe, did not exhibit any sign of toxicity even at doses as high as 2000 mg/kg and (Choo et al., 2012). Researchers are fascinated towards vitexin as it exhibits an array of pharmacological activities including antioxidant, cardioprotective and anti-inflammatory attributes. A study performed by Venturini et al., 2018 has demonstrated an anti-hypersensitive allergic potential of vitexin in asthmatic mice (Venturini et al., 2018). However, there appear to be distinct dearth of evidences pertaining to understanding whether vitexin could modulate the vicious loop between oxi-inflammatory aggravation, autophagy and apoptosis that would curtail progressive allergic lung damage. Moreover, it needs to be considered that still there is no information *per se* regarding the concomitant influence of vitexin on multiple signaling such as PTEN/PI3K/Akt/NF-κB, TGF-β-Smad/Caspase, GATA-3/STAT-3 and AMPK/Beclin1-LC3/p62 cascades in allergic asthma. Considering these apparent lacunae, the present study aimed to understand how vitexin affects deleterious pathologies including oxi-inflammatory aggravation, disrupted autophagy and apoptosis of allergic asthma. To ascertain the efficacy of vitexin, B cell homeostasis, histopathology, macrophage activation, Tregs and oxi-inflammatory facets, autophagy and structural defects sensing signaling pathways were assessed in OVA-LPS induced allergic asthma mice model. Here, our findings provided novel mechanisms of vitexin and suggested it could be a potential nutraceutical for allergic asthma.

Materials and methods

Chemicals

Vitexin (≥ 96.41%) was procured from MP Biomedicals, Ontario, USA. Ovalbumin, LPS, protease inhibitor cocktail (PIC) and RIPA lysis buffer were purchased from Sigma-Aldrich, USA. RNeasy mini kit and verso 1-step RT-qPCR Kit, SYBR green were acquired from Qiagen, USA and Thermo Fisher Scientific, Waltham, MA USA, respectively. Primary antibodies were supplied by Cell Signaling (Boston, MA, USA) or Santa Cruz Biotechnology (Dallas, Texas, USA). In addition, ELISA kits were obtained from BioLegend, USA. All other chemicals were offered by Sigma-Aldrich (St. Louis, MO, USA) and Hi-Media, Mumbai, India if not otherwise indicated.

Experimental design, administration and immunization of OVA-LPS

Six to eight weeks old female Balb/C mice (approximately 20–23 g) were randomized into five groups and maintained as reported by Tirpude et al., 2021 (Tirpude et al., 2021). All experiments were approved by the Institutional Animal Ethics Committee (Registration No. 1381/GO/ReRcBiBt/S/10/CPCSEA, Approval no. IAEC/IHBT/P-16/May 2019). To induce allergic asthma mice were sensitized and challenged with OVA and LPS as described by Tirpude et al., 2021 with slight modifications at selected time points as depicted Fig. 1A. Mice were divided into five groups ($n = 6$) viz, 1. control, 2. OVA-LPS control, 3. Dexamethasone (positive control; 0.5 mg/kg/b.w), 4–5 Vitexin (15 mg/kg/b.w and 30 mg/kg/b.w). The specific dose of vitexin and dexamethasone was selected from previous studies (Rosa et al., 2016; Venturini et al., 2018). At the end experiment, mice were euthanized using CO_2 asphyxiation and lungs, Bronchoalveolar lavage fluid (BALF) and spleen tissues were collected. A part of lung tissue was used for histopathology and special staining while remaining was used for evaluation of different cellular assays, gene and protein expression.

Cytokine determination in lung tissue homogenate

ELISA was performed to determine secretory levels of inflammatory cytokines and immunoglobulins as descried in manufacturer's protocols. Briefly, lung tissues (50 mg) were homogenized using 50 mM phosphate buffer (pH 6) having 0.5% hexadecyltrimethylammonium bromide and then centrifuged at 14,000 g for 15 min at 4 °C. Finally, recovered supernatant was used to evaluate TNF-α, IFN-γ, IL-10, IL-6 IL-4 and IgE levels relative to total protein concentration as assessed by Bradford method.

RNA isolation and gene expression analysis of inflammatory cytokines, chemokines, endopeptidase, and nitric oxide synthases in lung tissues

Lung tissues were washed using PBS. Qiagen RNeasy mini kit was used to extract total RNA**.** Gene expression analysis of selected target cytokines (Th1/Th2/Th17 and IL-15), chemokines, iNOS, MMPs was carried as per Sharma et al., 2018. qRT-PCR for different selected genes and reference gene (GAPDH) as was carried out using Verso SYBR Green 1-Step qRT-PCR ROX Mix kit as per manufacturer's instructions. The ΔΔCt method was used to calculate mRNA relative quantitation (Sharma et al., 2018). Primer sequences used for gene expression analysis and primary antibodies used are listed in Supplementary File Table 1.

Immunoblotting analysis

Total protein was extracted from lung tissue using RIPA supplemented with PIC as described previously (Tirpude et al., 2021). The immunoblotting was performed to validate several signaling cascades of oxi-inflammation, fibrosis, autophagy and apoptosis in lung tissues by following procedure described by Tirpude et al., 2021. Primary antibodies used are listed in Supplementary File Table 2. ImageJ software was used for the quantification of western blot image.

Histological and toluidine staining analysis

The extent of lung damage and inflammation was analyzed by H & E staining as described previously (Tirpude et al., 2021). Briefly, 5μm-thick sections of lung tissue embedded into paraffin, were fixed

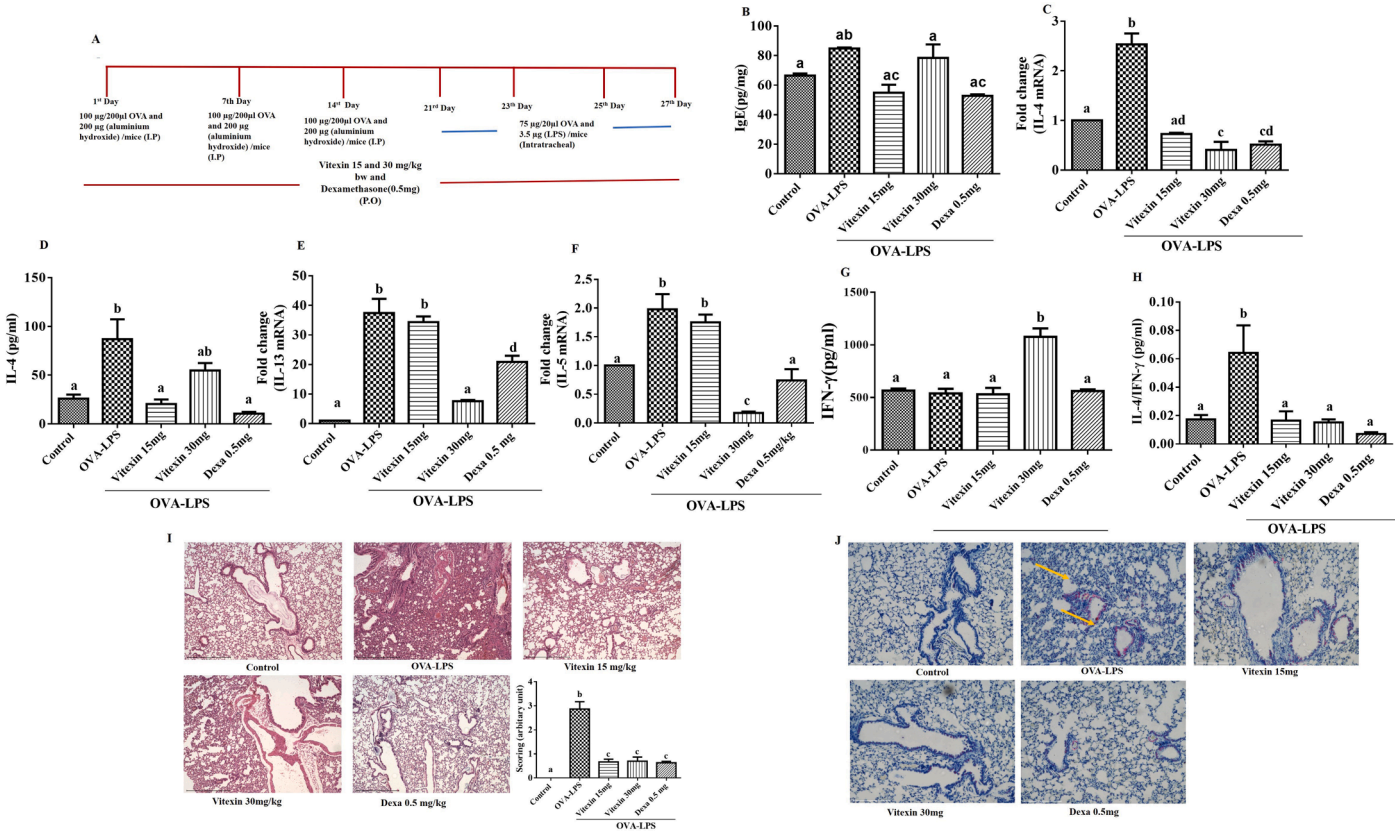


Fig. 1. Schematic study design (A) for assessing vitexin effects on (15 and 30 mg/kg b.w) in OVA-LPS-induced allergic asthma. (B-J) Vitexin mollifies inflammatory allergic response and lung damage in OVA-LPS challenged mice as evident by levels of (B) IgE (C-D) IL-4 (E) IL-13 (F) IL-5 (G) IFN-γ (H) IL-4/IFN-γ (I) Haematoxylin and eosin (J) Toluidine stained lung sections.Values are mean± SD, ($n = 3$–4). Different letters indicate statistically significant difference ($p \leq 0.05$) between different groups; groups having same letters are not significant statistically.

with 37% formaldehyde and were further deparaffinized using xylene, rehydrated and stained with hematoxylin for 8 min in incubator at 37 °C temperature. Next, sections were washed under tap water for 5 min followed by treatment with 1% acid alcohol until light brown color appears. Second washing under tap water for 5 mins was followed by incubation with 1% lithium carbonate until light blue color become visible and again sections were rinse with running tap water for 15 min. After that, sections were counterstained with eosin for 3 min, excess stain were removed using alcohol and finally mounted using dibutyl phthalate in xylene. A pathologist assesed extent of lung damage, efflux of inflammatory cell, congestion, bronchial wall thickness, alveolar collapse and fibrosis in a blinded manner using EVOS FL cell imaging system (Thermo Fisher Scientific, US). Scoring criteria of histopathological evaluation, is sub grouped into 1 to 4 (Minimal, Mild, Moderate and Severe).

Toluidine staining was carried out to detect mast cell activation in lung tissue. After deparaffinization and hydration, lung sections were stained with toluidine blue (0.25% w/v in 1% NaCl, pH 2.3) for 15 min followed by washing with tap water for 5 min. Finally, sections were dehydrated, cleared using xylene and mounted. The images were captured using EVOS FL cell imaging system. Red purple metachromatic staining indicated presence of mast cells.

Acridine orange, mdc staining and p62/SQSTM1 localization for autophagy induction analysis

Paraffin embedded lung sections of 5 µm was used for subsequent analysis for autophagy. Lung tissues were deparaffinized and stained with acridine orange (0.03% W/V in 1% glacial acetic acid) for 15 min at 37 °C for acidic vesicle organelles detection. Next, sections were dehydrated, cleared using xylene and mounted. The images were captured at 473 nm and 561 nm using EVOS FL cell imaging system to detect green and red fluorescence, respectively (Shin et al., 2012; Silveira et al., 2020).

MDC staining of lung tissue was also performed to detect autophagosomes and lysosome. Deparaffinized sections of lung tissue were stained with 200 µM of MDC for 25 min at 37 °C. Subsequently, tissues were washed twice with PBS (Shin et al., 2012; Cho et al., 2015).

Immunofluorescence assay for co-localization of p62/SQSTM1/Alexa Flour 488 was used to assess effect of vitexin on autophagosome degradation. Immunofluorescence was performed as per protocol described by Sharma et al., 2019, using ImmPRESS excel staining kit, Vector Laboratories, USA. Antibodies at a dilution of 1:50 was incubated for one and half an hour and washed twice with PBS followed by incubation with DAPI (5 µg/ml) for 5 min. The images were captured using EVOS FL cell imaging system.

BALF collection and analysis of myeloid cells markers CD11c, CD11b and F4/80 in BALF and lung tissue

BALF was collected and recovered with 0.9% (w/v) phosphate-buffered saline as described in our previous study (Tirpude et al., 2021). Briefly, mice were euthanized using asphyxiation. To perform bronchoalveolar lavage (BAL), 20-gauge polypropylene cannula was inserted in exposed tracheal tract and 4–5 ml of 0.9% (w/v) phosphate-buffered saline was instilled for recovery of fluid. After that, collected lavage fluid was centrifuged at 15,000 rpm for 8 min at maintained temperature conditions 4 °C. Finally, supernatant was discarded and cells were collected, reconstituted in appropriate vehicle and used for further assays.

Table 1
Docking result of vitexin and dexamethasone with selected proteins by using of Auto dock software (version 4.2.6).

Proteins	Molecule	Binding energy (kcal/mol) (neg)	Inhibition constant (μM)	Intermolecular energy (kcal/mol) (neg)	Dissolving energy (neg)	Electrostatic energy (kcal/mol) (neg)	Total Internal energy (neg)	Torsional energy	Docking energy (kcal/mol) (neg)
Beclin-1	Vitexin	7.85±0.08	1.83±0.25	10.82±0.08	10.51±0.08	0.31±0.00	3.76±0.01	2.98±0.00	14.58±0.09
	Dexamethasone	7.16±0.01	5.64±0.06	8.65±0.01	8.39±0.04	0.26±0.04	0.76±0.03	1.49±0.00	9.41±0.04
Smad4	Vitexin	6.15±0.13	32.64±7.40	9.13±0.14	8.70±0.14	0.43±0.01	4.06±0.18	2.98±0.00	13.19±0.31
	Dexamethasone	8.14±0.00	1.07±0.01	9.64±0.00	9.44±0.00	0.20±0.00	0.88±0.00	1.49±0.00	10.52±0.00
TGF-β	Vitexin	7.06±0.27	7.06±2.99	10.08±0.26	9.73±0.32	0.43±0.03	3.99±0.15	2.98±0.00	14.08±0.41
	Dexamethasone	6.43±0.07	19.63±2.43	7.92±0.07	7.62±0.24	0.31±0.17	1.27±0.04	1.49±0.00	9.19±0.11
Caspase - 3	Vitexin	6.18±0.01	29.70±0.50	9.16±0.01	8.67±0.07	0.50±0.07	4.06±0.00	2.98±0.00	13.23±0.01
	Dexamethasone	7.20±0.02	5.22±0.19	8.70±0.02	8.58±0.11	0.16±0.05	0.81±0.03	1.49±0.00	9.51±0.06
Bcl-2	Vitexin	4.93±0.01	244.88±4.42	7.91±0.01	7.69±0.00	0.22±0.01	4.34±0.02	2.98±0.00	12.25±0.03
	Dexamethasone	5.93±0.05	45.50±3.77	7.42±0.05	6.95±0.03	0.46±0.02	1.30±0.01	1.49±0.00	8.73±0.06
PTEN	Vitexin	6.46±0.04	18.28±1.45	9.45±0.05	8.91±0.07	0.51±0.12	3.85±0.17	2.98±0.00	13.30±0.22
	Dexamethasone	7.17±0.01	5.53±0.07	8.66±0.01	8.21±0.02	0.46±0.01	1.28±0.02	1.49±0.00	9.94±0.03
NF-κB	Vitexin	6.06±0.14	34.74±8.30	9.09±0.14	8.52±0.19	0.58±0.05	3.54±0.60	2.98±0.00	12.63±0.74
	Dexamethasone	6.94±0.02	8.24±0.23	8.43±0.02	8.33±0.01	0.10±0.01	1.10±0.03	1.39±0.14	9.53±0.05
GATA-3	Vitexin	6.06±0.03	36.38±1.77	9.04±0.03	8.87±0.14	0.18±0.11	4.22±0.42	2.98±0.00	13.26±0.45
	Dexamethasone	7.75±0.03	2.10±0.11	9.25±0.03	8.86±0.02	0.37±0.01	1.22±0.05	1.49±0.00	10.47±0.08
Akt	Vitexin	6.25±0.07	26.64±3.12	9.24±0.07	8.46±0.01	0.77±0.08	4.12±0.15	2.97±0.01	13.36±0.22
	Dexamethasone	8.92±0.10	0.29±0.04	10.42±0.10	10.20±0.04	0.22±0.06	0.17±0.04	1.49±0.00	10.59±0.14
PI3K	Vitexin	7.38±0.27	4.52±1.88	10.50±0.16	10.18±0.40	0.19±0.13	4.26±0.51	2.98±0.00	14.76±0.67
	Dexamethasone	8.05±0.02	1.27±0.05	9.54±0.02	9.35±0.03	0.22±0.01	1.07±0.06	1.47±0.03	10.61±0.09
Caspase - 9	Vitexin	6.94±0.24	9.29±3.47	9.92±0.24	9.33±0.26	0.60±0.02	4.35±0.00	2.98±0.00	14.27±0.24
	Dexamethasone	6.47±0.02	18.29±0.56	7.95±0.02	7.45±0.00	0.50±0.01	1.26±0.01	1.49±0.00	9.22±0.03
Bax	Vitexin	5.63±0.11	80.46±13.86	8.59±0.10	8.50±0.10	0.09±0.00	4.52±0.47	2.98±0.00	13.11±0.58
	Dexamethasone	7.52±0.36	3.64±2.21	9.01±0.36	8.64±0.25	0.37±0.12	0.82±0.35	1.49±0.00	9.84±0.71
Connexin 43	Vitexin	5.40±0.03	111.04±4.68	8.38±0.02	8.24±0.06	0.14±0.09	4.38±0.14	2.98±0.00	12.76±0.16
	Dexamethasone	7.31±0.03	4.43±0.24	8.80±0.03	8.61±0.04	0.20±0.01	1.00±0.20	1.49±0.00	9.80±0.23
Connexin 50	Vitexin	6.25±0.13	27.70±5.90	9.22±0.13	8.89±0.10	0.34±0.03	3.72±0.15	2.97±0.01	12.95±0.28
	Dexamethasone	6.49±0.02	17.48±0.38	9.48±0.01	9.23±0.00	0.25±0.02	4.57±0.04	2.98±0.00	14.05±0.05
Smad2	Vitexin	6.74±0.06	11.88±1.06	9.71±0.05	9.27±0.01	0.44±0.06	4.28±0.13	2.98±0.00	13.99±0.19
	Dexamethasone	8.01±0.04	1.37±0.09	9.50±0.04	9.33±0.02	0.17±0.01	1.22±0.02	1.49±0.00	10.72±0.05
Smad3	Vitexin	5.58±0.08	61.29±7.23	8.74±0.07	8.39±0.02	0.45±0.16	4.10±0.26	2.98±0.00	12.84±0.33
	Dexamethasone	7.71±0.08	2.27±0.31	9.20±0.08	9.00±0.07	0.21±0.01	1.21±0.01	1.49±0.00	10.41±0.09
c-Fos	Vitexin	3.29±0.30	4643.33±2082.07	6.28±0.30	5.91±0.17	0.35±0.13	4.12±0.12	2.97±0.01	10.40±0.42
	Dexamethasone	4.78±0.08	317.75±43.36	6.27±0.08	6.14±0.10	0.14±0.02	1.05±0.22	1.49±0.00	7.32±0.30
LC3A/B	Vitexin	7.94±0.07	1.49±0.19	10.91±0.06	10.24±0.03	0.69±0.03	4.05±0.28	2.98±0.00	14.96±0.34
	Dexamethasone	7.89±0.29	1.80±0.90	9.38±0.29	9.05±0.24	0.33±0.05	0.85±0.16	1.49±0.00	10.23±0.45
AMPK	Vitexin	5.66±0.11	72.41±13.70	8.65±0.11	7.88±0.04	0.78±0.07	3.90±0.31	2.98±0.00	12.55±0.42
	Dexamethasone	8.07±0.18	1.27±0.38	9.57±0.18	9.27±0.12	0.34±0.07	1.22±0.02	1.49±0.00	10.79±0.20
STAT-3	Vitexin	7.07±0.19	7.14±2.21	10.05±0.20	9.76±0.16	0.30±0.04	3.45±0.38	2.98±0.00	13.50±0.57
	Dexamethasone	7.36±0.02	4.05±0.17	8.85±0.02	8.57±0.10	0.28±0.07	0.95±0.33	1.49±0.00	9.80±0.35
p38	Vitexin	6.40±0.10	20.60±3.53	9.39±0.11	8.86±0.27	0.54±0.18	3.75±0.23	2.98±0.00	13.14±0.33
	Dexamethasone	8.35±0.00	0.76±0.01	9.84±0.00	9.73±0.02	0.11±0.01	1.27±0.03	1.49±0.00	11.11±0.03

(neg) indicates values are represented in negative.

Phenotypic characterizations of alveolar macrophages (AMs) for CD11b, CD11c, F4/80 markers amongst BALF were performed by AMNIS ImageStream®X Mark II Imaging Flow Cytometer (Merck Millipore, Germany). Briefly, BALF were incubated with APC-anti-mouse-F4/80, FITC-anti-mouse-CD11c and PE-anti-mouse-CD11b antibodies for 15–20 min at 4 °C. After that, cells were washed twice with PBS and resuspended in FACS buffer (PBS, pH 7.4, with 0.5% BSA and 0.1% sodium azide). CD11c+/F4/80+ and CD11b+/F4/80+ sub-population were quantified using IDEAS software, Germany. Forward and side scatter gating pattern were used to gate myeloid cells (AMs) population. Appropriate fluorescence minus one (FMO) control were used to accurately identify specific sub-population sets and for compensation purpose.

Immunofluorescence for PE-CD11b, FITC—CD11c, APC-F4/80 was performed as per protocol described by Sharma et al., 2019 using ImmPRESS excel staining kit, Vector Laboratories, USA. The images were captured using EVOS FL cell imaging system.

Flow cytometry and immunofluorescence assay of t cell sub-population in splenocytes and lung tissue

Spleen tissue were collected aseptically described previously via Sharma et al., 2018; Flow cytometry analysis for T cells subpopulation was performed as described previously (Sharma et al., 2018; Tirpude et al., 2021). Briefly, splenocytes were incubated at 4 °C for 15 min with FITC-anti-mouse CD3, PE-anti-mouse CD4, and APC-anti-mouse CD25 at particular dilution prepared in FACS buffer prepared using PBS, pH: 7.4 with 0.5% BSA and 0.1% sodium azide. AMNIS ImageStream RX Mark II Imaging Flow Cytometer (Merck Millipore, Germany) and ImageStream system software was used to perform Flow cytometry and data analysis, respectively. For gating of single cell population, forward and side scatter pattern was used. Appropriate FMO (fluorescence minus one) control were used to precisely measure specific sub- sets of T cell population and for compensating purpose. CD3+/CD4+ and CD4+/CD25+ sub-population were quantified using IDEAS software, Germany.

Immunofluorescence for FITC-anti-mouse CD3, PE-anti-mouse CD4, and APC-anti-mouse CD25 was performed as per protocol described by

Sharma et al., 2019, using ImmPRESS excel staining kit, Vector Laboratories, USA.

Molecular docking to validate immunoblotting of signaling cascade

Molecular docking was carried out to analyze the probable interactions of target proteins with vitexin and dexamethasone. Table 1 depicted the 3D structure of proteins retrieved from RCSB protein data bank (Berman et al., 2000) and for modeling of connexin-43, connexin-50 and LC3A/B structure an online software Phyre2 (Kelley et al., 2015) was used. Vitexin and dexamethasone structure was obtained from PubChem. Before performing docking using Autodock tools and Autodock 4.2.6 energy minimization of proteins and compounds were performed. Native binding pocket of proteins from homologous proteins were used for docking purpose and binding affinity of protein-ligand docked complex was confirmed using GROMACS. LigPlot and PyMol were used for to visualize protein-ligand docked complex interactions and representation of 3D cartoon structure, respectively.

Statistical analyses

Resource equation method was used to determine the group size needed for adequate statistical analysis. Statistical analyses were performed using one-way ANOVA followed by Tukey's test to measure the statistical significance amongst means. Differences with p-value ≤ 0.05 were considered as statistically significant. Different letters indicate statistically significant difference ($p \leq 0.05$) between different groups; groups having same letters are not significant statistically.

Results

Vitexin mollifies allergic inflammatory response and lung damage in ova-lps challenged mice

To assess the anti-allergic potential of vitexin, predominant mediator of type 1 hypersensitivity IgE and its central regulators IL-4 and IL-13 along with Th2 cytokine IL-5 were assessed. As depicted in Fig. 1B-F, noticeable increase in IgE, IL-4, IL-13 and IL-5 levels was observed in OVA-LPS challenged group in comparison to control group. On the other hand, vitexin treatment evidently alleviated the scenario of IgE and these Th2 cytokines. Further, decrease in IL-4/IFN-γ cytokine ratio in vitexin treated mice as compared to OVA-LPS sensitized mice suggest its preventive attributes in allergic airway inflammation (Fig. 1H).

Histopathological analysis confirmed allergen induced lung injury and inflammation (Fig. 1I). It was noticed that compared to control group, OVA-LPS challenge showed fibrosis, inflammatory cell

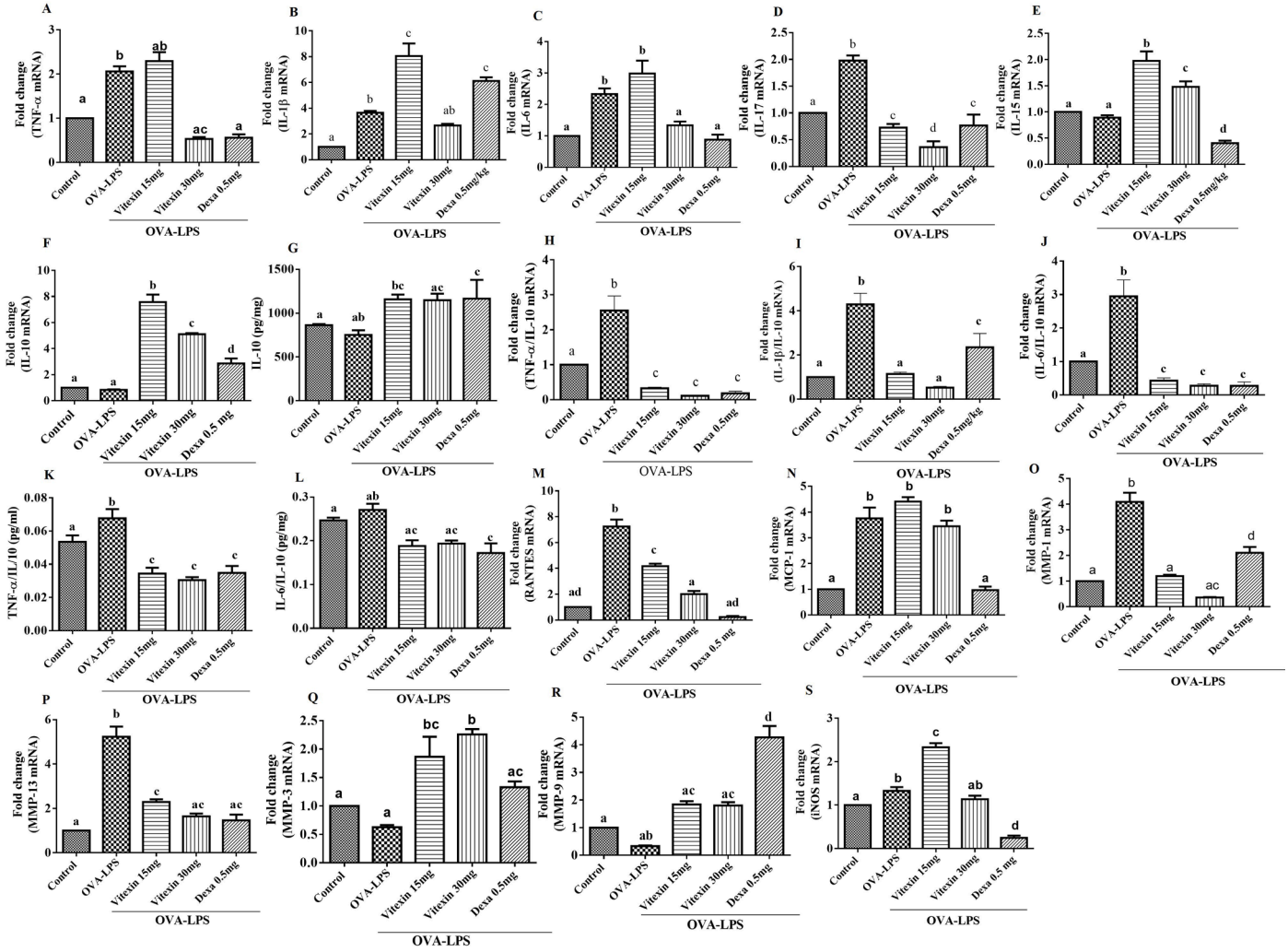


Fig. 2. Vitexin modifies inflammatory and redox homeostasis in OVA-LPS challenged mice as affirmed by changes in expression and levels of cytokines milieu, proteases levels and oxidative effectors. (A) TNF-α (B) IL-1β (C) IL-6 (D) IL-17 (E) IL-15 (F-G) IL-10 (H) TNF-α/IL-10 mRNA (I) TNF-α/ IL-1β mRNA (J) TNF-α/IL-6 mRNA (K) TNF-α/IL-10 (l) IL-6/IL-10 (M) RANTES (N) MCP-1 (O) MMP-1 (P) MMP-13 (Q) MMP-3 (R) MMP-9 (S) iNOS. Values are mean± SD, (n = 4–5). Different letters indicates significane level at $p \leq 0.05$.

infiltration, congestion, bronchial thickness, peribronchial inflammation and alveolar collapse indicating overall disruption in pulmonary architecture. However, vitexin treatment apparently reverted these pathologies as evident by normalization of alveolar structure and airway remodeling. Additionally, toluidine staining confirmed activation of mast cell which was also reversed by vitexin (Fig. 1J).

Vitexin modulates cytokines milieu, protease levels and redox homeostasis

Perturbation in cytokines milieu, proteases balance and redox homeostasis results in exaggerated inflammatory response which ultimately leads to tissue damage. Therefore, to corroborate and substantiate the impact of vitexin on allergic pulmonary inflammation, we measured levels of Th1/Th17/IL-15/IL-10 cytokine, chemokines, proteases (MMPs) and other inflammatory mediators.

Compared to control group, OVA-LPS challenge resulted in a significant ($p \leq 0.05$) enhancement of pro-inflammatory cytokines TNF-α, IL-1β, IL-6, IL-17 with concurrent slight reduction in IL-10 and IL-15 transcript levels (Fig. 2A-G). On contrary, vitexin treatments markedly countered these tendencies albeit at higher dose (30 mg/kg/b.w.). Intriguingly, lower dose of vitexin comparatively provoked mRNA expression of TNF-α, IL-1β and IL-6, while at the same time it maintains the ratio of pro-inflammatory/anti-inflammatory mRNA fold change as shown in Fig. 2A-C and Fig. 2H-J suggesting maintenance of cytokines homeostasis. Additionally, increased level of TNF-α/IL-10 and IL-6/IL-10 ratio in OVA-LPS group further indicates skewed pro-inflammatory-anti-inflammatory cytokines response which was significantly balanced via vitexin treatment (Fig. 2K-M).

Assessment of chemokines and proteases revealed that OVA-LPS challenge had stimulatory effect on RANTES, MCP-1, MMP-1 and MMP-13 transcript level, whereas inhibitory impact on MMP-3 and MMP-9 mRNA expression when compared to control (Fig. 2M-R). Concomitantly, OVA-LPS challenge group showed significant increase in iNOS expression in comparison to control group which further signifies disruption in inflammatory and redox homeostasis (Fig. 2S). In contrast, vitexin administered mice showed a significant reduction in transcript level of RANTES, MMP-1 and MMP-13, while a non-significant and marginal decrease in MCP-1, iNOS mRNA expression, albeit only at higher dose. Moreover, decline in MMP-3 and MMP-9 expression was significantly upregulated via vitexin. Fascinatingly, vitexin follow the same trends on above mentioned factors as observed in dexamethasone administered mice. These results imply that vitexin can modulates allergen specific distorted inflammatory and redox homeostasis.

Vitexin influences autophagic response by inhibiting autophagosome formation and degradation

Next, to verify the notion that ROS dependent inflammation activates autophagic response, we examined the autophagic mechanism in OVA-LPS as well as vitexin exposed lungs. Preliminary confirmation of autophagic response was noticed via acridine and MDC staining. As depicted in Fig. 3A-B, compared to control group, OVA-LPS sensitization apparently enhanced red fluorescence and green indicating presence of acidic vesicular organelles and induction of autophagy. On contrary, vitexin treatment effectively diminished these anomalies. For better interpretation regarding induction of autophagy process via allergen sensitization, next we assessed expression of AMPK-Beclin-1/LC3A/B-p62 markers of autophagy. Results revealed that OVA-LPS challenge effectively interrupted autophagy mechanism as evident by elevated AMPK activation, LC3A/B level and reduced Beclin-1 protein expression (Fig. 3C-E). Administration of vitexin appears to have significantly countered this scenario. Additionally, as shown in Fig 3F translocation of p62 or localization of p62-DAPI was noticeably deceased in OVA-LPS challenge which was impressively enhanced via vitexin treatment further signifying its role in maintaining cellular homeostasis.

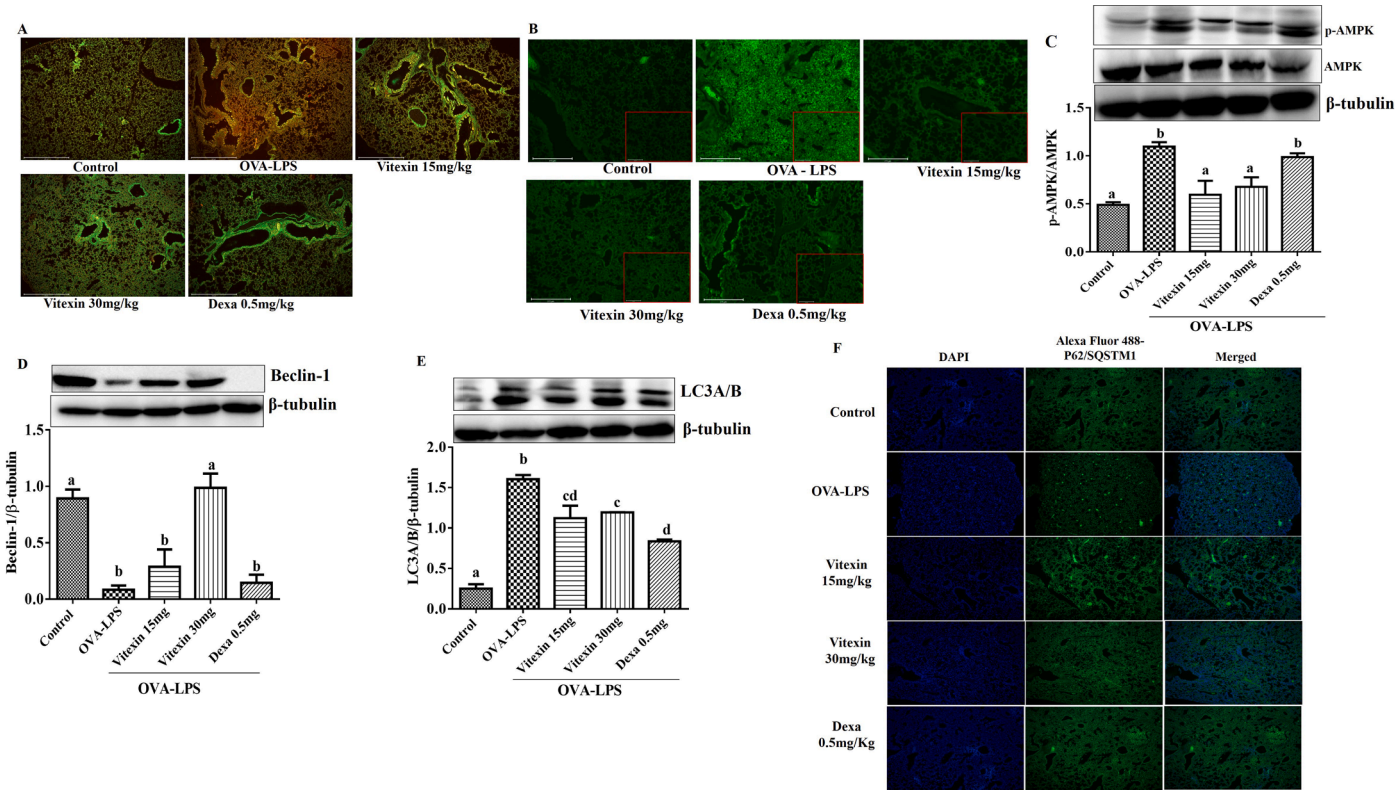


Fig. 3. Vitexin modulates OVA-LPS impaired autophagosome accumulation and degradation. (A) representative images of acridine-orange-stained for acidic vesicle organelles (AVOs) (B) Monodansylcadaverine (MDC) staining for autophagic vacuoles. (C) Protein expression of p-AMAPK/AMPK (D) Beclin-1 (E) LC3A/B and (F) Co-localization of DAPI-p62/SQSTM1. Values are mean± SD, ($n = 3$). Different letters indicates significane level at $p \leq 0.05$.

Vitexin reduces fibrosis and apoptosis in OVA-LPS challenge

Next, we examined anti-fibrotic and anti-apoptotic effects of vitexin on OVA-LPS sensitized lung tissue. It was observed that compared to control group OVA-LPS exposure markedly increased expression of TGF-β (Fig. 4A), a pivotal regulator of pulmonary fibrosis which was efficiently reduced on account of vitexin administration. Additionally, compared to control group, increased protein expression of Smad2/3, Smad4, caspase9, cleaved caspase3 and decreased Bcl2/Bax ratio, a crucial signaling bridge between oxi-inflammatory stress and apoptosis was observed in OVA-LPS challenge group (Fig. 4B-F). Nevertheless, vitexin treatment opposed expression of these proteins albeit at varying degree. It is worth mentioning that observed effects of vitexin was at par with dexamethasone (Fig. 4G).

Vitexin modifies inflammatory signaling cascade PTEN/ PI3K/Akt/NF-κB-c-Fos

Signaling cascades of inflammation are well established regulators of allergic asthma, therefore, to ascertain underlying molecular mechanism of vitexin in alleviation of allergen induced inflammatory aggravation, expression and activation of PI3K upstream kinases of Akt (central regulator of redox homoeostasis), PTEN and downstream classical inflammatory transcription factor NF-κB as well as c-Fos (regulator of degranulation) was assessed. Results revealed a dramatic decrease in the activation of PTEN (negative regulator of Akt) in allergen exposed group which, however, appears to be categorically enhanced on treatment with vitexin (Fig. 5A). Subsequently, we noticed elevated activation of PI3K and Akt in OVA-LPS challenge group which was further accompanied by a remarkable increase in activation vis-a-vis ratio of p-NF-κB/ NF-κB and p-c-Fos/c-Fos (Fig. 5B-E). On contrary, vitexin administered group eventually declined the activation of PI3K/Akt/NF-κB-c-Fos in dose-independent manner signifying suppression of allergen induced exaggerated inflammatory response and mast ecell degranulation (Fig. 5B-E).

Vitexin suppressive effect on GATA-3/STAT-3/p38/Connexins confers amelioration of oxi-inflammation and Th2 cytokines mediated asthmatic damage.

Overwhelming evidences signify role of GATA-3 and STAT-3 in regulation of Th2 cytokines that mainly mediate allergic airway inflammation. As, vitexin attenuated the levels of Th2 cytokines, we sought to determine whether vitexin could affect activation of GATA-3 and STAT-3 in OVA-LPS exposed mice. The expression of GATA-3 and activation of p-STAT-3 was noticeably increased on sensitization with OVA-LPS (Fig. 5F-G). As speculated, administration of vitexin markedly diminished protein levels of GATA-3 and STAT-3.

Indeed, together with these above-described upstream kinases and transcription factors, p38 MAPKs is one of the central signaling kinase essential for activation of redox-transcription factor NF-κB, GATA-3 and Th1/Th2/Th17 cytokines response in context of allergic asthma. As such, OVA-LPS challenge significantly ($p \leq 0.05$) enhanced activation as well as ratio of p-p38/p38, which was considerably suppressed via vitexin administration (Fig. 5H).

Furthermore, elevated p-connexin 43/connexin 43 and connexin 50 expression levels in OVA-LPS sensitized group indicate enhanced lung vascular permeability which was gradually annulled via vitexin treatment deciphering, at least in part, governing mechanism by which vitexin could mitigate allergen induced asthma (Fig. 5I-J).

Vitexin regulates myeloid cell population in balf and tregs subset in splenic lymphocytes

To further gain insight into modulatory effects of vitexin on regulation of immune cell lineage, myeloid cell population in BALF and Tregs subset in splenic tissue were assessed. Result revealed significant increase in F4/80+, CD11b+, CD11c+, F4/80+CD11b+ and F4/80+CD11c+ double positive cells frequency in OVA-LPS challenge (Fig. 6A-F). On the other hand, vitexin treatment significantly reduced double positive F4/80+CD11b+, F4/80+CD11c+, CD11b+, CD11c+ cells, whereas slight reduction was observed for F4/80+ positive which could not reach to statistically significant level (Fig. 6A-F). Immunofluorescence analysis also revealed similar pattern of F4/80+, CD11b+,

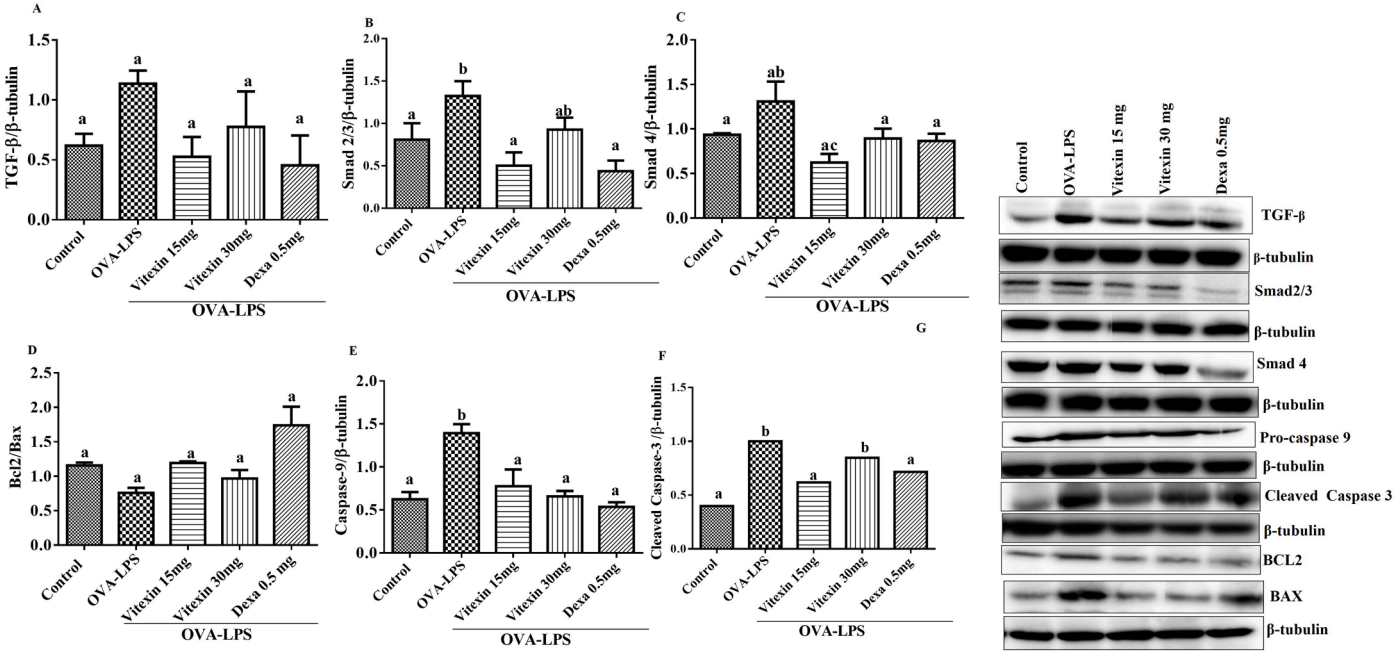


Fig. 4. Vitexin decreases fibrosis and apoptosis in OVA-LPS challenge as event by relative protein expression of (A) TGF-β (B) Smad2/3 (C) Smad4 (D) Bcl-2/Bax (E) Caspase-9 (F) Cleaved Caspase-3 (G) representative western blot images. Values are mean± SD, ($n = 3$). Different letters indicates significane level at $p \leq 0.05$.

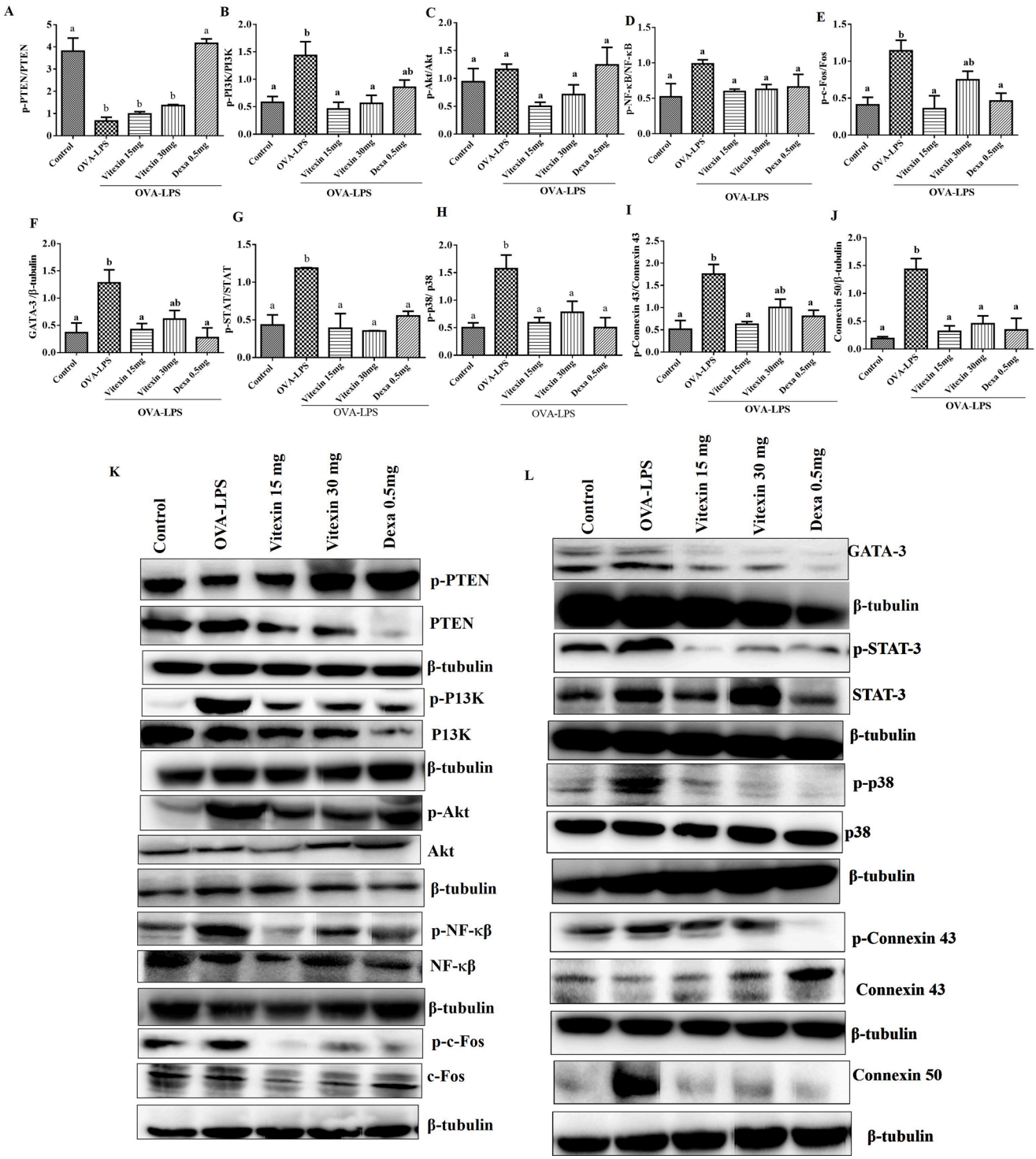


Fig. 5. Vitexin decreases oxi-inflammatory, Th2 response, lung vascular permeability and mast cell degranulation in OVA-LPS challenge as apparent by relative expression of (A) p-PTEN/PTEN (B) p-PI3K/PI3K (C) p-Akt/Akt (D) p-NF-κB (E) p-c-Fos/c-Fos (F) GATA-3 (G) p-STAT-3/STAT-3 (H) p-p38/p38 (I) p-Connexin 43/ Connexin 43 (J) Connexin 50. (K and L) Representative western blot images. Values are mean± SD, ($n = 3$). Different letters indicates significane level at $p \leq 0.05$.

CD11c+, F4/80+CD11b+ expressions in lung tissues with vitexin treatment (Fig. 6G). Analysis of T cell population in splenocytes illustrated a significant reduction in CD3+ and Tregs (CD4+CD25+) cells in OVA-LPS sensitized group as compared to control. On contrary, vitexin treatment significantly improved CD3+ and Tregs cell count at all used doses (Fig. 7A-D). These results were also confirmed using immunofluorescence-based detection in lung tissues with vitexin treatments (Fig. 7E). Nonetheless, dexamethasone showed same results for CD3+ cell count while opposite pattern with Tregs when compared with vitexin (Fig. 7A-E).

Molecular docking reveals interactions of vitexin with PTEN/PI3K-Akt/ NF-κβ, AMPK/ beclin/lc3/p62, TGF-β-Smad and GATA3-STAT3/ p38MAPKs

Based on above results, it is reasonable to hypothesize that vitexin

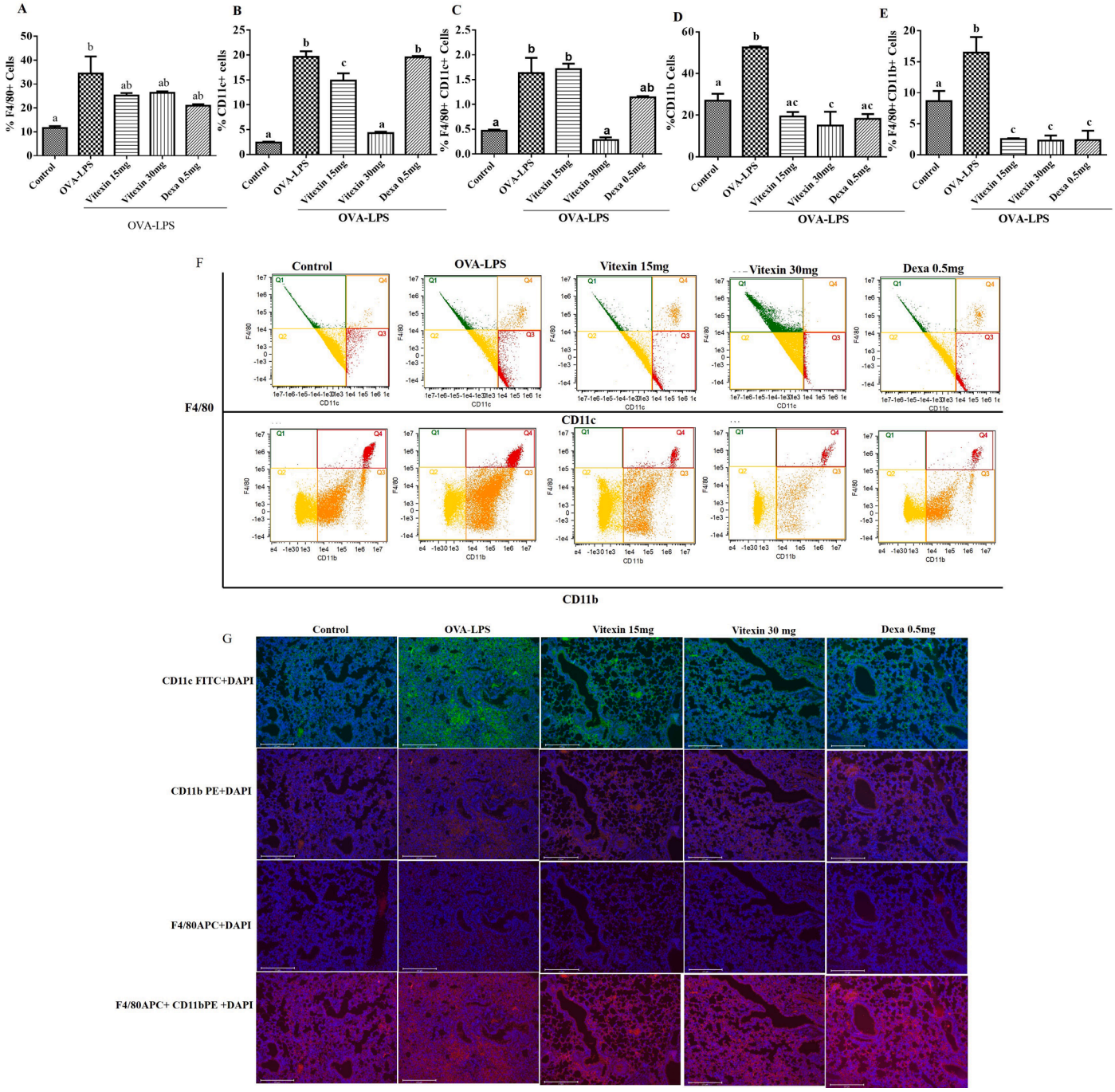


Fig. 6. Vitexin regulates myeloid cell population in BALF and lung. (A)%F4/80+ cells (B)% CD 11c+ (C)%F4/80+CD11c+ (D)% CD11b+ (E)%F4/80+CD11b+ (F) Representative flow cytometry plots of F4/80+ *vs.* CD11c+ and F4/80+ *vs.* CD11b+ (G) Expression of myeloid cell population markers F4/80+, CD11c+, CD11b+ and F4/80+CD11b+ in lung tissue. Different letters indicates significane level at $p \leq 0.05$. Values are mean± SD, ($n = 3$).

might interact with cascade proteins involved in regulation of oxi-inflammation, autophagy, fibrosis and apoptosis. An *in-silico* approach was followed to substantiate this hypothesized interaction and to validate binding affinity of vitexin as well as dexamethasone with these proteins (Figs 8-11, Supplementary File Table 3). Docking studies demonstrated that vitexin as well as dexamethasone bind with target proteins with variable binding affinity as shown in Table 1. The negative docking score (kcal/mol) is inversely proportional to binding affinity. Interestingly, the binding affinity of vitexin for these proteins was at par with dexamethasone. Collectively, *in silico* findings demonstrated that vitexin could act as potential modulator of these proteins, thereby altering regulation of these signaling cascades.

Discussion

Allergic asthma has long been thought as type 1 hypersensitivity reaction and Th2 cell- dominant disease (Lambrecht et al., 2019). Indeed, it usually occurs in individuals, who have genetic predisposition to produce IgE in response to environmental allergens, a mechanism mainly regulated by Th2 cytokines, IL-4 and IL-13 dependent B cells class switching. However, with substantial progress on allergic asthma pathogenesis, a new notion came out that it is a complex, polygenic, multifactorial pathology driven by several biological mechanisms which work in a vicious loop. Thus, key factors that mainly coordinate this vicious process can be considered as alterative drug targets for allergic

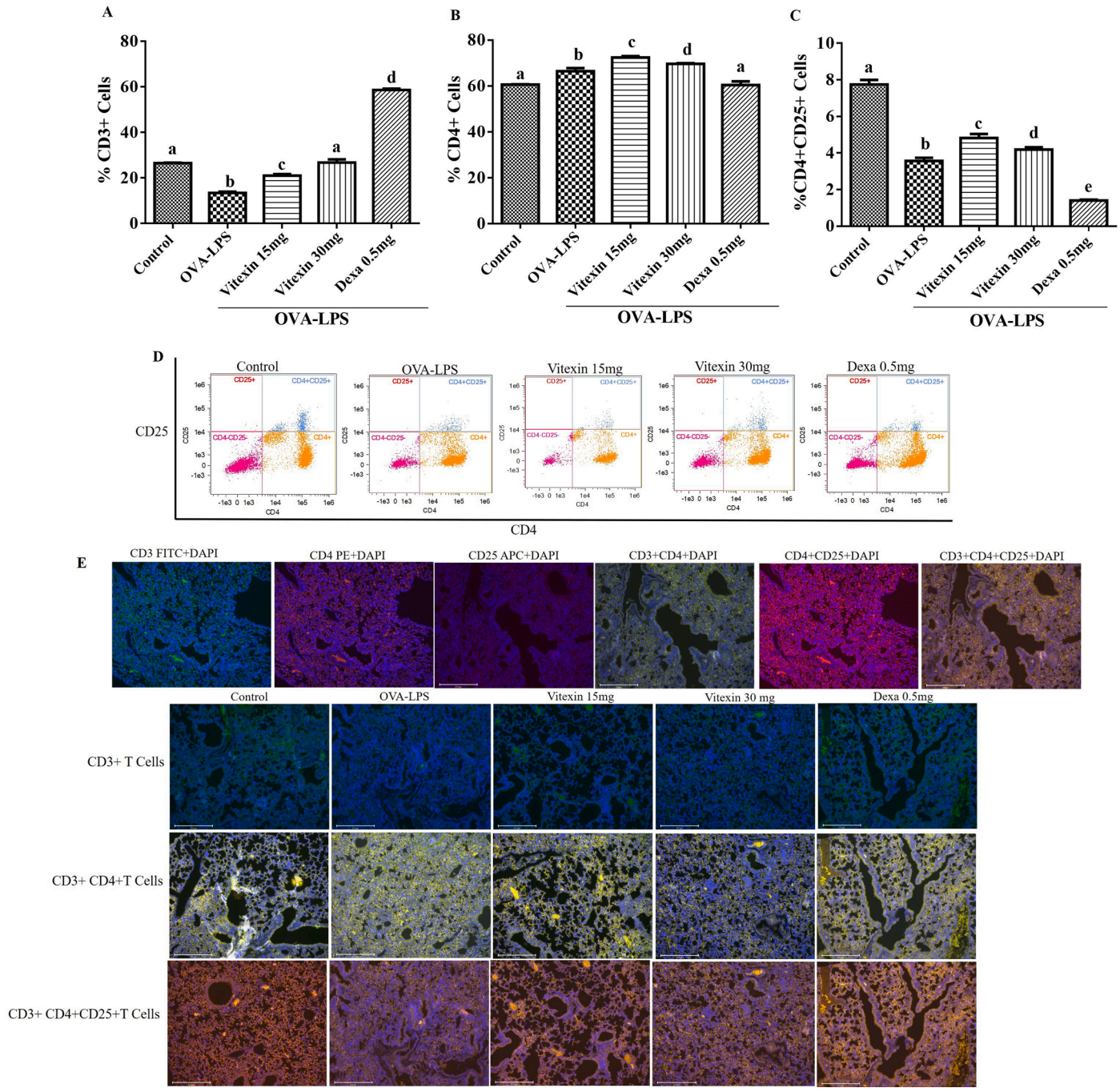


Fig. 7. Vitexin regulates splenic and lung Tregs sub-population. (A)% CD3+ cells (B)% CD4+ cells (C)% CD4+CD25+ cells (D) Representative scattered flow cytometry plots of CD4 *vs*. CD25 (E) Expression of CD3+ cells, CD4+ cells and CD4+CD25+ cells in lung tissue. Values are mean± SD, ($n = 3$). Different letters indicates significane level at $p \leq 0.05$. Values are mean± SD, ($n = 3$).

asthma. These factors encompass oxi-inflammatory aggravation, aberrant immune cells activation, Th1/Th2/Th17 dichotomy and autophagy mechanism vis-à-vis disruption in signaling kinases and cascades that mainly orchestrate allergic airway inflammation (Duecker et al., 2018). Venturini et al. (2018) studied anti-asthmatic potential of vitexin in context of OVA-induced Th2 cytokines response and hypersensitivity of immune cells. Although, vitexin is considered a potent nutraceutical, yet to best of our knowledge its therapeutic efficacy and underlying molecular mechanism in context of defective autophagy, perturbed cytokine, proteases milieu and redox-homeostasis during allergic asthma is not deciphered. In present study first time assessed the concurrent multifaceted attributes of vitexin in restoration of lung homeostasis by modulating PTEN/PI3K-AKT/NF-κβ-cFos, AMPK/Beclin/LC3/p62, TGF-β-Smad/Bcl-2/Caspase and p38MAPKs/GATA3-STAT3 cascade mediated ox-inflammation, autophagy, apoptosis, fibrosis and myeloid cells alteration in OVA-LPS induced allergic asthma. Furthermore, *in silico* studies confirmed the binding affinity of vitexin for these kinases and cascades indicating its potential in modulating expression of these pathogenic meditators of allergic asthma. OVA-LPS mice model is conductive to assess potential of any therapeutics or validating new targets for Th1/Th2/Th17 mediated oxi-inflammation associated allergic asthma (Fallon and Schwartz, 2020). In present investigation, specific dose of vitexin was selected from previous study wherein authors reported dose-independent and non-linear anti-inflammatory

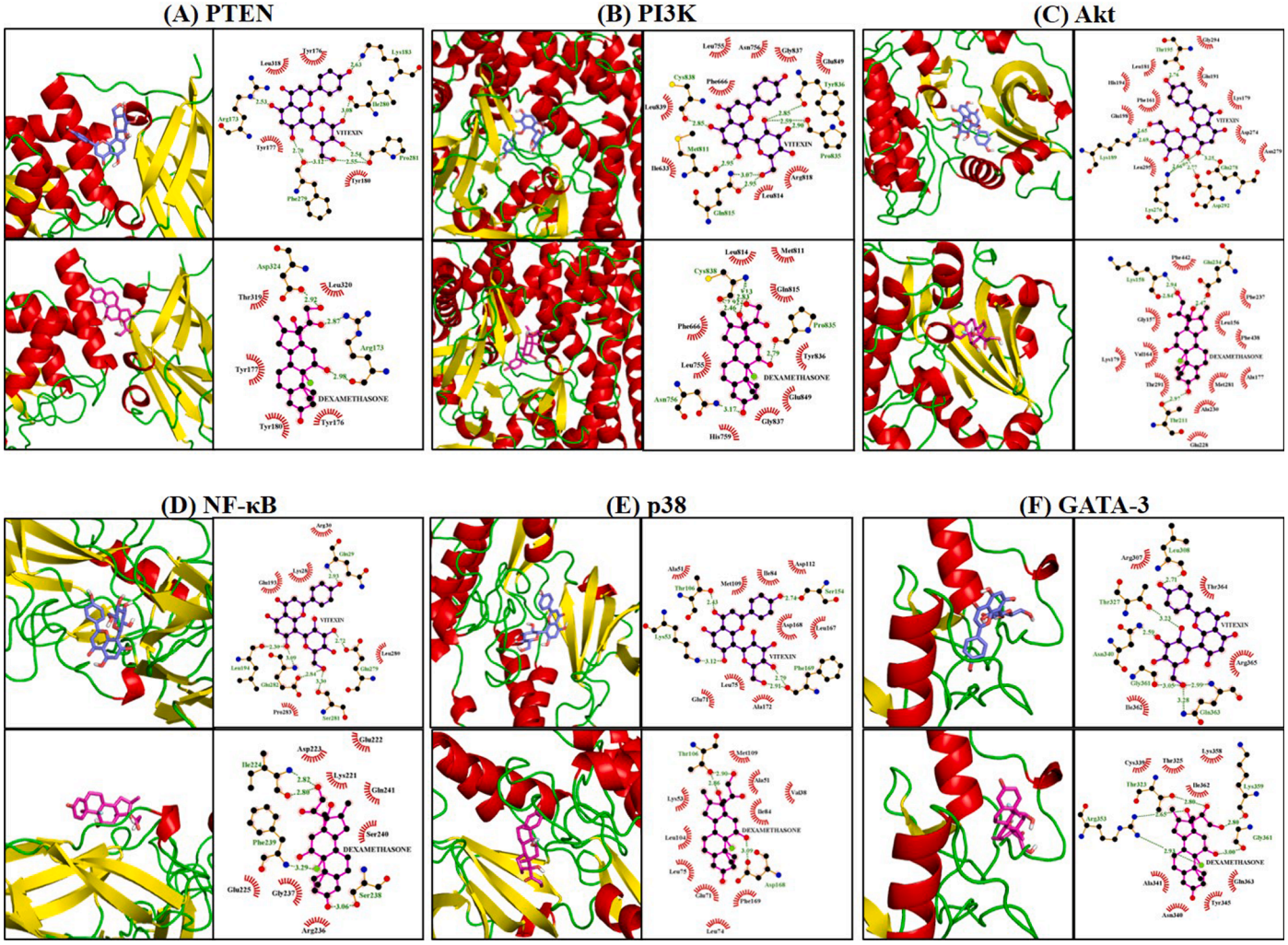


Fig. 8. Molecular binding interactions of proteins (A) PTEN, (B) PI3K, (C) Akt, (D) NF-κB, (E) p38, (F) GATA-3 with vitexin (purple color) dexamethasone (pink color) .

response and reduction in neutrophil migration with vitexin when administered orally at 5, 15, 30 mg/kg/b.w dose in a mouse model of induced acute peritonitis (Rosa et al., 2016.) Hallmark features of allergic asthma are airway remodeling, alveolar collapse, congestion, fibrosis, loss of alveolar structure, hyperactivation of immune cells particularly mast cells and eosinophils, inflammatory cells influx, goblets cells hyperplasia, mucous hypersecretion and chronic inflammation (Lambrecht et al., 2019; Shin et al., 2020; Tirpude et al., 2021). Indeed, histopathological, toluidine analysis and enhanced levels of IgE, its major driving cytokines IL-4 and IL-13 together with IL-5 affirmed establishment of allergic asthma and its hallmark pathologies. This scenario was efficiently reversed via vitexin administration, signifying its protective attributes in allergic asthma. It is also evident that allergen induced Th2 cytokine IL-5 and IL-13 release facilitates production of other inflammatory cytokines and chemokines which ultimately disrupt proteases and redox balance. Nonetheless, we also observed higher transcript level of TNF-α, IL-1β, IL-6, Th17, Chemokines, MMP-1, MMP-13, iNOS, pro-inflammatory/anti-inflammatory cytokines and IL-4/IFN-γ release with concomitant reduction in IL-15, MMP-9 and MMP-3. However, vitexin treatment impressively modulated the release and expression of aforementioned mediators in lung tissue as compared to OVA-LPS challenge, albeit independent of doses used thus further implying that vitexin could suppress airways hyperactivity, inflammatory aggravation and distorted redox milieu. Collectively, these results suggested anti-inflammatory potential of vitexin in wake of allergic asthma. These observations are in line with previous studies wherein authors reported modulation of these mediators and regulatory effect of IFN-γ, IL-15, and MMP-9 in allergic asthma (Mehra et al., 2010; Venkateshaiah et al., 2016; Shin et al., 2020; Wu et al., 2020; Tirpude et al., 2021).

Evidences reported close relationship between redox homeostasis and autophagy process in pulmonary diseases including allergic asthma. Of note, disrupted inflammatory and redox homeostasis are well known to impact on autophagy induction (Ornatowski et al., 2020; Tirpude et al., 2021). In support of these speculations and based on above results, subsequently we explored mechanism of vitexin for impaired autophagy. Enhanced acridine orange red and green fluorescence expression indicated induction of autophagy process which was further confirmed by increased p-AMPK, p-AMPK/AMPK, LC3A/B and decrease in beclin-1 expression and p62-DAPI colocalization. Fascinatingly, treatment with vitexin remarkably reversed OVA-LPS induced changes in autophagy markers albeit to varying degree, thereby suggested as prominent target, at least in part, for allergen elicited autophagy associated asthma (Tirpude et al., 2021). Collectively, these results imply that vitexin inhibited the autophagy induction as well as degradation. Interestingly, our results are in line with the notion that despite of autophagy induction, autophagic flux inhibition can also results in autophagosome accumulation (Meng et al., 2018; Zhao et al., 2019). Aiming to ascertain the molecular mechanism via which vitexin maintains allergic inflammatory homeostasis and protecting lung architecture, the regulation and

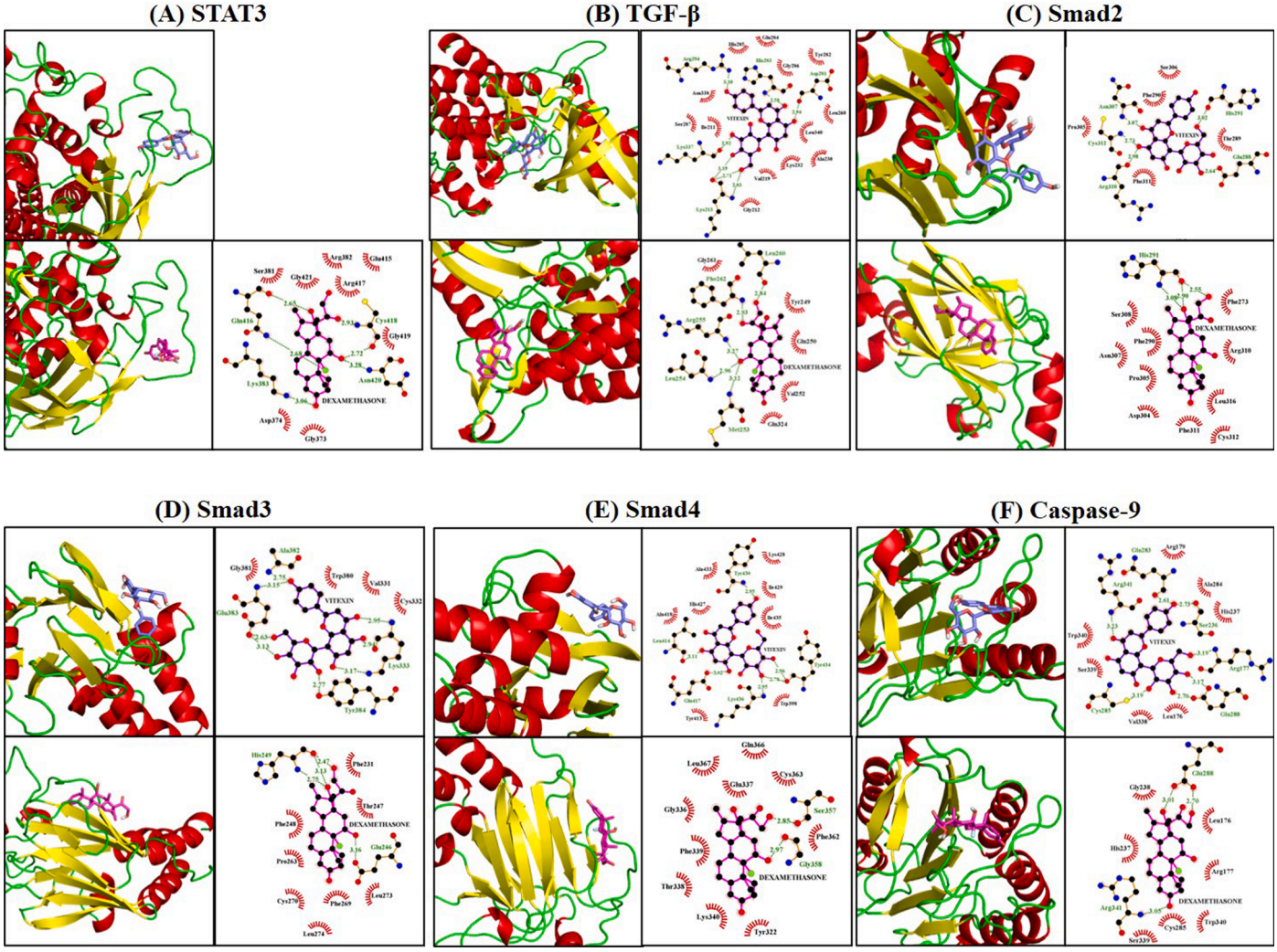


Fig. 9. Molecular binding interactions of proteins (A)STAT-3 (B) TGF-β (C) Smad2 (D) Smad3 (E) Smad4 (F) Caspase-9 with vitexin (purple color) dexamethasone (pink color).

expression of redox signaling cascades such as PTEN/PI3K/Akt/NF-κB and Akt-p38/GATA3-Stat-3 were assessed. Li et al., 2020 and Luo et al., 2019 in their study reported implication of p38MAPK/NF-κB and PI3K/Akt signaling axes in modulation of redox homeostasis, autophagy and apoptosis response (Luo et al., 2019; Li et al., 2020). p38 MAPKs cascade is one of most pivotal therapeutic targets for oxi-inflammation and is known to be activated with OVA-LPS sensitization in order to maintain inflammatory homeostasis and Th2 cytokines response. Evidences claimed its direct activation by central protein kinase B (Akt) which eventually results in regulation of downstream redox sensitive transcription factor NF-κB and Th2 cytokines differentiating transcription factor GATA-3 and Stat3 in allergic asthma (Simeone-Penney et al., 2007; Barnes, 2008; Shin et al., 2020; Wu et al., 2020). Noteworthy, in present study OVA-LPS challenge considerably instigated upregulation of GATA-3 and ratio of p-p38/p38, p-Akt/Akt, p-NF-κB/NF-κB, p-Stat3/Stat3 expression which were apparently downregulated by vitexin treatment. From these results, it is reasonable to speculate simultaneous down-regulation of PTEN (negative regulator of Akt) and up-regulation of PI3K (upstream positive modulator of PI3K). Of note, OVA-LPS challenged mice showed decline in expression of p-PTEN/PTEN ratio and increase in p-PI3K/PI3K ratio which was noticeably reversed by vitexin treatment. These observations are apparently in agreement with previous studies (Kwak et al., 2003; Barnes, 2016) where authors reported upregulation in phosphorylation of PTEN and downregulation of PI3K phosphorylation as defensive mechanism for allergic asthma. Intriguingly, effects of vitexin were almost followed the same trends as observed in dexamethasone treatment group. Furthermore, suppressive effect of vitexin on OVA-LPS induced expression of p-connexin 43, connexin 50 and p-c-FOS signifies its role in maintaining of lung vascular permeability and inhibiting mast cell degranulation as well as leukocytes migration to injured tissue. These results are in accordance with Freund-Michel et al., 2016 and Lee et al., 2004 (Lee et al., 2004; Freund-Michel et al., 2016). Collectively, these observations illustrated that vitexin could effectively mitigate OVA-LPS induced excessive oxidative stress and overwhelmed long term inflammatory aggravation and associated lung damage mainly dependent on activation of above-mentioned signaling axes. It is noteworthy that here in present study role of vitexin in modulation these signaling cascades, particularly in allergic asthma is being reported first time.

Considering evidences that impaired autophagy/redox homeostasis perturbation is associated with fibrosis and apoptosis, we further explored whether vitexin would modulates fibrosis and apoptosis mechanism. In light on this, attenuation of OVA-LPS induced TGF-β expression, a cellular sensor of autophagy, apoptosis and fibrosis as well as regulator of Smad/Caspase in vitexin administered mice indicates its inhibitory attributes for fibrosis and apoptosis (Conery and Luo, 2006; Tirpude et al., 2021). Intriguingly, this apparent effects of vitexin were further confirmed by reduction in Smad2/3, Smad4, caspase9, cleaved

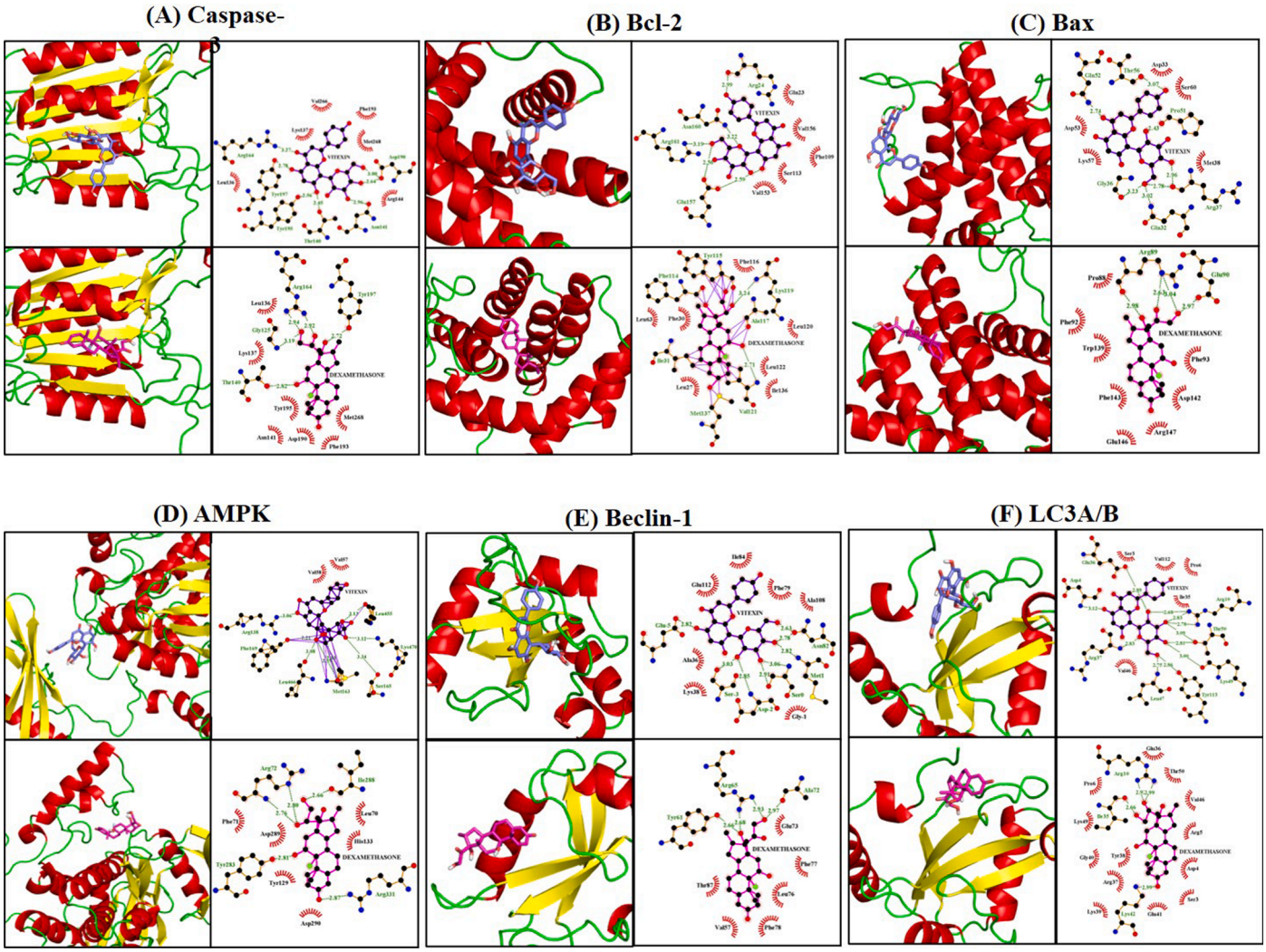


Fig. 10. Molecular binding interactions of proteins (A) Caspase-3 (B) Bcl-2 (C) Bax (D) AMPK (E) Beclin-1(F) LC3A/B with vitexin (purple color) dexamethasone (pink color).

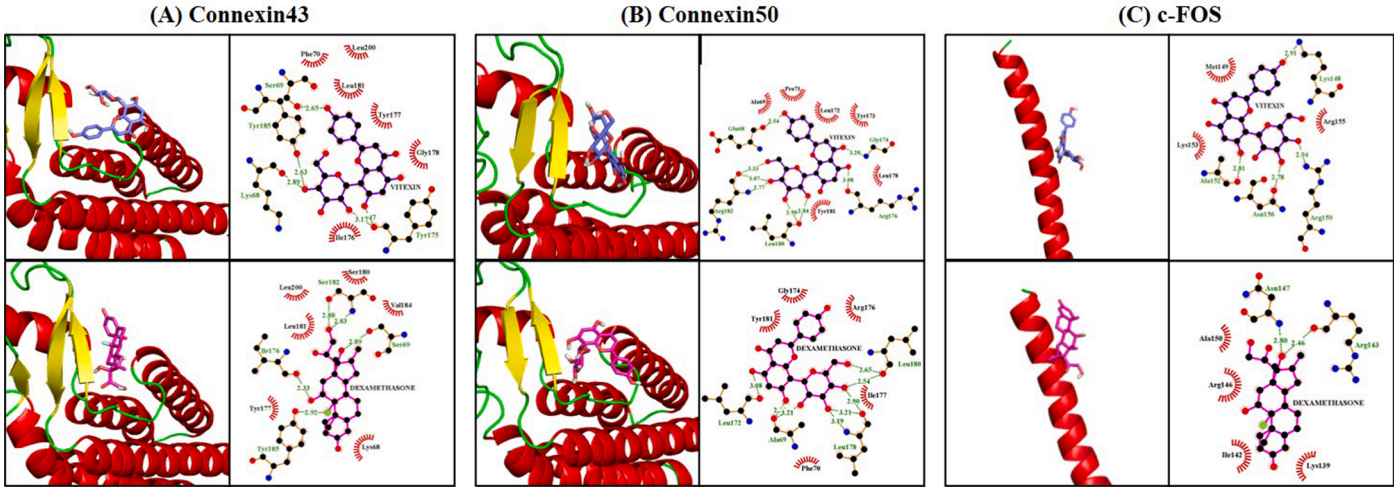


Fig. 11. Molecular binding interactions of proteins (A) Connexin43 (B) Connexin50 (C) c-Fos with vitexin (purple color) dexamethasone (pink color).

cascapse3, and increased Bcl2/Bax ratio as compared to OVA-LPS challenged group, suggesting abrogation of allergic inflammation pathologies such as fibrosis and apoptosis. These observations are well complied with our previous study where we also reported elevated level of TGF-β/Smad/Caspase9/3 with OVA-LPS sensitization (Tirpude et al., 2021). Binding affinity of vitexin with these proteins from *in silico*

studies further supported and validated the above observed findings and indicated its direct interaction could modulate expression of these proteins in airways inflammation.

Alveolar macrophages, myeloid cells population in lungs and splenic Tregs are well studies as crucial players for sustaining redox milieu and pulmonary remodeling (Maazi et al., 2012; Tirpude et al., 2021). Moreover, Xu et al., 2012 have reported splenic CD4+CD25+ T cells as an essential controller for the induction, maintenance and suppression of allergic asthma (Xu et al., 2012). Therefore, to gain insight into any modulatory role of vitexin on regulation of myeloid and lymphoid cell lineage in OVA-LPS challenge, we gauged myeloid cells and Tregs. As resulted via flow cytometry and immunofluorescence, OVA-LPS challenge revealed increase cell activation of Mϕ and total myeloid cells population as evident by increase in double positive F4/80+CD11b+, F4/80+CD11c+, CD11b+, CD11c+ cells which was considerably reduced via vitexin administration. Simultaneously, it was noticed that OVA-LPS reduced CD4+CD25+ Tregs in lung as well splenocytes which were also remarkably enhanced by vitexin treatment. Our findings are in agreement with studies performed previously, wherein authors reported that improving Treg balance and suppressing myeloid cells activation contributed to the re-establishment lung homeostasis during allergic asthma (Maazi et al., 2012; Ma et al., 2013; Tirpude et al., 2021). Altogether, results demonstrated that vitexin displayed considerable protection, at least in part, against allergen induced asthma via modulating innate as well we adaptive immune response. Overall, present study showed dose-independent and non-linear response of vitexin in mitigation of allergic inflammatory asthma, that could be attributed to pleiotropic nature of vitexin. Secondly, it indicates the possibility of concurrent induction of multiple physiological response mechanisms that may change its effective concentration along with compensatory mechanisms to maintain homeostasis. During pre-clinical studies such observations are often as overall effect lies on several facets of inflammatory and redox homeostasis and also reported with vitexin by other author previously (Rosa et al., 2016; Li et al., al.,2021).

Conclusion

As illustrated in Fig 12, present study findings showed concurrent multifaceted modulatory potential of vitexin for OVA-LPS induced perturbed and dysregulated oxi-inflammation, autophagy, apoptosis and fibrosis which are considered as prominent characteristic features of allergic asthma and also evident to be directly or indirectly involved in its pathology. Taken together, this work providing comprehensive understanding of governing molecular mechanism (s) of vitexin in wake of allergic asthma mediated by PTEN/PI3K-Akt/NF-κβ, AMPK/ Beclin/ LC3/p62, TGF-β-Smad and GATA3-STAT3/p38MAPKs axes. Although, present study assessed vitexin as convincing alternative therapeutic strategy for allergic airway, much more investigations with a focus on autophagy flux, apoptosis, airway resistance and physiology are required to be carried out. It would be more interesting to compare its potential on more realistic model of allergic asthma. Additionally, it would be fascinating to know whether vitexin penetrate cell membrane to get into cytoplasm or nucleus before it binds to those molecules by using immunoprecipitation or other functional assay such as enzymatic inhibition. To validate and confirm for better interpretation of *in-silico* molecular interaction, further binding studies using surface plasmon resonance, isothermal calorimetry or microscale thermophoresis can be performed.

Author contributions

NVT conceptualized and designed the study. NVT, AS, NB performed experiments. NVT and AS was involved in data analysis. MK performed *in silico* study. NVT wrote and edited manuscript. All data were generated in-house, and no paper mill was used. All authors agree to be accountable for all aspects of work, ensuring integrity and accuracy.

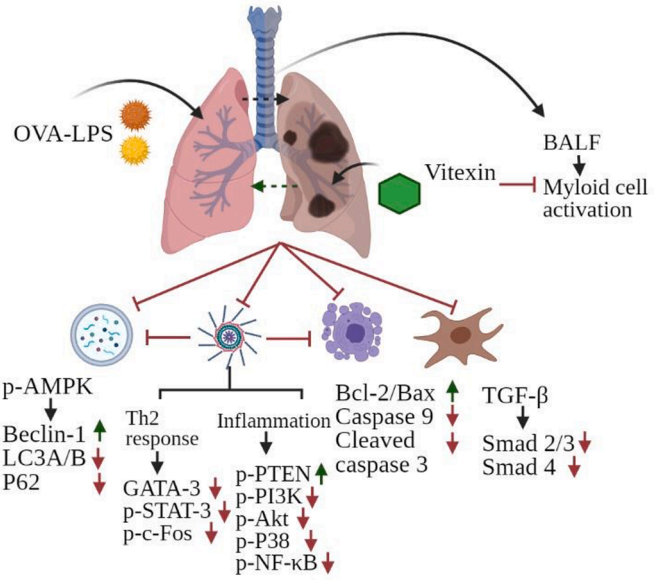


Fig. 12. Scheme concluding protective attributes of vitexin on OVA-LPS induced allergic asthma. Vitexin treatment impressively maintains lung homeostasis by modulating Th1/Th2/Th17 cytokines which regulates oxi-inflammation, associated autophagy, apoptosis and fibrosis that work in vicious loop.

CSIR IHBT manuscript number for this work is 4808.

Declaration of Competing Interest

The authors have no conflicts of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2021.153902.

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