Calcio-herbal formulation, Divya-Swasari-Ras, alleviates chronic inflammation and suppresses airway remodelling in mouse model of allergic asthma by modulating pro-inflammatory cytokine response

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\textbf{ABSTRACT}

Asthma is a chronic allergic respiratory disease with limited therapeutic options. Here we validated the potential anti-inflammatory, anti-asthmatic and immunomodulatory therapeutic properties of calcio-herbal ayurvedic formulation, Divya-Swasari-Ras (DSR) in-vivo, using mouse model of ovalbumin (OVA) induced allergic asthma. HPLC analysis identified the presence of various bioactive indicating molecules and ICP-OES recognized the presence of Ca mineral in the DSR formulation. Here we show that DSR treatment significantly reduced cardinal features of allergic asthma including inflammatory cell accumulation, specifically lymphocytes and eosinophils in the Broncho-Alveolar Lavage (BAL) fluids, airway inflammation, airway remodelling, and pro-inflammatory molecules expression. Conversely, number of macrophages recoverable by BAL were increased upon DSR treatment. Histology analysis of mice lungs revealed that DSR attenuates inflammatory cell infiltration in lungs and thickening of bronchial epithelium. PAS staining confirmed the decrease in OVA-induced mucus secretion at the mucosal epithelium; and trichrome staining confirmed the decrease in peribronchial collagen deposition upon DSR treatment. DSR reduced the OVA-induced pro-inflammatory cytokines (IL-6, IL-1β and TNF-α) levels in BALF and whole lung steady state mRNA levels (IL-4, -5, -33, IFN-γ, IL-6 and IL-1β). Biochemical assays for markers of oxidative stress and antioxidant defence mechanism confirmed that DSR increases the activity of SOD, Catalase, GPx, GSH, GSH/GSSG ratio and decreases the levels of MDA activity, GSSG, EPO and Nitrite levels in whole lungs. Collectively, present study suggests that, DSR effectively protects against allergic airway inflammation and possess potential therapeutic option for allergic asthma management.

\textbf{1. Introduction}

Asthma is a chronic allergic respiratory disease affecting more than 300 million people worldwide \cite{1} and its prevalence has been rising. It is characterized by chronic airway inflammation, excessive mucus production, episodic airway obstruction and airway wall remodelling driven by Th2 lymphocytes \cite{2}. Pathogenesis of asthma involves a complex interaction of genetic, environmental and immunological factors \cite{3}. Interaction between airway epithelium and components of innate and adaptive immune system play a very crucial role in orchestrating the pathogenesis and progression of asthma \cite{2,4,5}.

Immunologically, allergic asthma involves infiltrating of a variety of inflammatory cells, including eosinophils, mast cells, T-lymphocytes, neutrophils, and macrophages among others \cite{2,5}. The Th2 cell biased immune responses in asthma includes, infiltration of Th2 cells into lung and secretion of cytokines like IL-4, -5, -9, -13 and -33 \cite{4}. These cytokines call for eosinophilic inflammation, isotype switching of B cells leading to allergen specific IgE production \cite{6} and tissue damage, resulting in airway hyper responsiveness (AHR) and release of additional inflammatory mediators \cite{2,7}.

Oxidative stress plays a critical role in the pathogenesis of asthma \cite{8} due to an imbalance between the production of pro-oxidants and...
antioxidant defence mechanisms in the lungs. In asthma, oxidative stress can increase mucus hypersecretion, induce smooth muscle contraction and airway hyper-responsiveness [9,10]. Oxidative stress activates various signalling pathways in immune cells resulting in progression of asthma pathology [9].

While there is significant understanding about the pathophysiological processes underlying asthma, current treatments fail to cure the disease, imposing high social and economic costs. Though, use of combination of inhaled corticosteroids and β2-adrenergic agonists can control the pathological features of asthma, in 5–10 % of patients, the disease is refractory to corticosteroid treatment, which leads to hospitalization [2]. Further, blocking antibodies against specific cytokines have recently been approved for patients with severe asthma with limited responders [4]. Consequently, identifying new anti-asthmatic therapies that are cost effective, well-tolerated and effectively mitigate the symptoms of asthma are needed.

The practice of complementary or alternative medicine as adjunct in asthma treatment and as anti-inflammatory therapy has been increasing [11]. Natural products contain structurally diversified bioactive chemical combinations [12,13], offering valuable targets for novel drug discovery. Natural products and their derivatives account for 34 % of new medicines approved by the US Food and Drug Administration (FDA) between 1981 and 2010 [13]. A number of plant-based herbal formulations and purified natural compounds have shown promising results in preclinical and clinical studies of asthma in reducing clinically relevant symptoms [14,15,11].

Divya-Swasari-Ras (DSR) is a calcium containing herbal formulation that has been prescribed in India by ayurvedic practitioners, as a remedy for common cold, chronic cough, asthma and phlegm accumulation in chest. However, its anti-asthmatic and anti-inflammatory properties have never been explored scientifically till date. In the present research work, we attempted to validate potential anti-asthmatic therapeutic properties of DSR in-vivo using mouse model of ovalbumin (OVA) induced allergic asthma. In addition, HPLC and ICP-OES analysis were undertaken to identify chemical fingerprints present in DSR formulation and their co-relation with observed biological responses.

2. Methods

2.1. Experimental animals

All the animal care and treatment procedures were performed in adherence to the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India guidelines; and were approved by the Institutional Animal Ethical Committee of Patanjali Research Institute (IAEC approval number: PRIAS/LAF/IAEC-062). Male BALB/c mice (20–25 g) of 8–10 weeks’ age were procured from Charles River Laboratory licensed supplier, Hylasco Biotechnology Pvt. Ltd, Hyderabad, India and were housed in a vivarium facility with a 12:12-h light-dark cycle. The animals were supplied with standard pellet diet (Purina Lab Diet, St. Louis, MO, USA) and sterile filtered water ad libitum.

2.2. Dose calculation for in-vivo experiments

The animal equivalent doses of DSR for mouse studies were estimated based on the body surface area of the animals. The human recommended dose of the DSR powder is 1000 mg, twice a day. Accordingly, total human dose is 2000 mg/60 kg/day (33.33 mg/kg/day). Animal equivalent doses (mg/kg) for mouse were calculated by multiplying human equivalent dose (mg/kg) by factor of 12.3 [16]. Resultant therapeutic equivalent doses for mouse were found to be 410 mg/kg. Therefore, 400 mg/kg (round off) has been taken as mouse doses (human equivalent dose), and taken as the mid-dose for pharmacological studies.

2.3. Establishment of asthma model and drug treatment

Healthy mice were selected and randomized based on body weight and divided into six different (Fig. 1) groups namely, 1. Normal Control (NC); 2. Disease Control (DC); 3. 0.5 mg/kg Dexamethasone (DEXA); 4. 135 mg/kg Divya-Swasari-Ras (DSR); 5. 400 mg/kg DSR; and 6. 1200 mg/kg DSR.

All groups except the Normal Control (NC) mice were sensitized by intraperitoneal (i.p.) injection of 25 μg and 100 μg OVA (emulsified with 2 mg Aluminum hydroxide in 200 μl phosphate buffered saline (PBS) (pH 7.4)) on day 0 and day 7, respectively. Subsequently, mice were challenged with intra-nasal (i.n.) installation of 100 μg OVA in 25 μl PBS on day 14, 21, 28, 35, 42, 49, 54, 56 and were euthanized on day 58. Whereas, mice in NC group were sensitized and challenged with equal volume of PBS only.

Corticosteroid drug, Dexamethasone (DEXA), has been used for positive control as standard for inhibiting features of allergic asthma [17]. OVA-sensitized mice were treated with DSR, orally, from day 11 till the end of experiment and DEXA (0.5 mg/kg) by i.p., 2 h before OVA challenge, respectively. The mice in OVA-challenged (DC) group and the NC group were treated with carboxyl methyl cellulose (CMC) only by oral administration, similar to DSR group.

2.4. Bronchoalveolar lavage (BAL) phenotyping and harvesting lungs

The mice were humanely sacrificed, and the left lung was ligated and the right lung was subjected to broncho-alveolar lavage with 3 vol (1 ml / 25 g of ice-cold PBS by inserting a catheter in the trachea. The BAL fluid collected from the lungs of the mice was centrifuged 3000 rpm for 3 min at 4 °C, and the supernatant was retained for further.
analysis [18]. The red blood cells in cell pellets were lysed in 1% acetic acid, and BAL cells were re-suspended in PBS. Subsequently, the differential leukocyte counts were measured after Wright’s staining [19]. The lobes of right lung were resected; and flash-frozen in liquid nitrogen for RNA and protein isolation later.

2.5. Determination of cytokines levels

Enzyme-linked immunosorbent assay (ELISA) of BALF was performed to determine the levels of IL-6, TNF-α (BioLegend Inc., San Diego, USA) and IL-1β (Invitrogen, USA), according to the manufacturer’s instructions. ELISA plates were read at 450 nm using the Envision microplate reader (Perkin Elmer, USA).

2.6. RNA isolation and real-time quantitative RT-PCR of lung tissue

Resected lung samples were homogenized in TRIzol regent (Invitrogen, USA), and total RNA was extracted according to the manufacturer’s protocol. Tissue RNA was re-purified and rendered DNA-free using the RNeasy mini kit (Qiagen, USA). RNAs were reverse-transcribed using the Verso cDNA synthesis kit (Thermo Fisher Scientific, USA). Quantitative real-time PCR (qPCR) analysis was performed using the TProfessional thermocycler (Biometra, Germany) using SYBR green chemistry probes (Applied Biosystems, USA). Gene expression levels were calculated relative to PpiA (Cyclphin A) using the ddCT method as described previously [18,20]. The primers sequences used were as follows:

PpiA Fwd:5′-GGTGTGACCTTTACACGCCA-3′
PpiA Rev:5′-TCTCCGTAGATGGACCTGCC-3′

IL-4 Fwd:5′-GGTCTCAACCCCAAGCTGAGT-3′
IL-4 Rev:5′-GCGGTATGCTCTCAGTAGT-3′

IL-5 Fwd:5′-CTGGTTGCAAGCAGAATGAGG-3′
IL-5 Rev:5′-TCTTCAGTATGTCTAGCCCCTG-3′

IL-6 Fwd:5′-TCCAACCTCAAGATTTCGCCG-3′
IL-6 Rev:5′-CATCGTAGACATGGCAAGAA-3′

IFN-γ Fwd: 5′-ATGAAAGCGCTACACCTGCATC-3′
IFN-γ Rev: 5′-CCATCTCTGGGACCTTCCTC-3′

IL-6 Fwd:5′-CCTCTCTGACAGAGCTTCCC-3′
IL-6 Rev:5′-AGTCTCTCCGCGAGCTTG-3′

IL-1β Fwd:5′-CCTCTGTGAGGAGGTGGTGG-3′
IL-1β Rev:5′-TGGTCTGCTGTTGAGTGGTG-3′

2.7. Histological examinations

Lung tissue sections were fixed in 10 % (v/v) neutral-buffered formalin, dehydrated in various concentrations of ethanol, embedded in paraffin and cut into 5 μm thick sections. Then, the sections were deparaffinized and rehydrated by passing through a series of xylene and graded alcohol and were stained with Hematoxylin and Eosin (H&E), Periodic-Acid-Schiff (PAS) and Mason-Trichrome (MT) stains. Low-magnification and high-magnification history images of the lung tissue sections were obtained at 100× and 400×, respectively, by using a bright-field microscope. H&E stained lung sections were analysed for inflammatory cell infiltration at bronchi and at alveoli, along with changes in bronchial epithelium. Mucous secretion and goblet cell hyperplasia at bronchial epithelium was assessed by histological analysis of PAS-stained sections. MT stain was used for assessing peri-bronchial collagen deposits. The severity of histopathological changes was determined using 5-point score system [21,22] as follows: 0 = No Abnormality Detected (NAD), 1 = Minimal, 2 = Mild, 3 = Moderate, 4 = Moderately Severe, 5 = Severe; and distribution was recorded as focal, multifocal and diffused.

2.8. Assessment of anti-oxidative biochemical parameters

2.8.1. Assay of antioxidant enzymes (SOD, CAT, GPX and EPO activity assay)

SOD activity was assayed as per Beauchamp and Fridovich (1971) [23] by measuring the ability of SOD to inhibit the photochemical reduction of NBT (Nitro blue tetrazolium chloride). The activity of enzyme is expressed as unit/ml lung tissue. The Catalase (CAT) activity assay was performed using spectrophotometric determination of Hydrogen Peroxide (H₂O₂) [24] which form stable complex with Ammonium Molybdate. CAT activities are expressed as kilo unit per Liter (kU/L). Glutathione peroxidase (GPx) activity was assayed according to the procedure of Rotruck et al. (1973) [25] with minor modifications in a 96 well format using DTNB reagent and the enzyme activity is expressed in terms of U/mg weight of lung tissue. Eosinophil Peroxidase (EPO) activity using 3,3′,5,5′-Tetramethylbenzidine [26,27] (TMB, Sigma) was measured as described before by Suzuki et al., 1983 [28]. Envision microplate reader was used (Perkin Elmer, USA) to measure the absorbance at 560 nm for SOD activity, 405 nm for CAT activity, 420 nm for GPX activity and 450 nm for EPO activity.

2.8.2. Oxidative stress and responses of antioxidant enzyme (MDA, GSH, GSSG and nitrite) assays

Lipid peroxidation was estimated by measuring the levels of malondialdehyde (MDA). MDA estimation assay is based on the reaction of MDA with Thio-Barbituric acid (TBA); forming a MDA-TBA adduct that absorbs strongly at 532 nm (Heath & Packer, 1968) [29]. The levels of GSH & GSSG were measured by the procedure of Hisin & Hill (1976) [30] and the fluorescence was read at Ex 350/Em 420 nm. Total nitrite content was measured according to Griess, 1879 [31] and the absorbance was measured at 548 nm in Envision microplate reader (Perkin Elmer, USA). A standard curve was generated with sodium nitrite in concentrations from 1 to 100 mmol/l.

2.9. Preparation of DSR sample

DSR was sourced from Divya Pharmacy, Hardiwara, India (Batch number A-SSR 020) and were used for all the experiments. The composition of DSR was given in Table 1. As per the manufacturer’s information, DSR was prepared by pulverising and blending of cleaned and dried plant materials (serial number 1–9; Table 1). The remaining components (serial number 10–13; Table 1) were mixed with above; and passed through sieve before aseptic packaging. For HPLC analysis, 0.50 g of DSR sample was mixed with 10 ml of methanol and sonicated for 45 min. This solution was centrifuged at 10000 rpm for 5 min and filtered using 0.45 μm nylon filter (Solution A). 1.0 ml of solution A was diluted to 5 ml with methanol (Solution B) and diluted to 10 ml with same solvent (Solution C). Solution A, B and C were used for the analysis of chemical signatures present in the sample using their respective standards with purity ≥ 98.0%.

The quantification of signature compounds was performed using High Performance Liquid Chromatography (Waters Corporation, USA) equipped with binary pump (1525), PDAD (2998) and auto-sampler (2707) using a reverse phase Sunfire C18 (5 μm, 4.6 × 250 mm) column with a flow rate of 1.0 ml/min at 37 °C. For HPLC, mobile phase A (0.1 % ortho-Phosphoric acid in water adjust pH 2.5 with Diethylamine) and mobile phase B (0.1 % ortho-Phosphoric acid in a mixture of acetonitrile and water in the ratio of 88:12, adjust pH 2.5 with Diethylamine) were used. For Cinnamic acid, Glycyrrhizin, Eugenol, 6-Gingerol and Piperine, gradient program was 35 % of mobile phase B from 0–5 min, 35–50 % mobile phase B from 5–15 min, 50–80 % mobile phase B from 15–25 min, 80–90 % mobile phase B from 25–30 min, 90–35 % mobile phase B from 30–31 min, 35 % mobile phase B from 31–35 min. For Gallic acid and Ellagic acid, the gradient program was 5–10 % of mobile phase B from 0–10 min, 10–30 % mobile phase B from 10–30 min, 30–90 % mobile phase B from 30–31 min, 90–95 % mobile phase B from 31–35 min.
Table 1
Composition of Divya-Swasari-Ras (DSR).

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Constituent’s Scientific Name</th>
<th>Traditional Name (In Hindi)</th>
<th>Plant Part Used</th>
<th>Quantity in DSR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Glycyrrhiza glabra L.</td>
<td>Mulethi</td>
<td>Root</td>
<td>12.7</td>
</tr>
<tr>
<td>2</td>
<td>Syzgium aromaticum (L.) Merr. &amp; L.M.Perry</td>
<td>Lavang</td>
<td>Flower bud</td>
<td>6.3</td>
</tr>
<tr>
<td>3</td>
<td>Cinnamomum zeylanicum Blume</td>
<td>Dalchini</td>
<td>Bark</td>
<td>6.3</td>
</tr>
<tr>
<td>4</td>
<td>Pasania integerrima J. L. Stewart ex Brandis</td>
<td>Kakdansingi</td>
<td>Gall</td>
<td>12.7</td>
</tr>
<tr>
<td>5</td>
<td>Cremos cretica (L.)</td>
<td>Rudarni</td>
<td>Fruit</td>
<td>12.7</td>
</tr>
<tr>
<td>6</td>
<td>Zingiber officinalis Rosco</td>
<td>Sonth</td>
<td>Rhizome</td>
<td>8.5</td>
</tr>
<tr>
<td>7</td>
<td>Piper nigrum (L.)</td>
<td>Marich</td>
<td>Fruit</td>
<td>8.5</td>
</tr>
<tr>
<td>8</td>
<td>Piperlongum (L.)</td>
<td>Chhoto pipal</td>
<td>Fruit</td>
<td>8.5</td>
</tr>
<tr>
<td>9</td>
<td>Anacycluspyrethrum (L.) Lag.</td>
<td>Akarkur</td>
<td>Root</td>
<td>6.3</td>
</tr>
<tr>
<td>10</td>
<td>Herbaly processed calcined mica ash</td>
<td>Abbraka bhasma</td>
<td>–</td>
<td>4.4</td>
</tr>
<tr>
<td>11</td>
<td>Herbaly processed calcined shell of Pearl oyster ash</td>
<td>Mukta-shukti bhasma</td>
<td>–</td>
<td>4.4</td>
</tr>
<tr>
<td>12</td>
<td>Herbaly processed calcium rich gum ash</td>
<td>Godanti bhasma</td>
<td>–</td>
<td>4.4</td>
</tr>
<tr>
<td>13</td>
<td>Herbaly processed calcium covry shell ash of Cypraea moneta Linn.</td>
<td>Kapardak bhasma</td>
<td>–</td>
<td>4.4</td>
</tr>
</tbody>
</table>

% mobile phase B from 31 – 35 min, 95-5 % mobile phase B from 35 – 36 min, 5 % mobile phase B from 36 – 40 min. While, solution A was used for the analysis of Cinnamic acid and Ellagic acid, solution B was used for the analysis of Eugenol, Glycyrrhizin, 6-Gingerol, Piperine and Solution C was used for the analysis of Gallic acid. Ten microliter of standard solutions and test solution was injected during the analysis. Chromatographs were recorded at 278 nm wavelength (for Cinnamic acid, Eugenol, Gallic acid and Ellagic acid), 250 nm wavelength (for Cinnamic acid) and 280 nm wavelength (for Gallic acid and Ellagic acid).

2.10. ICP-OES analysis

100 mg of Divya-Swasari-Ras sample was digested in HNO3/HClO4 (2:1) acid solution until a white residue was obtained. The residue was dissolved by adding 2% Nitric acid and filtered. The filtrate was reconstituted to 100 ml by 2% Nitric acid and 10 ml of above solution was further diluted ten-fold in 2% Nitric acid. Blank sample was prepared without DSR. Standard solution of Calcium (1 ppm, 2 ppm and 5 ppm) was prepared in 2% Nitric acid from stock standard solution. Calibration curve is obtained by using blank solution and three different concentration of standard solution using ICP-OES (Varian 720-ES) (Agilent, Inc, USA).

2.11. Statistical analysis

All data are expressed as the Mean ± Standard Error of the Means (SEM). Statistical analysis was done using GraphPad Prism version 7.0 software (GraphPad Software, San Diego, CA, USA). A one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison test was used to calculate the statistical difference. Differences in mean values were considered significant at p < 0.05.

3. Results

3.1. HPLC analysis identified indicating compounds in DSR

HPLC analysis identified the presence of Cinnamic acid, Eugenol, 6-Gingerol, Piperine, Glycyrrhizin, Gallic acid and Ellagic acid as indicating components of DSR (Fig. 2), upon comparing the chromatograms with chromatograms obtained from pure substances. The analytical curve showed linearity in the concentration range used as standard (data not shown). HPLC analysis revealed that 0.039 μg/ mg Cinnamic acid (at 13.49 min), 8.647 μg/ mg Eugenol (at 20.43 min), 0.460 μg/ mg 6-Gingerol (at 22.70 min), 3.925 μg/ mg Piperine (at 24.23 min) (Fig. 2A), 2.458 μg/ mg Glycyrrhizin (at 18.56 min) (Fig. 2B), 5.161 μg/ mg Gallic acid (at 7.19 min) and 0.158 μg/ mg Ellagic acid (at 28.39 min) (Fig. 2C) were present in DSR.

3.2. ICP-OES analysis identified calcium present in DSR

ICP-OES analysis of DSR for the presence of minerals identified the presence of Calcium in herbal formulation. In DSR the level of Calcium was discovered to be 4.60 %. (Fig. 2D).

3.3. DSR reduces inflammatory cell accumulation in the BALF in OVA-induced allergic lung inflammation

Activation of immune cells and infiltration into lungs is a hallmark of allergic airway inflammation and a measurement of its severity. In order to assess the therapeutic benefit of DSR on inflammatory cell infiltration, we evaluated the number of inflammatory cells, including eosinophils, neutrophils, macrophages, and lymphocytes, in broncho-alveolar lavage fluid (BALF).

As shown in the Fig. 3A, total number of inflammatory cells were significantly increased in chronic OVA challenged Disease Control (DC) mice, compared to Normal Control (NC) (p < 0.05). Oral treatment of mice with low (135 mg/kg) medium (400 mg/kg) and high dose (1200 mg/kg) of DSR markedly reduced the number of inflammatory cells present in BALF (p < 0.05). Further, DEXA-treated mice also had significantly fewer inflammatory cells (p < 0.05) than DC.

Differential count analysis of inflammatory cells identified that OVA-induced a significant influx of Lymphocytes (Fig. 3B) and Eosinophils (Fig. 3C). Compared to NC, significantly modest increase in the lymphocyte percentage (37 % vs. 46.8 %, p < 0.005) (Fig. 3B) and a robust increase in eosinophil percentage (2% vs 12.5 %, p < 0.005) (Fig. 3C) was noticed in DC. Interestingly, while a significant decrease in macrophage percentage (49 % vs 32 %, p < 0.005) (Fig. 3D) was noticed upon chronic OVA-challenge in DC, non-significant changes in neutrophil percentage was identified (Fig. 3E). Previous reports have also shown similar effects on neutrophil levels upon prolonged OVA challenge [22]. Treatment with DEXA significantly attenuated the OVA-induced lymphocyte and eosinophil percentage (Fig. 3B and C). A dose dependent decreases in the lymphocyte percent (46.8 % vs. 37.8 %, p < 0.005) and eosinophil percent (12.5 % vs. 4.0 %, p < 0.005) (Fig. 3B and C) was observed upon DSR treatment of mice receiving OVA with maximum response was noticed at highest dose of DSR (1200 mg/kg). Conversely, DSR treatment significantly increased the macrophage percentage in a dose dependent manner with a maximum response at the highest dose (32.4 % vs. 48.0 %, p < 0.005), and at the highest dose it is comparable to that of NC (49.8 % vs. 48.0 %) (Fig. 3D). Previous reports showed that alveolar macrophages have a suppressive role in allergic airway inflammation [33,34]. These results indicated the therapeutic benefit of DSR in inhibiting OVA-induced infiltration of inflammatory cells.
DSR sample was analysed on HPLC using reference standards. The chromatographs were recorded at 250 nm (Glycyrrhizin, Gallic acid and Ellagic acid) and 278 nm (Cinnamic acid, Eugenol, 6-Gingerol and Piperine) wave-lengths. By comparing with chromatographs of pure standards, HPLC analysis identified the presence of bio-active compounds namely, A) Glycyrrhizin B) Gallic acid and Ellagic acid and C) Cinnamic acid, Eugenol, 6-Gingerol and Piperine in the DSR. The structures of the respective compounds were given along with chromatogram. * indicate the particular chromatographic peak used for quantification for the given metabolite. D) ICP-OES analysis identified the presence of calcium mineral in DSR.
3.4 DSR alleviates OVA-induced histo-pathological changes during allergic lung inflammation

In order to assess the pathological changes upon OVA challenge and subsequent beneficial and anti-asthmatic effect of DSR treatment, H&E stained lung tissue sections were evaluated (Fig. 4) for the features of pathological signs namely, inflammatory cell accumulation and thickening of bronchial epithelium. From the results it is evident that, OVA stimulation induced a multifocal inflammatory cell infiltration in the lung tissues (Fig. 4A, B and E). Whereas, pharmacological treatment with DSR significantly modulated the features of this pathology with very focal inflammatory cell accumulation in lungs, similar to the DEXA treatment (Fig. 4A, B and E). Whereas, no abnormality was detected in NC group. Further, OVA-induced increase in bronchial thickening was significantly decreased upon DSR treatment (Fig. 4C and E). Collectively, critical evaluation of pathological features identified that chronic OVA-challenge induced a mild to moderate pathology in DC. Whereas treatment with DSR significantly decreased the features of pathology (Fig. 4D and E).

3.5 DSR suppresses airway remodelling in asthmatic mice

Goblet cell hyperplasia, peri-bronchial collagen deposition and airway wall thickening represents the hallmarks of pathological
Fig. 4. DSR alleviates airway inflammation and histo-pathological changes.
Histopathological analysis was performed to beneficial and anti-asthmatic effect of DSR treatment. A) H&E stained lung tissue sections of a) NC b) DC c) DEXA d) 135 mg/kg DSR e) 400 mg/kg DSR f) 1200 mg/kg DSR. H&E stained lung tissue sections were evaluated for B) Inflammatory cell infiltration in bronchi, C) Inflammatory cell infiltration in alveoli, D) Thickening of bronchial epithelium and E) Total pathological score. Arrow indicates either inflammatory cell accumulation and bronchial wall thickening. Data are present the means ± S.E.M (n ≥ 5). **, ##, p < 0.005 by one-way ANOVA.; ## Represents significant compared to NC and ** Represents significant compared to DC. (40X magnification).
changes in asthma, and are commonly used to evaluate the severity of airway remodelling [4,35]. To evaluate mucus hypersecretion by bronchial airways, lung sections were stained with Periodic Acid–Schiff (PAS) stain (Fig. 5A) and quantified in a double-blind manner using a numerical scoring system (Fig. 5B). Compared to NC, OVA-challenge significantly increased mucus hypersecretion and goblet cell hyperplasia in the airways (p < 0.05). Further, OVA induced moderately severe mucus hyper secretion was significantly alleviated in a dose dependent manner in DSR treated mice (vs. DC, p < 0.05) and in DEXA treated mice (vs. DC, p < 0.05).

To evaluate peri-bronchial collagen deposition and airway wall thickening, Masson’s trichrome staining was performed and evaluated. As shown in Fig. 6A, collagen deposition and airway wall thickness were significantly increased in the OVA treated DC (vs. NC, p < 0.05). Compared with the DC, DSR treatment slightly alleviated collagen deposition and airway wall thickening. Whereas, DEXA treatment significantly inhibited collagen deposition (vs. DC, p < 0.05) (Fig. 6B).

3.6. DSR reduced OVA-induced pro-inflammatory cytokine levels in BALF

Various cytokines have been shown to play a crucial role in inflammatory cell accumulation and the development of allergic inflammation [4,35]. BAL fluid analysis for the pro-inflammatory cytokine protein levels (Fig. 7) identified that, compared to NC, OVA sensitization and challenge in DC, markedly increased the protein levels of IL-6 (NC, 4 pg/ml vs. 57 pg/ml DC, 14 fold, p < 0.05), IL-1β (NC, 1.7 pg/ml vs. 10.4 pg/ml DC, 6 fold, p < 0.05) and TNF-α (NC, 6.5 pg/ml vs. 35.1 pg/ml DC, 5.3 fold, all p < 0.05) (Fig. 7A-C). Whereas, treatment with DSR significantly reduced the OVA-induced pro-inflammatory cytokine levels of IL-6 (DC, 57 pg/ml vs. 15.9 pg/ml -19.7 pg/ml, DSR, all p < 0.05), IL-1β (DC, 10.4 pg/ml vs. 4 pg/ml -5.9 pg/ml, all p < 0.05) and TNF-α (DC, 35.1 pg/ml vs. 10.2 pg/ml -10.9 pg/ml) (Fig. 7D-F).

Fig. 5. DSR suppresses mucus accumulation in asthmatic mice.

PAS staining of histological sections was performed and evaluated for mucus accumulation in airways. Compared to NC, DC showed mucus accumulation and DSR treatment significantly alleviated mucus accumulation. A) NC B) DC C) DEXA D) 135 mg/kg DSR E) 400 mg/kg DSR F) 1200 mg/kg DSR. E) Quantification mucus accumulation at bronchial mucosal epithelium. Arrow indicates mucus accumulation in airways. Data are present the means ± S.E.M (n ≥ 5). **, ##, p < 0.005 by one-way ANOVA.; ** Represents significant compared to NC and ## Represents significant compared to DC. (40X magnification).

Fig. 6. DSR suppresses collagen deposition in asthmatic mice.

Masson’s trichrome staining was performed and evaluated the peri-bronchial collagen deposition. Compared to NC, DC showed collage deposition around airways and DSR treatment significantly alleviated collage deposition. A) NC B) DC C) DEXA D) 135 mg/kg DSR E) 400 mg/kg DSR F) 1200 mg/kg DSR. E) Quantification peri-bronchial collagen deposition. Arrow indicates collage deposition. Data are present the means ± S.E.M (n ≥ 5). **, ##, p < 0.005 by one-way ANOVA.; ** Represents significant compared to NC and ## Represents significant compared to DC. (40X magnification).
ml, all \( p < 0.05 \) (Fig. 7A–C). Treatment with DEXA significantly decreased the levels of IL-6 (DC, 57 pg/ml vs. 13 pg/ml, \( p < 0.05 \)), IL-1β (DC, 10.4 pg/ml vs. 4.7 pg/ml, \( p < 0.05 \)) and TNF-α (DC, 35.1 pg/ml vs. 9 pg/ml, \( p < 0.05 \)) (Fig. 7A–C). Decrease in cytokine levels upon DSR treatment at highest dose was comparable to DEXA treatment.

3.7. DSR reduces expression of asthma related pro-inflammatory cytokines in asthmatic lungs

Activation of Th2 cells and production of Th2 cytokine (IL-4, IL-5 and IL-13) and Th2 oriented cytokines (IL-6, IL-33) contribute significantly to the pathophysiology and lung dysfunction in allergic asthma [36,37,4]. Steady state mRNA expression analysis of whole lung identified the expression of Th2 related (IL-4, IL-5 and IL-33) and Th1 cytokine (IFN-γ) (Fig. 8).

Fig. 8. DSR reduces the mRNA expression of pro-inflammatory cytokines in whole lung tissue.

Whole lung tissue RNA was analysed for the steady state mRNA expression levels of cytokines A) IL-4, B) IL-5 C) IL-33 D) IFN-γ E) IL-6 and F) IL-1β. Data are present the means ± S.E.M (n ≥ 5). **, ##, \( p < 0.005 \) and * \( p < 0.05 \) by one-way ANOVA.; ## Represents significant compared to NC and ** Represents significant compared to DC.

OVA-challenge in DC induced the expression of IL-4 (vs. NC, 3.3 fold, \( p < 0.05 \)), IL-5 (vs. NC, 2.7 fold, \( p < 0.05 \)), IL-33 (vs. NC, 3.6 fold, \( p < 0.05 \)), IFN-γ (vs. NC, 2 fold, \( p < 0.05 \)), IL-6 (vs. NC, 3.9 fold, \( p < 0.05 \)), and IL-1β (vs. NC, 16.1 fold, \( p < 0.05 \)) (Fig. 8A–F). Further, either treatment with DEXA (\( p < 0.05 \)) or DSR (vs. DC, all \( p < 0.05 \)) significantly attenuated the OVA allergen induced expression of these cytokines in a dose dependent manner (IL-4, IL-33, IL-6 and IL-1β). The attenuated mRNA expression levels were comparable to DEXA treatment groups at low and medium DSR dose. Remarkably, treatment with high dose of DSR of OVA-sensitized & challenged mice appeared to reduce the expression of IL-4, IL-33 and IFN-γ to even below that of non-sensitized NC mice (Fig. 8A, C and D). At its highest dose (1200 mg/kg), DSR significantly attenuated the expression of IL-4 (DC, 3.3 fold vs. 0.7 fold, DSR, \( p < 0.05 \)), IL-5 (DC, 2.7 fold vs. 0.9 fold, DSR, \( p < 0.05 \)), IL-6 (DC, 2.7 fold vs. 0.9 fold, DSR, \( p < 0.05 \)), and IL-1β (vs. NC, 16.1 fold, \( p < 0.05 \)) (Fig. 8A–F). Further, either treatment with DEXA (\( p < 0.05 \)) or DSR (vs. DC, all \( p < 0.05 \)) significantly attenuated the OVA allergen induced expression of these cytokines in a dose dependent manner (IL-4, IL-33, IL-6 and IL-1β). The attenuated mRNA expression levels were comparable to DEXA treatment groups at low and medium DSR dose.

Fig. 7. DSR administration reduced OVA-induced pro-inflammatory cytokine protein levels in BALF.

BALF was analysed for the pro-inflammatory cytokine protein concentrations using ELISA and expressed as pg/ml. A) IL-6 B) IL-1β and C) TNF-α. OVA induced cytokine levels were significantly supressed upon DSR administration. Data are present the means ± S.E.M (n ≥ 5). **, ##, \( p < 0.005 \) and * \( p < 0.05 \) by one-way ANOVA.; ## Represents significant compared to NC and ** Represents significant compared to DC.
DSR, p < 0.05) and IFNγ(DC, 2.1 fold vs. 0.5 fold, DSR, p < 0.05), IL-6 (DC, 3.9 fold vs. 0.9 fold, DSR, p < 0.05), IL-1β (DC, 16.1 fold vs. 1.5 fold, DSR, p < 0.05) (Fig. 8A–D). The decreased expression of cytokines correlates with the decreased infiltration of inflammatory cells specifically, eosinophils and lymphocytes as described above (3A–B), suggesting a beneficial effect of DSR on modulating airway inflammation.

3.8. DSR modulates antioxidant biochemical markers in OVA-Induced allergic asthmatic mice

Antioxidants play a very important role in defence mechanisms of the cells against oxidative stress [8]. Suppressed activity of key antioxidant enzymes had been found to be associated with bronchial asthma and the oxidant/antioxidant imbalance is believed to be a key event in asthma [38]. To test the role of oxidative stress and subsequent protective effect of DSR, lung homogenates were tested for key antioxidant biochemical markers. From the analysis it is evident that compared to NC, OVA-sensitization and challenge in DC significantly altered various oxidant/antioxidant parameters. However, treatment with DSR or DEXA reversed OVA-induced alterations (Fig. 9).

Superoxide dismutase (SOD) plays an important role in scavenging superoxide radicals during oxidative stress. OVA induced allergic asthma in DC, decreased the activity of antioxidant enzymes, SOD (2.3 U/mg vs. 1.0 U/mg, p < 0.05). Whereas, treatment with DSR, significantly restored the activity (vs. DC, all p < 0.05), similar to DEXA (vs. DC, p < 0.05) (Fig. 9A).

Catalase enzyme detoxifies H2O2 produced under physiological conditions by the action of superoxide dismutase. Compared to NC, allergic asthma in DC decreased the activity of catalase (84.8 U/mg vs. 30.3 U/mg, p < 0.05). Conversely, either treatment with DEXA (vs. DC, p < 0.05) or DSR significantly restored the catalase activity at medium and high dose (p < 0.05) (Fig. 9B).

GPx enzyme, reduces the organic peroxide H2O2, and prevents the peroxidation of cell membrane lipids and subsequent instability. In DC, the levels of GPx were significantly decreased (1.1 U/mg vs. 0.3 U/mg, p < 0.05) compared to NC. Treatment with DEXA (vs. DC, p < 0.05) or DSR partially restored the activity of GPx (Fig. 9C).

Malondialdehyde (MDA), the major end product of lipid peroxidation and indicator of damage to membrane lipids was significantly elevated DC (1.4 μM/mg vs. 4.5 μM/mg, p < 0.05). DEXA (p < 0.05) or DSR significantly decreased the OVA induced of MDA levels (vs. DC, 2.5 μM/mg-3.0 μM/mg, all p < 0.05) (Fig. 9D).

Glutathione (GSH), is an abundant airway antioxidant [39]. Upon oxidation, it is converted to GSSG. The ratio of GSH/GSSG is an indicator of cellular redox homeostasis. Compared to NC, in DC, the levels of GSH were significantly decreased (62.7 μM/mg vs. 32.0 μM/mg, p < 0.05) (Fig. 9E) and the levels of GSSG were elevated (11.2 μM/mg vs. 22.3 μM/mg, p < 0.05) (Fig. 9F) hence decreasing the GSH/GSSG ratio (4.8 vs. 1.5, p < 0.05) in DC (Fig. 9G). DSR treatment (vs. DC, all p < 0.05) and DEXA (vs. DC, p < 0.05) treatment restored the levels of GSH by supressing the levels of GSSG, significantly restoring the GSH/GSSG ration in a dose dependent manner (vs. DC, all p < 0.05) (9E-G). Interestingly, the studied inflammation markers are correlated positively (Figs. 7 and 8) with MDA levels and negatively with the antioxidant markers (SOD, CAT, GPX and GSH).

EPO is a biomarker that reveals the extent of cellular injury. Challenge with OVA-significantly increased the EPO activity in DC by 2 fold (NC, 0.7 vs 1.2, DC, p < 0.05). Treatment with DSR significantly decreased the OVA-induced EPO activity (vs. DC, p < 0.05). Similar results were achieved upon DEXA treatment (vs. DC, p < 0.05) (Fig. 10A).

The levels of nitrate and nitrite determine the intensity of nitrogen radicals and involvement of NO signalling pathway. OVA treatment significantly increased the levels of nitrite in lungs of DC (NC, 0.7 μM/mg vs. 2.7 μM/mg, DC, p < 0.05). DSR treatment significantly abrogated (vs. DC all, p < 0.05) the OVA-induced increases (vs. DC, ≥ 60%, p < 0.05) in the nitrite levels. These decreases were comparable to DEXA (p < 0.05) (Fig. 10B).

4. Discussion

Asthma pathophysiology is extremely complicated and interaction between cells of innate and adaptive immune system such as eosinophils, macrophages and lymphocytes and structural cells like airway epithelial cells and smooth muscle cells plays an important role orchestrating its pathology [2,36,40,41]. Ovalbumin is a well-studied...
allergen used to induce experimental asthma in mouse model of disease, as it mimics some of the features of human asthma allowing feasible method to study the immunological and non-immunological mechanisms involved in the pathogenesis of asthma and facilitate studies of potential therapeutic agents [42–44].

In the present study, OVA sensitization and challenge successfully induced asthma like feature in BALB/c mice namely, elevated eosinophils and lymphocytes in BAL fluid, histopathological signatures like inflammatory cell accumulation in lungs, bronchial epithelial thickening, mucus secretion by bronchial epithelial cells, peri-bronchial collagen deposition, BAL and whole lung pro-inflammatory profile and dysregulated oxidative/anti-oxidative balance. These results were in line with several asthma models [41,44,45]. Further, treatment with calcio-herbal formulation, DSR significantly ameliorated the pathological signs of Asthma in a dose dependent manner in-vivo by reducing infiltration of inflammatory cells into the airways and lung, attenuated airway remodelling and decreasing the pro-inflammatory gene and protein levels, restoring anti-oxidative stress capacity demonstrating its prominent therapeutic effect on regulation of inflammation in asthma (Fig. 11).

Glucocorticoids and β2-adrenergic agonists have been in use for the improvement of airway remodelling and features of asthma. However, patients with refractory asthma are poor responded to this treatment [2]. Consequently, identifying cost effective and reliable anti-asthmatic therapies that can mitigate the symptoms of asthma are needed. In the present study, we have used chronic model of allergic asthma as, such models have been shown to recapitulate hall mark features more closely to human asthma like eosinophilia, Th2-dependent allergic inflammation and remodelling of the airways [46,47,48,44]. Mucus hypersecretion may result in airway obstruction, leading to the morbidity and mortality in asthma [49,50]. Further, components of extra cellular matrix (ECM), particularly collagen, account for the formation of sub epithelial fibrosis [51]. Our results confirmed that DSR treatment markedly reduced mucus secretion, collagen deposition and subsequent fibrosis, indicating that compounds in DSR may have an inhibitory role in asthmatic airway remodelling.

Asthma is a complex chronic inflammatory airway disease and a number of studies have demonstrated aberrant expansion of Th2 lymphocytes and secrete type-2 cytokines, interleukin-4 (IL-4), IL-5, IL-9 and IL-13, and Th2 oriented cytokines, like IL-6 and IL-33 induce recruitment of inflammatory cells, promote eosinophilia of airways, airway mucus over secretion, bronchial hyper responsiveness (BHR), and immunoglobulin E (IgE) synthesis. Specifically, IL-4 induces Ig isotype switching and induces the production of IgE and IgG1 by B cells, IL-5 promotes eosinophil survival, differentiation and migration. Whereas, IL-13 induces mucus metaplasia and airway hyper responsiveness [51,2,52,36,37,4]. Recently it is becoming apparent that lung epithelial cells produce specific cytokines and that these cytokines can favour Th2 cell differentiation [37,53]. IL-33 is produced by lung epithelial cells and promotes IL-5 production and subsequent eosinophilia. IL-6 has been considered a general marker of inflammation along with TNF-α and IL-1β, two other classical inflammatory cytokines. IL-6 regulates CD4 T cell fate [54], promotes IL-4 production during Th2 differentiation and inhibits Th1 differentiation. Elevated levels of IL-6 have been found in BAL fluid of asthmatics, consistent with our in-vivo studies [55].

Attempts to block selective cytokines and chemokines and associated adaptive immune pathways has resulted in only a limited success in a subset of population [56,4]. In the present research work, a significant increase in the mRNA levels of IL-4, IL-5, IL-33 and IL-6 in whole lung and protein levels of IL-6, in BALF were identified. Further, significant decrease in the pro-inflammatory molecules parallels the decrease in eosinophil counts in BAL and airway lumen and mucus secretion in airway. These results suggest the role of DSR in regulating pro-inflammatory signalling mediators and subsequent inflammatory processes.

IL-1β is causally involved in both Th1 and Th2 features of asthma exacerbations. Sputum levels of IL-1β has recently been shown to play a very crucial role in severe asthma [57] and exacerbations of chronic obstructive pulmonary disease (COPD) and asthma [58]. Further, elevated levels of IL-1β may contribute to neutrophilic lung inflammation and asthma in both humans [59] and animal models of asthma [60–62] and contribute to steroid resistance [61]. Further, IL-1β has been shown to increase the expression of Th2 cytokines, IL-33 [63] and IL-6 [64]. Contrastingly, in our study we have not seen parallel increases neutrophilia along with increases in the BAL IL-1β. Further, DSR treatment significantly decreased the levels of IL-1β indicating its therapeutic potential.

Pro-inflammatory cytokine TNF-α has been implicated in many aspects of the airway pathology in asthma including AHR [65] probably due to its direct effect on airway smooth muscle cells [66] or by the release of the cysteinyl-leukotrienes LTC4 and LTD4 [67]. Further, TNF-α has recently been identified as important factor in refractory asthma [68]. Decrease in the levels of TNF-α upon DSR administration suggests, DSR may be an effective therapeutic target or complementary alternative medicine in refractory asthma.

Oxidative stress plays a crucial role in the pathogenesis of asthma. Airway inflammation is results in enhanced generation of reactive species by various immune cells specifically, activated macrophages, eosinophils, and neutrophils exogenously or endogenously resulting in elevated oxidative stress, causing severe macromolecular damage, altered enzymatic activities and functional changes in cells [9]. The role of oxidative stress and impaired antioxidant defence system in asthma pathology is evident from various studies [8,38]. Oxidative stress induces the activation of numerous genes involved in antioxidant,
cytoprotective, detoxification and anti-inflammatory functions including SOD-3, Catalase, GPx, MDA, GSH, NAD(P)H-quinone oxidoreductase (NQO1), Heme oxygenase-1 (HO-1) via nuclear erythroid 2 p45-related factor 2 (Nrf2) transcription factor dependent mechanism [69,70]. Previous reports suggest the use of herbal formulations for modulating oxidative stress in ameliorating asthma have been reported [70,71]. In the present investigation, elevated levels of EPO activity, nitrite species, MDA activity and decreased activity of SOD, Catalase, GPx and GSH confirms the aberrant oxidative/anti-oxidative defence mechanism. Importantly, DSR mediated regulation of anti-oxidative defence parameters confirms the protective role of DSR in asthma pathology by modulating these parameters.

World Health Organization (WHO) has estimated that around 70 %–95 % world population, especially in developing countries, depends essentially on plants based products and herbal preparations/medicines for health care needs [72]. Use of poly herbal or mono herbal formulations for asthma treatment have been reported [70,72,74]. DSR consists of roots of Glycyrrhiza glabra L. (Liquorice), buds of Syzygium aromaticum (L.) Merr. & L.M.Perry (Cloves), bark of Cinnamomum zeylanicum Blume (Cinnamon), galls of Pistacia integerrima J. L. Stewart ex Brandis (Zebrawood), fruits of Cressa cretica (L.), rhizomes of Zingiber officinale Rosco (Ginger), fruits of Piper nigrum (L.) (Black pepper), fruits of Piperlongum (L.) (Long pepper), roots of Anacycluspyrethrum(L.) Lag. (Spanish chamomile) (Table 1). HPLC analysis identified the presence of Cinnamic acid, Eugenol, 6-Gingerol, Piperine, Glycyrrhizin, Gallic acid and Ellagic acid as phyto-metabolites present in DSR. Glycyrrhiza glabra L. (Liquorice), contains glycyrrhizin as major bioactive ingredient, along with glycyrrhretinic acid, flavonoids, iso-flavonoids, and chalcones as active compounds. Its roots have been traditionally used for cough, colds, asthma, and COPD [75]. In line with our findings, it alleviated the allergic asthma in OVA-induced experimental mouse model by decreasing the levels of Th2 IL-4 and IL-5 levels and eosinophil and interfered with IgE by decreasing the IgE-stimulating cytokines, AHR [76] and OVA-induced oxidative stress [77], mucus production [78]. Further, it decreased LPS induced TNF-α and IL-1β levels in lungs [79].

Eugenol is a phenylpropanoid phenolic compound constituting 45–90 % of its essential oil of Syzygium aromaticum [80]. It has anti-oxidative, free radical scavenging [81] and anti-inflammatory [80] properties. In lungs, eugenol inhibited eosinophilia and the levels of IL-4 and IL-5 in a NF-κB pathway dependent manner [82]. Further, it has also increased the SOD, CAT, GPx, and GST activity [83] and decreasing MPO, MDA activity, improving antioxidant capacity [84] and it modulates NF-κB, ERK1/2, and p38 MAPK signalling pathways [85]. The barks of Cinnamomum zeylanicum contains trans-cinnamaldehyde, eugenol, and linalool, which represent 82.5 % of the total composition [86]. Cinnamon has anti-oxidant [86] properties [87] and suppresses IL-1β, IL-6 and TNF-α in LPS-activated macrophages in MAPK dependent pathway [88] and bleomycin-induced idiopathic pulmonary fibrosis [89].

In India, essential oil of P. integerrima is used for the treatment of asthma, chronic bronchitis, and other ailments for the respiratory tract. However, its pharmacological properties have not yet fully explored [90]. It exerts anti-asthmatic activity by reducing the levels of TNF-α, IL-4, and IL-5 and regulating AQP expression levels [91]. Ethyl gallate present in galls attenuates acute lung injury through Nrf2 signaling [92] and inhibits cell adhesion molecules by blocking AP-1 transcription factor [93]. Cressa cretica L exhibits Bronchodilatory and mast cell stabilising activity [94] and its aerial parts contain nootcaconol-1, β-sitosterol, 6-hydroxy-3,4-dimethyl coumarin, 6-methoxy-7,8-methylene
diosy coumarin, β-sitosterolglucoside, quercetin, kaempferol and rutin and showed protection from CCl4-induced liver damage and antioxidant parameters modulation [95].

Rhizome of *Zingiber officinale* has been used in the treatment of cold, asthma, and bronchitis [75] and it has antiviral activity against respiratory syncytial virus [96] n-gingerol present in ginger prevents Th2-mediated immune responses in a mouse model of airway inflammation and 6-gingerol was sufficient to suppress eosiinophilia [97] and inhibit the production of TNF-α, IL-1β, and IL-12 from LPS-stimulated macrophages [98]. *Piper longum* and *P. nigrum* is used for treating bronchial diseases, tuberculosis, due to its anti-oxidant, anti-inflammatory, antibacterial, anti-tumor properties [99–101]. The fruits of *P. longum* contains the alkaloids and amides; piperidine, piperine, piperlongumine, pipiltarine, and aristolactams [100,102,100]. Piperine is a major component of *Piper nigrum* and *P. longum*. *Piper longum* exhibited endothelial barrier protective effects and leukocytes migration in-vivo, suppressed IL-6 and TNF-α levels by acting upon NF-kB and ERK1/2 pathways [103]. Fruits of *P. longum* inhibited the release of Th2-mediated cytokines, eosinophil infiltration in mouse model of asthma [104]. *P. Nigrum* inhibited allergic Inflammation by Inhibiting Th2/Th17 responses and mast cell activation and regulated the balance of cytokines production from Th1, Th2, Th17 and Treg cells, and inhibited GATA3, IL-4, IL-6, IL-1β, RORγt, IL-17A, TNF-α expression and increased the secretions of IL-10, INF-γ [105]. Piperine also reduced histologic damage and myeloperoxidase (MPO) activity in the pancreas [106].

The roots of Anacyclus pyrethrum abundantly contains N-isobutyldienynamide and polysaccharides [107] along with aponis, sesamin, inulin, gum and traces of essential oil [108,109]. This plant exerts immune modulatory and immune stimulating properties [110] along with anti-inflammatory and antioxidant properties [111]. Polyherbal formulation, DSR may contains additive properties of all the above mentioned plant materials with proven anti-inflammatory or anti-asthmatic potential and hence may serve as superior formulation for treatment of asthma, correlating with the observed biological responses.

Bhasma are unique ayurvedic metallic/minerals preparation, treated with herbal juice or decoction and exposed for Ayurveda, which are known in Indian subcontinent since 7th century A.D. Bhasma is an ash obtained through incineration [112,113]. DSR contains kapardak bhasma, abhraka bhasma, godanti bhasma and mukta shukti bhasma. Kapardak bhasma is prepared from shell of sea animal *Cypraea moneta* Linn [114] and has reported anti asthmatic properties [115]. Abhraka bhasma is a calcined mica ash that has anti-inflammatory properties and provides relief from chronic and incessant cough and asthma [116] and hepato protective function [117]. Godanti bhasma has a potent combination of calcium rich gypsum and *Aloe vera* juice, heated at high temperatures in earthen pots. It has anti-inflammatory function [118] and significant gastro protective and antipyretic activity [119]. Mukta shukti bhasma is prepared from Pearl oyster and it has been traditionally used for its anti-inflammatory properties [120] and lung diseases [121], antacid, anti-pyretic, and also as a source of calcium [122]. Use of bhasma for treatment of asthma has been reported previously and demonstrated that bhasma have mast cell stabilizing & anti-inflammatory activity [123]. Hence, the bhasma and herbal components present in DSR formulation might be modulating various pathological parameters of asthma either by acting upon immune cells, structural cells and inhibiting the inflammatory processes and providing the overall therapeutic efficacy.

5. Conclusions

The present study demonstrates the therapeutic efficacy and disease modifying properties of the Indian traditional calcio-herbal formulation, Divya-Swasari-Ras by inhibiting airway inflammation, airway remodelling and modulating various cellular, molecular and biochemical parameters associated with asthma pathology. Hence, the use of Divya-Swasari-Ras containing various indicating ingredients as complementary or alternative medicine as adjunct in asthma treatment could alleviate the asthma pathology.

Author contributions

A.B. provided broad direction for the study, identified and prepared the test formulations, generated resources and gave final approval for the manuscript; M.T. performed analytical chemistry experiments; S.K.S. and H.S. performed the biology study and analysed the data, S.K.S performed data curing, and wrote the manuscript; A.V. conceptualized and supervised overall studies, generated resources, critically reviewed and finally approved the manuscript.

Data availability

The data is available on request.

Declaration of competing interest

The authors declare no competing interests in the publication of the data in this manuscript.

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