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Original Article

Daidzein Attenuates Allergic Asthma via NF-κB1 Downregulation and Cytokine Modulation: A Comprehensive Preclinical Evaluation

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ABSTRACT

Background: Bronchial Asthma (BA) is a chronic inflammatory airway disorder strongly associated with immune system dysregulation. Daidzein, a natural isoflavone found in soy, exhibits well-documented anti-inflammatory and immunomodulatory activities, including inhibition of dendritic cell maturation and regulation of effector cell functions. **Objectives:** To investigate the anti-asthmatic potential of Daidzein through diverse *in vitro* and *in vivo* experimental models, including an Ovalbumin (OVA)-induced allergic asthma rat model. **Materials and Methods:** Daidzein was evaluated for multiple pharmacological activities: antioxidant potential using the DPPH radical scavenging assay; antihistaminic activity using an isolated goat tracheal chain; mucolytic activity by measuring egg white viscosity; and mast cell-stabilising effects through clonidine-induced mast cell degranulation and passive paw anaphylaxis. *In vivo* anti-asthmatic efficacy was assessed in OVA-sensitised rats by analysing body weight changes, Bronchoalveolar Lavage (BAL) fluid cellular infiltration, and levels of IL-4, TNF-α, and IgE in serum and BAL fluid. Lung histopathology and NFKB1 gene expression analysis were also performed. **Results:** Daidzein demonstrated significant antioxidant, antihistaminic, mucolytic, mast cell-stabilising, and anti-anaphylactic activities in all *in vitro* and *in vivo* screening models. In OVA-induced asthmatic rats, Daidzein pretreatment reduced body weight loss, significantly decreased total inflammatory cells in BAL fluid, and markedly lowered IL-4, TNF-α, and IgE levels. Histopathological studies revealed reduced inflammatory infiltration, decreased goblet cell hyperplasia, and attenuation of airway remodelling. Daidzein also strongly downregulated NFKB1 gene expression, indicating inhibition of NF-κB-mediated inflammatory pathways. **Conclusion:** Daidzein exhibits strong anti-asthmatic efficacy by regulating inflammatory cytokines, reducing IgE levels, suppressing NF-κB activation, and protecting lung structure. These findings support Daidzein as a promising and safe multi-target therapeutic candidate for asthma management.

Keywords: Asthma, BAL fluid, Cytokines, Daidzein, Inflammation, NFKB1.

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INTRODUCTION

Asthma, from the Greek name signifying "breathlessness," is an inflammatory airway disease of long-standing that is characterised by reversible airway obstruction, bronchial hyperresponsiveness, and airway remodelling (Lambrecht and Hammad, 2012). It may occur in individuals of any age, but with increased prevalence in children and young adults. Globally, it is estimated that 300 million individuals are affected by asthma, with prevalence rates of 1-18% among the population (Singh *et al.*, 2022). India alone accounts for about 13.09% of the global burden of asthma, affecting about 34.3 million persons (Masoli *et al.*, 2004). The increasing global prevalence of asthma is primarily attributed to environmental factors and altered lifestyle trends. Pathologically, asthma is a heterogeneous inflammatory disorder characterised by reversible airway narrowing, bronchial hyperresponsiveness, and airway wall structural remodelling, including epithelial injury, subepithelial fibrosis, smooth muscle hypertrophy, and excessive mucus production (Holgate, 2008). It is regulated by immune dysregulation, where T-helper 2 (Th2) cytokines (IL-4 and IL-5) are released by T helper 2 cells, inflammatory eosinophils, IgE production, and mast cell degranulation. Increased IgE and mast cell degranulation enhance the inflammatory cascade, whereas pro-inflammatory cytokines such as TNF-α augment inflammatory cell recruitment

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(Holgate, 2012). At the molecular level, transcription factors NF-κB1 and STAT1 regulate genes involved in inflammation, contributing to chronic airway remodelling and oxidative stress, key characteristics of long-standing asthma (Menzel *et al*., 2022).

With our current knowledge of the pathogenesis of asthma, the clinical treatment is principally directed towards anti-inflammation and bronchodilatation (Liu *et al*., 2023). At present, Inhaled Corticosteroids (ICS) are successful for long-term asthma management, and combining long-acting β2-agonists is helpful in controlling asthma. Yet, they have exhibited intense side effects such as toxicity to the kidney, liver, heart, GI system, immune system, and the development of different types of cancer. They are not, however, directed at critical aspects of the pathogenesis of asthma and are not effective in all patients (Shaik *et al*., 2015). Thus, new, efficacious, and safe anti-asthmatic drugs are still being sought, and attention has focused on traditional approaches, including phytoconstituents, especially flavonoids, many of which have been shown to have an array of profiles of anti-inflammatory activity for the treatment of asthma and other allergic disorders.

One of the phytoisoflavones, small molecules, Daidzein (4',7-dihydroxyisoflavone), has been widely utilised to treat a variety of illnesses, including infectious and non-infectious inflammation. High concentrations of physiologically active isoflavones, like Daidzein, are found in soybeans, which are widely consumed in Asian nations (Ahmad *et al*., 2024). Daidzein has been found in both glycoside and aglycone forms in kudzu root (*Pueraria radix*), a well-known Chinese folk medicine used as a dietary supplement (Shin and Jeong, 2015). Daidzein exhibits potent antioxidant and anti-inflammatory properties, which suggest that it may be a potential drug in a broad range of diseases such as cardiovascular diseases, cancer, diabetes, osteoporosis, and neurodegeneration. Daidzein in the reports greatly decreased the NO and IL-6, and mRNA due to LPS-stimulated cells of RAW264.7 (Choi *et al*., 2012). The preclinical research has examined the impact of daidzein on the activation of human Dendritic Cells (DC) using Lipopolysaccharide (LPS) stimulation and the resulting efficacy of DC-mediated effector cell activity in the context of *in vitro* and *in vivo* mouse models in the study of upper airway inflammation. Daidzein suppressed the DC maturation markers (CD83, CD80, and CD86) expression in mice previously sensitised with ovalbumen, as well as the expression of the MHC class-I molecules on the mucosal immune response (Yum *et al*., 2011; Wei *et al*., 2012). It has been proven by other works that the anti-inflammatory effect of daidzein is achieved through pro-inflammatory chemokine Cxcl2 transcription inhibition, under the action of TNF-stimulated murine lung epithelial cells, through a depression of the PARP-1 activity (Li *et al*., 2014).

Although several studies have demonstrated the antioxidant and anti-inflammatory activities of Daidzein in different disease models, few studies have examined the anti-asthmatic properties of Daidzein in laboratory animals. In particular, its effect on airway inflammation or cytokine modulation. Furthermore, limited studies have examined the role of Daidzein on other aspects of modulating inflammatory genes, such as NF-κB1, particularly as it relates to asthma. We aim to fill this gap by examining the impact of Daidzein in pre-clinical models of asthma, with this study evaluating the impact of Daidzein with a focus on track airway inflammation, TH2 cytokines, IgE levels, and inflammatory gene expression.

MATERIALS AND METHODS

Drugs and Chemicals

Test drug Daidzein (purity>98%) was purchased from Dhamtec Pharma and Consultant (Mumbai, Maharashtra) and used as received based on the certificate of analysis provided by the supplier. 0.5% DMSO, Egg albumin, Histamine, RPMI buffer medium, and ovalbumin (Grade V) were purchased from Sigma-Aldrich, and Clonidine, Dexamethasone, and Sodium Cromoglycate (Cipla Ltd., India) were procured from a commercial source. The present study involved the use of analytical grade reagents and solvents.

Experimental animals

The National Institute of Biosciences in Pune provided the female Wistar rats, weighing between 150 and 250 g. The animals were kept in cages made of opaque plastic (polypropylene) at 24±2°C and 45 to 55% humidity. Every animal was given free access to water and a commercial pelleted feed (Nutrivet Life Sciences, Pune, India). They were maintained on a 12-hr light/dark cycle, which is the usual biological clock. Prior to the experiments, the rats were given 14 days to get used to the lab environment. Being a study of animal care, the protocol of the study was approved by the Institutional Animal Ethical Committee (IAEC) (established by the Committee for Control and Supervision of Experiments on Animals (CPCSEA), Government of India), (DYPCOP/IAEC/2025/16/07).

Antioxidant activity

In vitro DPPH radical scavenging activity

The radical scavenging property of Daidzein at various doses could be measured in terms of hydrogen-donating or radical scavenging ability against the stable free radical 1,1-diphenyl-2-picryl hydrazyl (DPPH) (Gulcin and Alwasel, 2023; Gulcin, 2020). The BHT serves as a standard. Test solutions were prepared at dosages of 25, 50,75,100, and 125 μg/mL. The experiment was fulfilled using a freshly made 70 μM DPPH solution in ethanol. A 1 mL sample solution and a 3 mL DPPH solution were combined for each concentration. By mixing 1 mL of ethanol with 3mL of the DPPH solution, Ethanol was utilised as a control. After giving the mixtures a good shake, they were permitted to rest

at room temperature for half an hour in the dark. Following incubation with blank methanol, the absorbance was recorded at 517 nm using a spectrophotometer. The absorbance of the reaction mixture will decrease as the free radical scavenging activity increases. Radical scavenging ability was ascertained as a percentage Effect (E%) as in the formula:

$$\%\ Inhibition\ = \frac{Abs\ control\ -\ Abs\ sample}{Abs\ control} \times\ 100$$

Where Absc = absorbance of the control,

Abss = absorbance of the sample.

In vitro Model

Isolated goat trachea chain preparation

At the slaughterhouse, the isolated adult goat tracheal tissue was immediately removed from the killed animal. To create a chain, the trachea was divided into several rings and fastened together in succession. The trachea was hung in an organ bath loaded with Krebs solution and continuously aerated at 37±0.5°C. The histamine dose-response relationship was investigated in plain Krebs and Krebs medium with 1000 µg/mL Daidzein. To create a histamine dose-response curve with and without Daidzein, the % maximal contractile reaction was plotted (Patil *et al*., 2008).

Mucolytic Activity test using Egg-white Model

The mucus solution was synthesized to a mixture of egg white and Phosphate-Buffered Saline (PBS) (20:80). In the test groups, Daidzein was carefully weighed group wise (67 mg, 133 mg, 200 mg, 267 mg and 333 mg correspond to 5%, 10%, 15%, 20% and 25% respectively) and carefully dissolved in a minimal amount of 0.5% DMSO after which the volume was raised to 20 mL using DMSO to ensure that Daidzein was fully dissolved. Each prepared 20 mL Daidzein solution was then added to 80 mL of the simulated mucus solution (prepared by mixing 16 mL of egg white and 64 mL of PBS) to obtain a final volume of 100 mL per group. The 20 mL of egg white and 80 mL of PBS in the negative control group, and 20 mL of egg white, 80 mL of PBS, and 0.2% N-acetylcysteine in the positive control group were added. All prepared solutions were transferred into 100 mL beakers, and viscosity measurements were performed using a Brookfield viscometer with spindle number 2 at 12, 30, and 60 rpm, with viscosity recorded in Centipoise (cP) at room temperature (Deswati *et al*., 2018).

Acute toxicity test (Determination of LD_{50})

The acute oral toxicity of Daidzein was evaluated in female Wistar rats as per OECD guideline 423 (OECD, 2002). Rats were divided into three groups (consisting of 3 rats each) and were exposed orally to Daidzein at the following doses: 5 mg/kg, 50 mg/kg, and 500 mg/kg. The 5 mg/kg and the 50 mg/kg doses did not show any manifestation of toxicity or death after the 14-day observation period. In the 500 mg/kg group, there was the death of 1 rat on day 3. To verify, three more rats have been used to administer the 500 mg/kg dose, and there was no sign of further death or toxicity during the 14 days. Judging by these results, Daidzein was considered safe up to 500 mg/kg, with very little toxicity. Therefore, doses of 50 mg/kg, 100 mg/kg, and 200 mg/kg were selected as low, medium, and high doses for subsequent *in vivo* studies.

In vivo Models

Clonidine-induced mast cell degranulation in rats

Female Wistar rat was divided into five groups (*n*=6/group): normal control, standard group, and three groups of Daidzein administration (50 mg/kg, 100 mg/kg, and 200 mg/kg). Seven consecutive days were used in applying treatments once a day. The standard group received sodium cromoglycate (50 mg/kg, i.p.), and the normal control group received distilled water (5 mL/kg, p.o.). The test groups were dosed with Oral Daidzein (50 mg/kg, 100 mg/kg, and 200 mg/kg). Following the last treatment, 2 hr later, all animals received 10 mL of normal saline via intraperitoneal injection, and the abdomen was gently massaged for 5 min to recover cells from the peritoneal cavity. Following the opening of the peritoneal cavity, the peritoneal fluid was extracted and put into centrifuge tubes with 7-10 mL of RPMI 1640 buffer (pH 7.2-7.4). The samples were centrifuged at 400-500 rpm for three cycles, and the supernatant was discarded each time. The mast cell pellets obtained were washed twice with RPMI 1640 buffer. After adding clonidine (0.5 µg/mL) to the mast cell suspension, it was stained with 1% toluidine blue. The stained preparations were examined with a light microscope (45X). A total of 100 mast cells in different fields were counted, and ratios of intact and degranulated cells were recorded and used to determine the percentage of mast cell protection (Kumar *et al*., 2010; Kumar *et al*., 2011).

Passive paw anaphylaxis in rats

Wistar rats were sensitised on days 1st, 3rd, and 5th by subcutaneous administration of egg albumin (100 µg) adsorbed on aluminium hydroxide gel (12 mg) suspended in 0.5 mL of saline to enhance antiserum production against egg albumin (Kumar *et al*., 2011). On the 10th day, blood was obtained via the retro-orbital plexus of each rat under light ether anaesthesia. Serum was separated by centrifuging the blood at 1500 RPM for 20 min. To achieve passive sensitisation, 0.1 mL of the serum obtained from the blood was injected into the left hind paw of each rat. Saline was injected using an equal volume into the contralateral hind paw of each rat. After 24 hr, the animals were randomly assigned to five groups (*n*=6 per group) and thereafter administered 10 mL/kg distilled water orally (control group), 0.5 mg/kg dexamethasone intraperitoneally (standard group), and 50, 100, and 200 mg/kg Daidzein. After a 1-hr post-treatment, the rats were subjected to a challenge involving the injection of 10 µg of egg albumin in 0.1 mL of saline into the sub-plantar region of the left hind

paw. The paw volume was assessed at 0, 1, 2, 3, and 4-hr using a plethysmometer, and the variations in the paw volume defined the oedema response.

$$\%\ inhibition = \left\{1 - \frac{Mean\ relative\ change\ in\ paw\ volume\ (Test)}{Mean\ relative\ change\ in\ paw\ volume\ (Control)}\right\} \times 100$$

OVA-induced allergic asthma in Rats

Six groups (n=6 per group) of Wistar rats were randomly assigned: three daidzein-treated groups (50, 100, and 200 mg/kg), a normal control, an induction control, and a standard (Mahajan and Mehta, 2011). On the first day, 20 µg of Ovalbumen (OVA) and 2 mg of aluminium hydroxide were given intraperitoneally to the rats in 200 µL of PBS (pH 7.4) to sensitise them. The control group was given PBS only. The same sensitisation was administered again on day 14. A treatment of daidzein was administered orally on days 15 - 26 at 50, 100, and 200 mg/kg, with the standard group receiving dexamethasone (2 mg/kg i.p.). On days 27, 28, and 29, the rats were given 100 µg of OVA (50 µg of PBS) intranasally, whereas animals in the control group received an equal volume of PBS using the intranasal instillation technique. On day 30 (i.e., 24 hr post final OVA challenge), the rats were euthanised to collect blood and Bronchoalveolar Lavage (BAL) fluid and to collect lung tissues to be used to evaluate histopathology (Ninave and Patil, 2019).

Collection of blood and BAL fluid

All of the deceased rats had their vena cava punctured and their trachea cannulated to obtain blood and BAL fluid, respectively. The blood is centrifuged at 3000 rpm for 10 min at 4°C to create the serum, which is then kept at -40°C until analysis. A catheter was inserted into the trachea, and 0.5 mL PBS aliquots were lavaged twice to get the BAL. After mixing the lavaged fluid, it was centrifuged for 10 min at 4°C and 1500 rpm. 1 mL of cold PBS was added to the collected remnant cells, and it was used to count total and differential cells. The BAL fluid was also stained with trypan blue to estimate the total number of inflammatory cells quantifiable by millilitre. Subsequently, the cells were counted by using a hemocytometer under a light microscope as per standard procedures. For differential cell counting, 150 µL of BAL fluid was stained with a modified Leishman stain on a sanitised glass slide. A light microscope was used to view these dyed slides, and a differential cell count was conducted (Ninave and Patil, 2019).

Estimation of IL-4 and TNF-α in Serum and BALF

The concentrations of certain cytokines, specifically IL-4 and TNF-alpha, were measured in the serum and Bronchoalveolar Lavage (BAL) fluid by a rat Enzyme-Linked Immunosorbent

Table 1: % Inhibition and IC_{50} Value of Daidzein and standard with DPPH.

Concentration µg/mL	% inhibition	
	BHT	Daidzein
25	20.49	17.81
50	38.89	32.93
75	53.41	46.29
100	64.81	59.50
125	73.21	68.26
150	81.07	73.85
IC_{50}	69	82

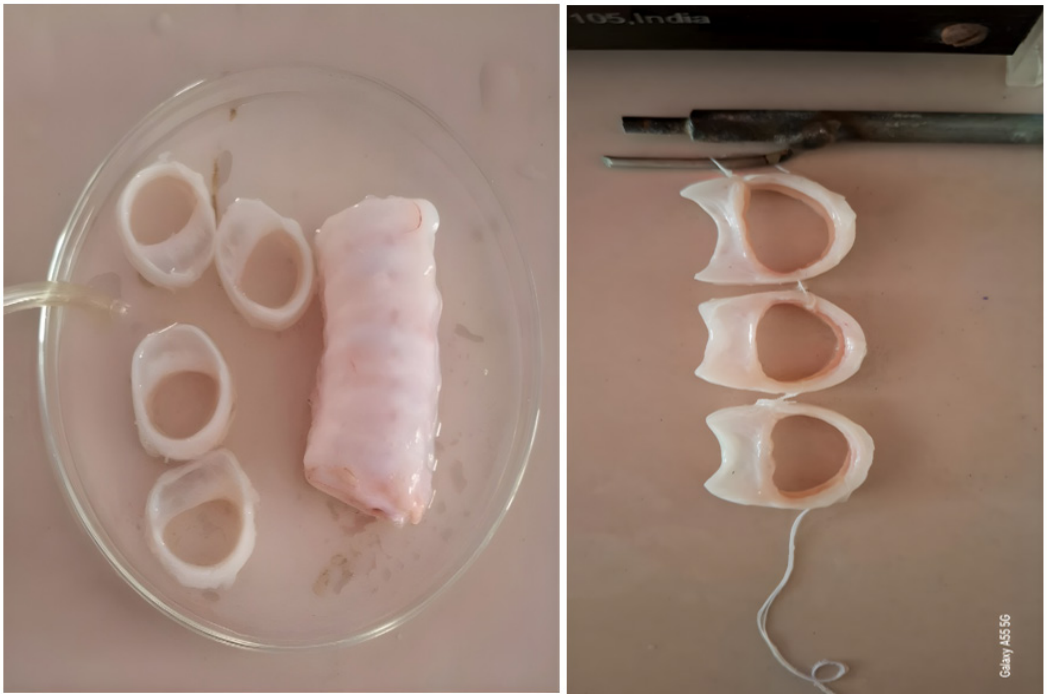

Figure 1: Goat tracheal chain preparation.

Assay (ELISA) kit. Blood samples in rats were collected as serum, and the BAL fluid was collected by cannulating the trachea after euthanasia. The collected samples were sent to Scitesla Lab, Research and Development, Navi Mumbai, for the estimation of IL-4 and TNF-α.

Determination of OVA-Specific IgE Levels

OVA-specific IgE quantity was measured in serum and Bronchoalveolar Lavage (BAL) fluid, with the help of a rat Enzyme-Linked Immunosorbent Assay (ELISA) kit. Blood samples in rats were collected as serum, and the BAL fluid was collected by cannulating the trachea after euthanasia. The collected samples were sent to Scitesla Lab, Research and Development, Navi Mumbai, for the estimation of OVA-specific IgE levels in both serum and BAL fluid.

Quantitative Real-Time PCR for NFKB1 Gene Expression

Samples of the lung tissues were given to PreclinBio Laboratory, Pune, to analyse NFKB1 gene mRNA expression using quantitative real-time PCR (qPCR) after being stored in a container with dry ice, after euthanasia, to preserve the quality of RNA.

Histopathological analysis of lung tissue

A tissue of the lung was sampled after euthanasia and fixed in 10% neutral-buffered formalin (pH 7.0) and embedded in paraffin blocks. Histological Analysis was done by cutting thin sections (3-5 µm), mounting them on clean glass slides. Hematoxylin-Eosin (H&E), Periodic Acid-Schiff (PAS) staining were done on deparaffinized sections to evaluate the inflammatory cell infiltration and goblet cell hyperplasia, and mucus production, respectively (Wang *et al.*, 2017). Semi-quantitative evaluation of the intensity of inflammatory cell infiltration in the light microscope was rated as mild, moderate, and severe. The abundance of goblet cells was scaled as follows: no goblet cell 1 =<25%, 2=25-50%, 3=50-75%, and 4=>75% respectively on the PAS-stained sections of the airway epithelium. The scoring was done in three fields on each section of the lungs.

Statistical analysis

To describe the data, the mean±SEM was used. To statistically compare the experimental groups, a one-way ANOVA with the Dunnett test was used. *p<0.5, **p<0.01, and ***p<0.001 were deemed statistically significant results (Figure 1).

RESULTS

Antioxidant Activity: DPPH Radical Scavenging Assay

DPPH free radical scavenging was carried out to investigate the ability of Daidzein and BHT (Standard) as scavengers of a free radical. In Table 1 and Figure 2, a per cent inhibition of the DPPH radicals increases, as a concentration-dependent response (25-150 µg/mL) for both compounds, was observed. Values of IC_{50}, calculated using linear interpolation, are defined as concentrations that inhibit 50% of DPPH radicals, which for BHT was approximately 69.13 µg/mL and for Daidzein, 82.03 µg/mL. Based on these findings, BHT is shown to have an increasingly greater antioxidant capacity, whereas Daidzein also exhibits a considerable free radical scavenging activity.

Effect of Daidzein on histamine-induced contraction in goat tracheal chain preparation

In the current research, histamine (20 g/mL) exerted a dose-dependent effect on the isolated goat tracheal chain preparation, as shown in the graph showing the proportion of the greatest contractile response to a negative log molar histamine concentration. The physiological salt solution, which was modified with Daidzein (Daidzein 1000 µg/mL), showed a significant (p<0.001) inhibitory effect on the contractile effect of histamine. as shown in Table 2 and Figure 3. The maximum percentage contraction produced by histamine in the control group was 100±0.43%, which was reduced to 65.79±1.03% in the presence of Daidzein.

Table 2: Effect of Daidzein (1000 µg/mL) on histamine-induced contraction in isolated goat tracheal chain preparation.

Sl. No.	Dose of Histamine (20 µg/mL)	-Log Molar Conc. of Histamine	% Maximum Contraction	
			Control	Daidzein
1.	0.1	7.86	20.45±0.99	9.82±1.15
2.	0.2	7.56	31.8±0.81	19.68±0.75
3.	0.4	7.26	45.2±0.63	24.82±0.97
4.	0.8	6.96	72.5±1.01	36.19±1.36
5.	1.6	6.66	84.43 ±1.15	48.30±0.36
6.	3.2	6.35	92.18 ±0.68	53.1±0.92
7.	6.4	6.05	100±0.43	65.79±1.03

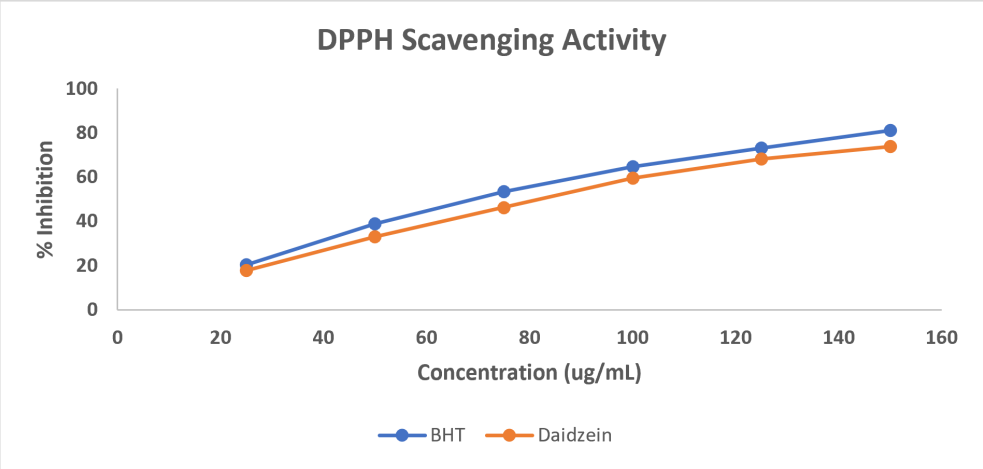

Figure 2: DPPH assay of BHT and Daidzein.

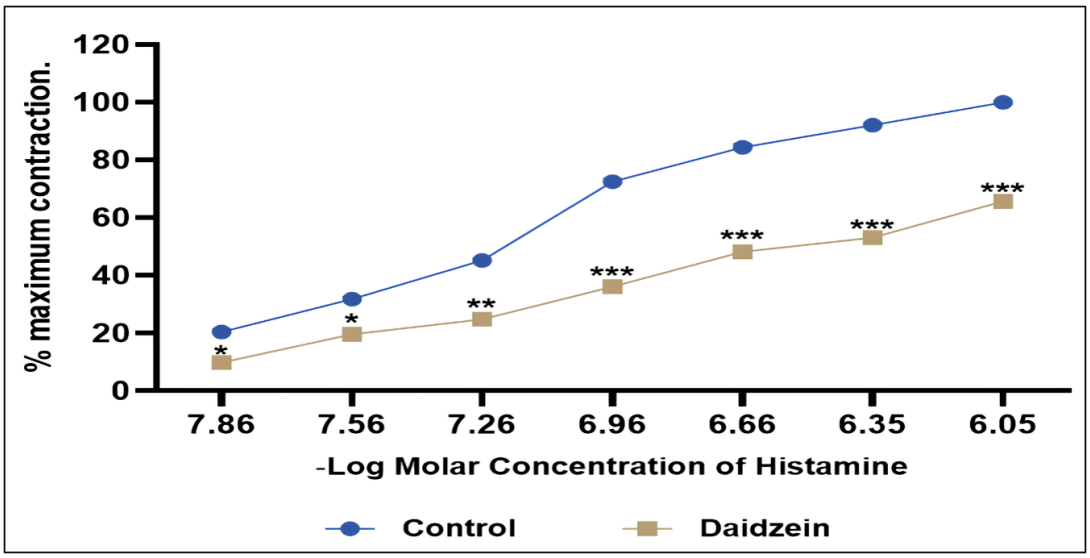

Figure 3: Effect of Daidzein on histamine-induced contraction of isolated goat tracheal chain preparation Values are expressed as Mean±SEM. Where *n*=3. Control=D.R.C. in the absence of Daidzein. DDZ=D.R.C. in the presence of Daidzein (1000 µg/mL). Statistical analysis was carried out by using Student's *t*-test (*p<0.05, ** p<0.01, *** p<0.001), significantly different from the control.

Effect of Daidzein on the Viscosity of Egg White: An *in vitro* Model for Mucolytic Activity

The mucolytic potential of Daidzein was determined as the viscosity reduction of an egg white solution at three shear rates (12 rpm, 30 rpm, and 60 rpm) using a Brookfield viscometer. The action of Daidzein was contrasted both with the negative control (solution of egg white phosphate buffer) and the positive control (N-Acetylcystine (NAC), known as a mucolytic agent). The results indicated that the viscosity of the negative control was 40.48±1.07 cP, and the positive control was fairly lower, which is 23.50±0.23 cP at 12 rpm, which confirmed the mucolytic effect of NAC. Daidzein treatment produced a dose-dependent reduction in viscosity, with the lowest dose recording a value of 33.61±0.65 cP (Dose 1), 24.28±0.89 cP (Dose 2), and 21.87±0.21 cP (Dose 5) when compared to the negative control. Dose 5 recorded a lower viscosity as compared to the positive control. At 30 rpm, the viscosity of the negative control was 36.14±0.39 cP, and that of the positive control was 21.56±0.61 cP. Daidzein also showed a typical dose-dependent decrease in the viscosity, estimated as 30.00±0.91 cP (Dose 1) and 19.26±0.68 cP (Dose 5), which is further evidence of significant mucolytic action (Table 3 and Figure 4). The viscosity of the negative control at 60 rpm was 27.82±0.53 cP, and the positive control measured 19.32±0.26 cP. In the Daidzein-treated groups, viscosity ranged from 24.26±1.06 cP to 18.08±0.41 cP, where Dose 5 again had a greater viscosity reduction than the standard.

Description

X±SEM: Mean Viscosity Value of Egg White Solution±SEM (Standard Error Mean).

Negative Control (Comparison of 20% egg white solution with phosphate pH 7 (20 80)).

Positive Control (Acetylcysteine white solution Eggs 20% in phosphate pH 7 (20: 80)).

Dose 1 (Daidzein 5% plus 20% egg white solution in Phosphate buffer pH 7).

Dose 2 (Daidzein 10% plus 20% egg white solution in Phosphate pH 7).

Dose 3 (Daidzein 15% plus 20% egg white solution in phosphate, pH 7).

Dose 4 (Daidzein 20% plus 20% egg white solution in phosphate, pH 7).

Dose 5 (Daidzein 25% plus 20% egg white solution in phosphate, pH 7).

Effect of Daidzein on mast cell degranulation in Rats

In this study, animals pre-treated with Daidzein showed a significant protection concerning the degranulation of mast cells after challenge with clonidine. Daidzein's suppression of mast cell degranulation ($p<0.01$ to $p<0.001$) would appear to indicate a stabilising effect on the mast cell biomembrane, suggesting mast cell stabilising activity comparable to that displayed in Table 4 and Figure 5. Daidzein dosages of 50, 100, and 200 mg/kg (p.o.) resulted in 32%, 43%, and 53% protection of mast cells, respectively, as compared to sodium cromoglycate (50 mg/kg, i.p.), the standard drug, which showed 59% protection against degranulation.

Effect of Daidzein on Passive Paw Anaphylaxis in Rats

In the current investigation, the challenge with egg albumin significantly accelerated the anaphylactic reaction, resulting in a steady rise in the volume of paw oedema in the control group's sensitised rats that lasted for 4 hr. However, the paw oedema volume was significantly decreased in a dose-dependent manner by Daidzein therapy at all investigated dosages (50, 100, and 200 mg/kg, p.o.) (Table 5). Significant inhibition of worsened oedema volume of the paws was observed at all periods assessed. The results obtained showed that maximum inhibition was at the 4-hr point with the per cent inhibition of 44.6%, 48.21%, and 57.14% of Daidzein doses of 50, 100, and 200 mg/kg, respectively, whereas the standard group showed 60.71 per cent inhibition (Table 6 and Figure 6).

Table 3: Viscosity Measurement Result.

Groups	The viscosity of the egg white Solution (cPois)		
	12 rpm	30 rpm	60 rpm
Negative group	40.48±1.07	36.14±0.39	27.82±0.53
Positive group	23.50±0.23	21.56±0.61	19.32±0.26
Dose 1	33.61±0.65	30.00±0.91	24.26±1.06
Dose 2	30.32±0.54	29.82±0.72	23.62±1.18
Dose 3	28.68±0.79	27.78±0.44	22.36±0.63
Dose 4	25.82±1.24	23.34±1.16	21.54±1.50
Dose 5	21.87±0.21	19.26±0.68	18.08±0.41

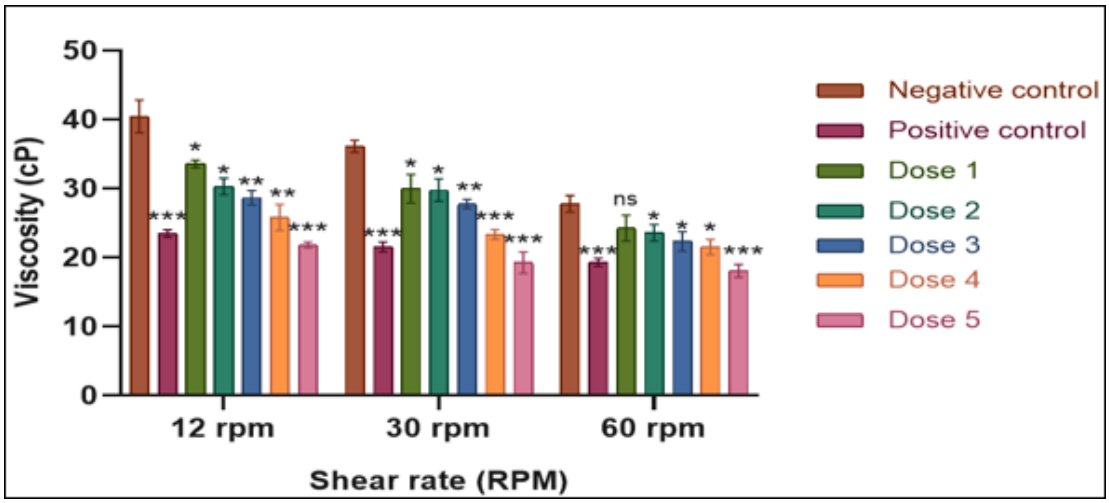

Figure 4: Effect of daidzein on the viscosity of Daidzein. All values are expressed in Mean±SEM (n=5). Data were analysed using a two-way ANOVA test followed by a Tukey Multiple comparison test. * $p<0.05$, ** $p<0.01$, and *** $p<0.001$ are considered statistically significant, and ns $p>0.05$ is considered non-significant in comparison with the negative control group.

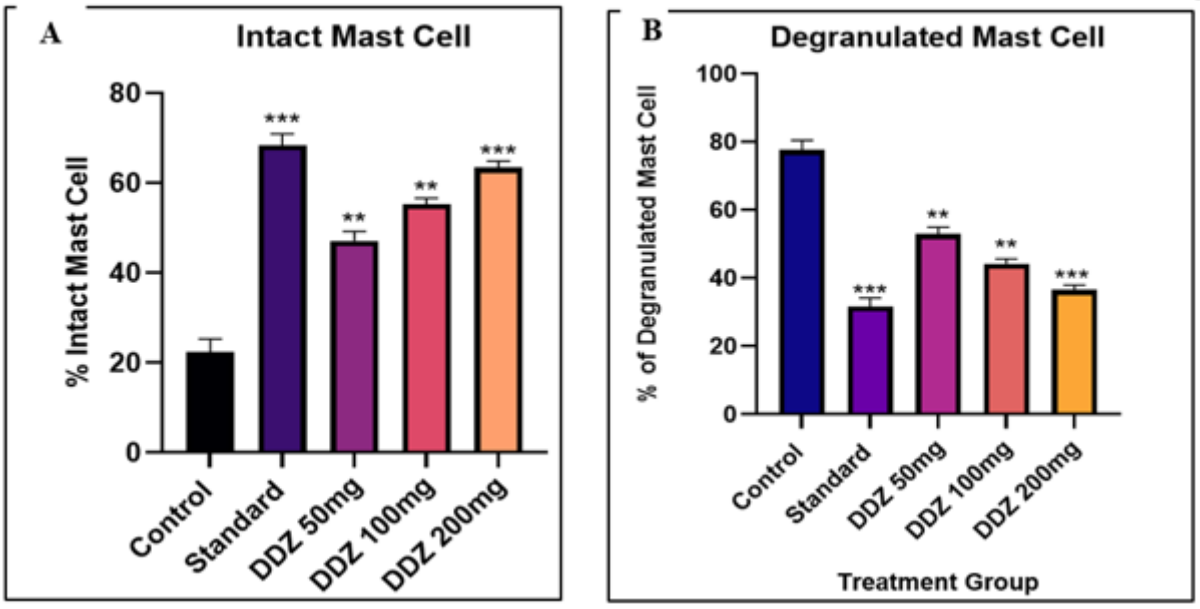

Figure 5: Effect of Daidzein on (A) Intact Mast Cell (B) Degranulated Mast Cell. Values are expressed in Mean±SEM (*n*=6). Data were analysed using a one-way ANOVA test followed by Dunnett's test of Multiple comparisons. Level of Significance *** $p<0.001$, ** $p<0.01$ in comparison with the control group.

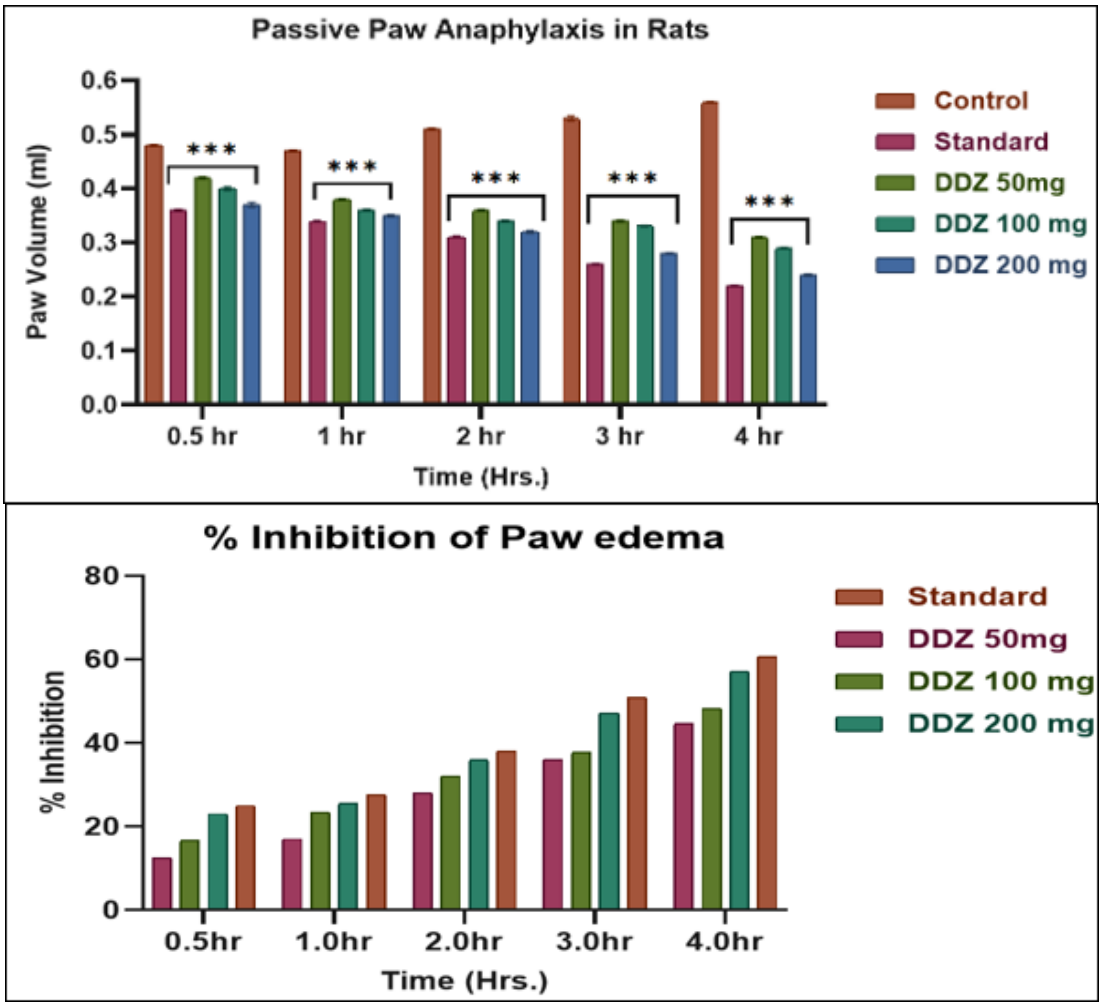

Figure 6: Effect of Daidzein on (A) Passive Paw Anaphylaxis in Rats (B) Per cent inhibition of paw oedema. All values are expressed as Mean±SEM, n=number of rats per group (6). Data analysed by a Two-way ANOVA test followed by Dunnett's Test of multiple comparisons. Level of significance *** $p<0.001$, when compared with the normal control group.

Table 4: Effect of Daidzein on Total Intact and Degranulated Mast Cell (Mean±SEM).

Group	Treatment	Mast cell %		
		Intact Mast Cell	Disrupted Mast Cell	% Protection
I	Control	22.4±2.87	77.6±2.87	-
II	Standard	68.4±2.59	31.6±2.59	59%
III	DDZ 50 mg/kg	47.16±2.09	52.84±2.09	32%
IV	DDZ 100 mg/kg	55.33±1.27	44.17±1.46	43%
V	DDZ 200 mg/kg	63.45±1.42	36.55±1.42	53%

OVA-induced allergic asthma in rats

Effect of Naringenin on the Bodyweight of Rats

Based on statistical findings conducted on different groups, no significant change was found in the body weight of the individual groups on Day 0 and Day 15, except for the OVA-sensitised group, which decreased significantly relative to the control group. The body weight was also significantly reduced in OVA group as compared to the control on Day 29, but on treatment with Dexamethasone (DEX, 1 mg/kg) and Daidzein (50, 100 and 200 mg/kg). A notable increase in body weight was noted in comparison to the OVA group demonstrating the protective role of Daidzein on ova-induced body weight loss in the chronic asthma model (Figure 7).

Effect of Daidzein on the recruitment of inflammatory cells in BAL fluid

The effects of Daidzein on the recruitment of inflammatory cells in the lungs were evaluated by counting the total and differentiated cells in the BAL fluid. OVA-challenged rats had a considerably greater total inflammatory cell count in BAL fluid than the control group, as was to be expected. In contrast to the OVA-challenged group, Daidzein dose-dependently inhibited the rise in inflammatory cell counts at all examined doses (Figure 8). The BAL fluid of OVA-challenged rats had significantly higher levels of eosinophils, neutrophils, macrophages, and lymphocytes than the control group, according to a study on differential cell count analysis. Administration of Daidzein and the standard drug dexamethasone significantly inhibited the rise in these inflammatory cell populations compared to the OVA-challenged group.

Effect of Daidzein on Cytokine Levels

Following the assay process, the OVA-challenged group's serum and BALF levels of IL-4 and TNF-alpha were significantly greater than those of the normal control group. Daidzein at 100 mg/kg and 200 mg/kg also markedly decreased the elevated cytokine levels, much like the standard treatment. Daidzein 50 mg/kg suppressed IL-4 and TNF-alpha in serum and IL-4 in BALF significantly as compared to the induction group (Figure 9).

Effect of Daidzein on IgE level of serum and BAL fluid

Both serum samples and BAL fluids showed a significant increase in IgE levels (as was seen in the induction group). The daidzein treatment showed a dose-related reduction of serum and BAL fluid IgE levels. The greatest dose of daidzein of 200 mg/kg exhibited a significant IgE reduction, by 54.9% in serum and 61.1% in BAL fluid, compared to their respective induction groups. Similarly, 100 mg/kg of daidzein also demonstrated a significant reduction of IgE levels by 36.6% in serum and 35.8% in BAL fluid. Lastly, the 50 mg/kg, while showing similar decreases, did so at only moderate levels of 9.6% in serum and 22.8% BAL fluid. The treatment with standard drug dexamethasone also showed significant IgE decreases in both the serum and BAL fluids at similar levels to the 200 mg/kg dose (Figure 10).

Effect of Daidzein on NFKB1 mRNA expression in lungs

The mRNA expression of NFKB1 was significantly higher in the OVA-induced (induction) group than in the control group. Daidzein treatment decreased NFKB1 mRNA expression in a dose-dependent manner when compared to the induction group. Specifically, daidzein at 50 mg/kg, 100 mg/kg, and 200 mg/kg significantly reduced NFKB1 mRNA expression. The standard drug, dexamethasone, also significantly reduced NFKB1 mRNA expression levels compared to the induction group (Figure 11).

Effect of Daidzein on histopathological changes in lung tissue

We observed severe infiltration of inflammatory cells surrounding the bronchial area and enlargement of the airway epithelium in the OVA-challenged group when contrasted with the control group, as shown in the H&E-stained lung sections. The PAS-stained lung sections showed a significant increase in goblet cell number, as well as excessive mucus production in the OVA-challenged group. Pre-treatment with daidzein, a phytoestrogen, had a suppressive effect on inflammatory cell infiltration based on a dose-response relationship. Daidzein also reduced goblet cell hyperplasia and mucus secretion compared to the OVA group, with moderate treatment reduction at 100 mg/kg and a marked reduction at 200 mg/kg. The group pre-treated with dexamethasone had mild inflammatory infiltration, but had reduced goblet cell hyperplasia

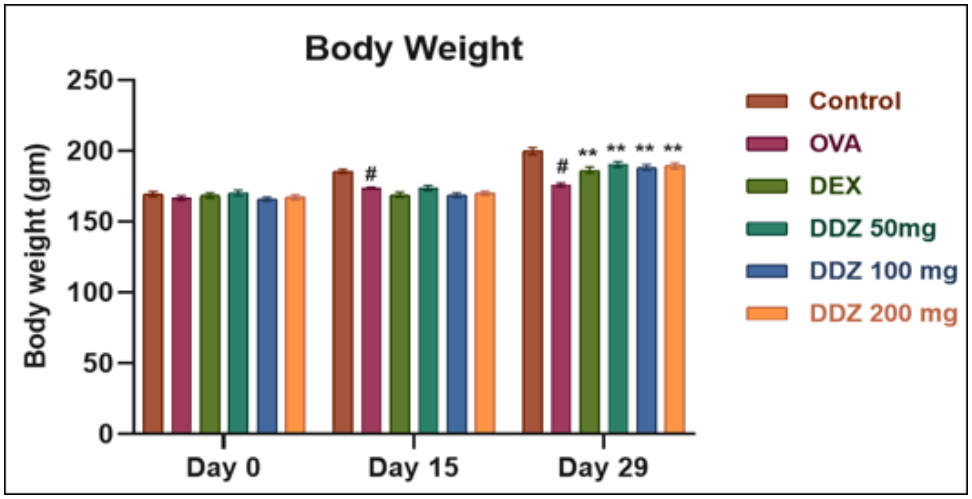

Figure 7: Body weight of rats in OVA-induced Asthma. Values are stated as mean±SEM; *n*=6; Data analysed by Two-way ANOVA followed by Dunnett's test of multiple comparison. Level of significance ###p<0.001, in comparison with the normal control group, also *p<0.05, **p<0.01, ***p<0.001 when compared with the induction group.

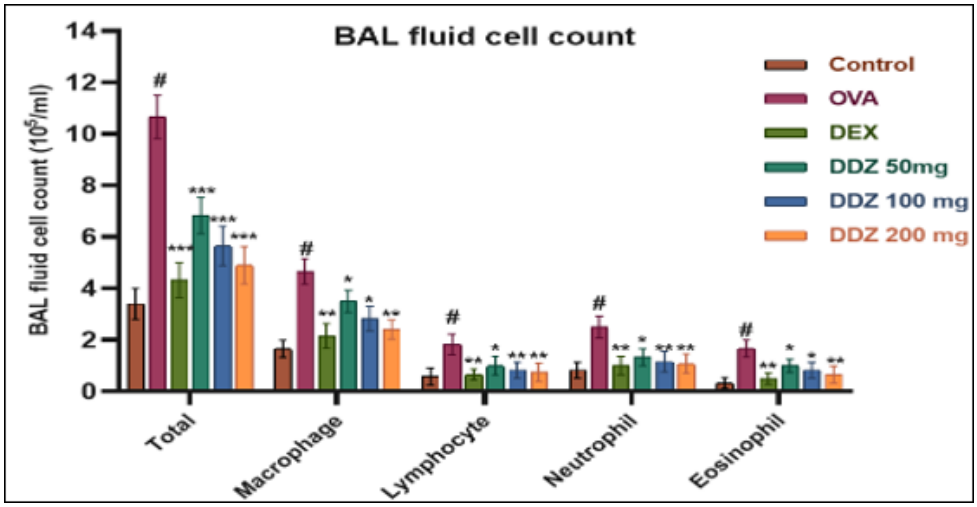

Figure 8: Effect of Daidzein on Inflammatory Cells in BALF. Values are stated as Mean±SEM; *n*=6; Data were analysed by two-way ANOVA test followed by Tukey test of multiple comparison. Level of significance: ###p<0.001, in comparison with the normal control group, and *p<0.05, **p<0.01, ***p<0.001 when in comparison with the induction control group.

and mucus secretion, similar to the 200 mg/kg dose of daidzein, as shown in Figure 12.

RESEARCH DISCUSSION

Asthma is a multifactorial disorder, an inflammatory disorder of the airways, and is associated with the influx of different inflammatory cells into the lungs, including eosinophils, neutrophils, and numerous types of lymphocytes. Asthma is also characterised by drastic bronchospasm, overproduction of mucus, production of IgE, and the release of multiple inflammatory mediators. Given the escalating prevalence of asthma and limitations of current therapeutics, there is an urgency for alternative therapies with safe profiles. Herbal medicine has been used as a treatment for hundreds of years for asthma (Chung and Adcock, 2001). However, with regard to the use of herbal medicines in asthma in traditional settings, there are few reported scientific evaluations of the anti-asthmatic potential of only a few plant-derived agents or their active constituents. The most significant could be considered ephedrine from the plant *Ephedra* (Barger and Dale, 1910). Theophylline, as a component of tea, is also another agent, and another example could be Sodium Cromoglycate from Khellin (Cox, 1967). More directly relevant to the extensive scientific studies, there are abundant accounts on the biological activities of flavonoids, especially isoflavonoids, in the context of asthma in animal models. Recently, there has been increasing research that shows Daidzein can inhibit the maturation and function of dendritic cells, which are the key cells in producing airway inflammation, as they present antigens and activate the T-cell response.

In the present investigation, we conducted a systematic evaluation to assess Daidzein's antiasthmatic efficacy using an array of animal models, through its different mechanisms of action.

Antioxidant potential of Daidzein was evaluated using the DPPH radical scavenging assay, which assesses the capacity of compounds to donate hydrogen atoms to accumulate free

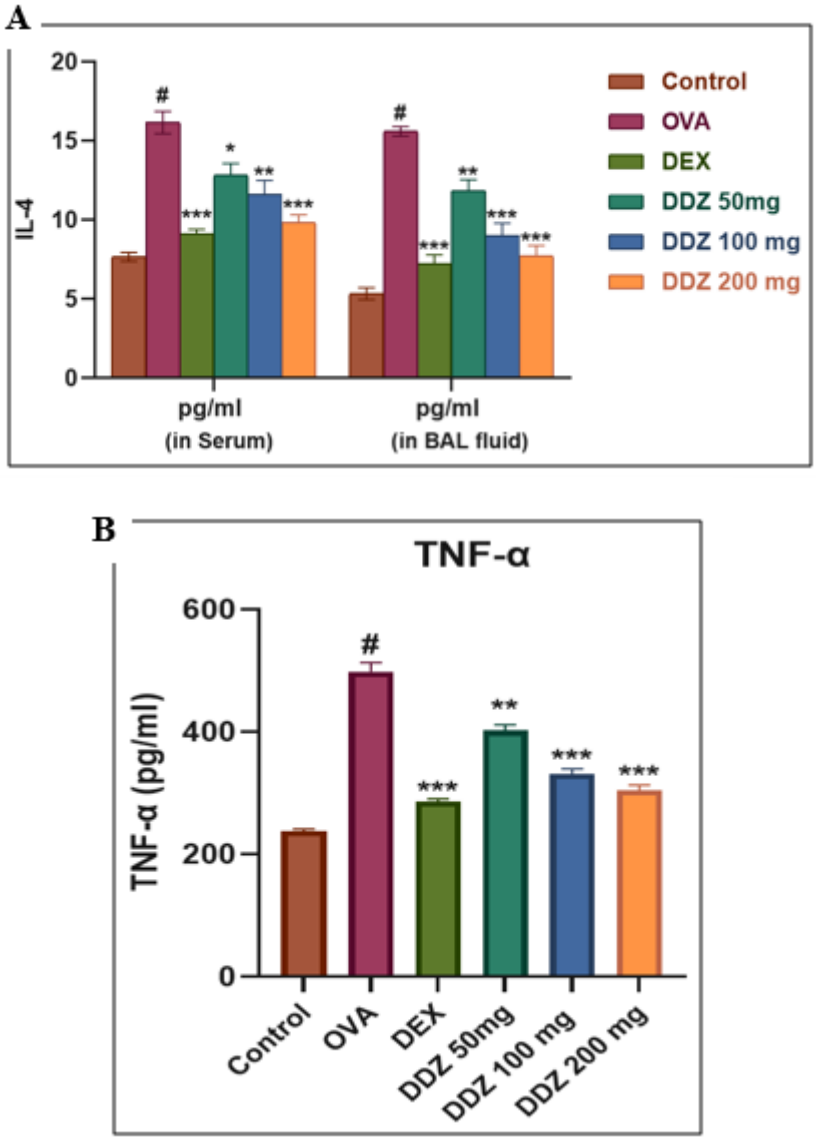

Figure 9: Effect of DDZ on the (A) level of IL-4 in serum and BALF, (B) TNF-α level. Values are presented as Mean±SEM (n=6). Statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparisons. $p<0.001$ indicates a significant difference compared to the normal control group. *p<0.05, **$p<0.01$, ***$p<0.001$ denote significant differences compared to the induction control group.

Table 5: Effect of Daidzein on Passive paw anaphylaxis of rats.

Group (*n*=6)	Paw Oedema Volume (mL)				
	0.5 Hr	1 Hr	2 Hr	3 Hr	4 Hr
Control	0.48±0.0015	0.47±0.0015	0.51±0.0102	0.53±0.0044	0.56±0.013
Standard	0.36±0.0018***	0.33±0.0012***	0.31±0.0016***	0.26±0.0013***	0.22±0.0014***
DDZ 50 mg	0.42±0.0021***	0.38±0.0018***	0.36±0.0018***	0.34±0.0022***	0.31±0.0018***
DDZ 100 mg	0.40±0.0032***	0.36±0.0017***	0.34±0.0016***	0.33±0.0012***	0.29±0.0015***
DDZ 200 mg	0.37±0.0039***	0.35±0.0022***	0.32±0.0024***	0.28±0.0015***	0.24±0.0022***

Table 6: Effect of Daidzein on Percentage Inhibition of Paw Oedema.

Group (*n*=6)	% Inhibition of Paw Oedema				
	0.5 Hr	1 Hr	2 Hr	3 Hr	4 Hr
Standard	25	27.65	38	50.94	60.71
DDZ 50 mg	12.5	17.02	28	36	44.6
DDZ 100 mg	16.66	23.40	32	37.77	48.21
DDZ 200 mg	23	25.53	36	47.16	57.14

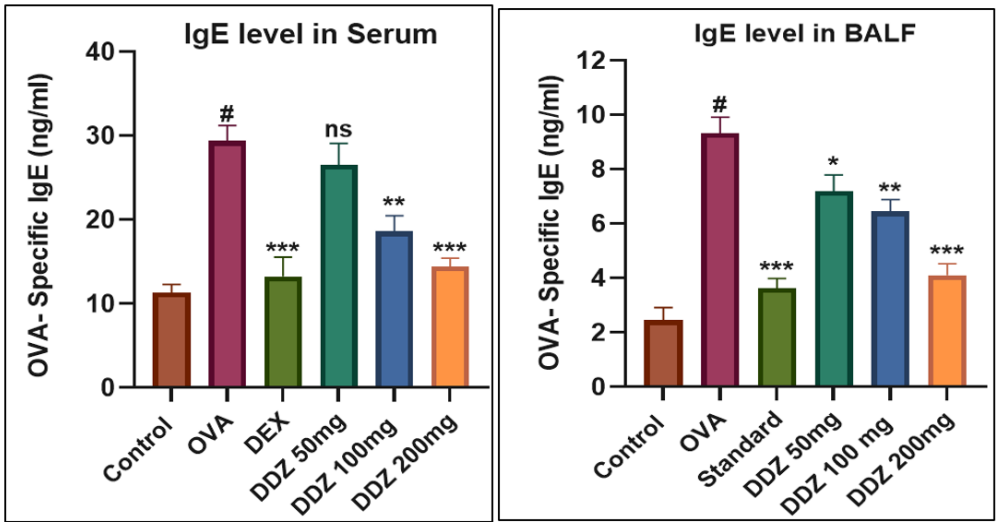

Figure 10: Effect of DDZ on the IgE level in serum and BALF. Values are expressed as Mean±SEM; n=6: Data analysed by One-way ANOVA test followed by Dunnett test of multiple comparisons. Level of significance: ### p<0.001, in comparison with the normal group, and ns $p>0.05$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, when in compared with the induction group.

radicals with resulting in an associated decrease in absorbance at 517 nm. Daidzein exhibited dose-dependent DPPH scavenging activity with an IC_{50} of 82.03 µg/mL compared to the standard antioxidant BHT at 69.13 µg/mL, which indicates significant antioxidant capacity with still significant but slightly less activity as compared to BHT. The free radical scavenging response to Daidzein directly correlates to its structural features, which include phenolic hydroxyl groups in its isoflavonoid composition, and the action is underpinned by its free radical quenching mechanism associated with its hydroxyl groups that neutralise reactive species. Collectively, these results suggest that Daidzein is a significantly potent antioxidant compound and a potentially useful naturally occurring product in the treatment of oxidative stress.

One of the key players in allergy, inflammation, and bronchoconstriction is histamine. Antihistaminic treatment for asthma includes targeting histamine, either by blocking its release from mast cells or by using histaminergic receptor antagonists (Uvnas, 1999). Preparations using goat tracheal muscle are simpler to handle and make. Additionally, it is more delicate than the tracheal chain of guinea pigs. Pharmacologically, the trachea and bronchi react similarly, and histologically, they share a common kind of cartilage and muscle. In the current research, Daidzein causes the histamine Dose-Response Curve (DRC) of the isolated goat tracheal chains preparation to shift to the right, which is indicative of an antiasthmatic effect.

Cough is a protective and defensive reflex to clear mucus, a foreign mass, and infection of the larynx, trachea, and large bronchi. Physiologically, coughing is further found to be the mechanism best able to clear the upper respiratory tract (Lucanska *et al.*, 2020). Sputum, particularly that from the lower respiratory tract, becomes less viscous when mucolytic drugs are used. Hence, altering the physical aspect of mucus chemistry causes a decrease in the viscosity, and thus, coughing can be facilitated easily (King and Rubin, 2002). The mucolytic activity of Daidzein was documented in an *in vitro* egg white viscosity model, where it showed a reduction in viscosity in a dose-dependent manner with all different measured shear rates (12, 30, and 60 rpm). Daidzein had significantly less viscosity than the negative control, and at the highest dose, the mucolytic activity exceeded the activity of the standard drug N-acetylcysteine. These data would suggest that Daidzein increases mucus fluidity, most likely by disrupting the mucin structure or breaking disulfide linkages, therefore supporting its potential for use as a natural mucolytic agent in respiratory diseases characterised by thick mucus secretions.

Among a number of immunological stimuli, antigen-antibody reactions that have expressed themselves on the surface of Mast cells are the most significant factors that result in Mast cell

degranulation (Kapoor *et al.*, 2011). Following degranulation, numerous mediators, including histamine, are released from mast cells. Mobilisation of intracellular Ca^{2+} needs to occur for the release of histamine from mast cells, which is of particular importance (Takei *et al.*, 1992). The current study shows that Daidzein is a dose-dependent mast cell stabiliser. The significant decrease in disrupted mast cells at all doses indicates that Daidzein reduces clonidine-induced mast cell degranulation. Importantly, Daidzein at 200 mg/kg had the highest protection at 53% compared to standard (59%), showing its potential as an anti-allergic or anti-inflammatory agent. The mast cell stability effect of Daidzein is also reinforced by the dose-dependent increase in intact mast cells, which can possibly be due to the isoflavonoid chemical structure that is known to alter inflammatory activities.

The administration of egg albumin (antigen) in the passive paw anaphylaxis model leads to an increase in the egg albumin antibodies. Rats become passively sensitised when these antibodies are injected into their paws. The allergy presented by the egg albumin later provoked a severe antigen-antibody response in the paw, leading to the inflammation of the paw (oedema) due to the release of various inflammatory mediators (Dai *et al.*, 2002). Immunomodulators also play a crucial role in regulating allergic disorders because they suppress antigen-antibody interaction and block the release of inflammatory molecules. In this experiment, the findings indicated that Daidzein pretreatment provides protection of the rats against inflammation of the paws caused by antigen-antibody reactions. The study's findings in this model imply that Daidzein's advantageous effects might be secondary to its strong anti-inflammatory, anti-allergic, anti-anaphylactic, and immunomodulatory properties, which prevent the antigen-antibody response.

Allergic asthma is characterised by persistent airway inflammation, which is led by protein allergens, such as Ovalbumen (OVA), pollen, and house dust. The OVA-induced asthma model in rodents is a closely resembling model of human asthma. The model is widely exploited to determine the potential of compounds in the anti-asthmatic efficacy. In the OVA asthma model, the animals are sensitised with OVA and an adjuvant, and they receive multiple airway challenges with OVA. These operations result in airway inflammation, which includes airway remodelling and the infiltration of neutrophils, lymphocytes, and eosinophils (Choi *et al.*, 2009). In the context of the molecular mediators involved in asthma pathogenesis, several have been shown to come together at a genetic level, including the NFKB1 gene, which encodes the p50 subunit of the NFKB signalling cascade, that modulates pro-inflammatory responses, such that pro-inflammatory responses may lead to transcription of cytokines and chemokines, which can lead to recruitment of immune cells within the airways. Further, NFKB translocation can result in the Th2 cytokines IL-4, IL-5, and IL-13, which function in IgE production, airway hyper-responsiveness, etc. (Menzel *et al.*, 2022). In our research work, we determined the effects of Daidzein on the inflammatory markers and lung histopathology in the OVA-induced allergic asthma model in rats. OVA challenge resulted in the loss of significant body weight, enlargement of inflammatory cells in the BALF, and higher production of IL-4, TNF-alpha, and IgE, which may be referred to as systemic inflammation and allergic sensitisation. Daidzein treatment, at

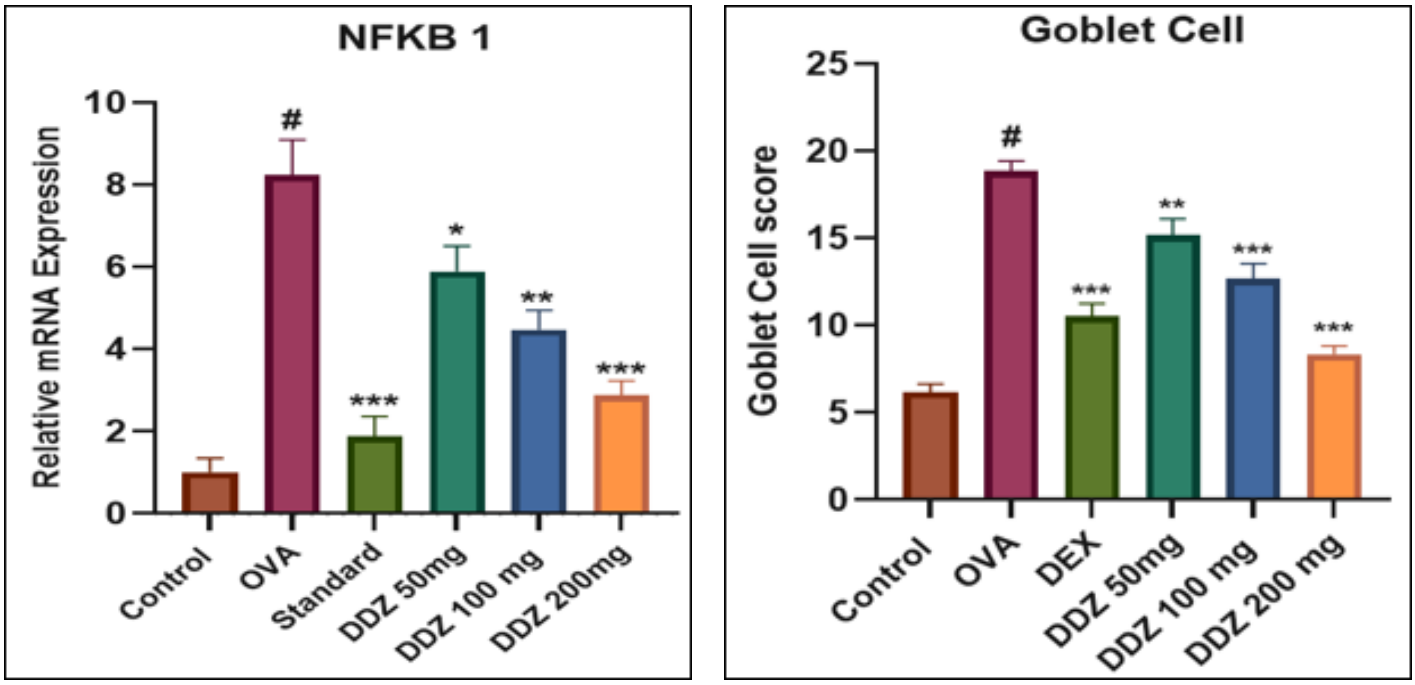

Figure 11: Statistical analysis of the effect of Daidzein treatment on mRNA expression of the NFKB1 gene. Values are expressed as Mean±SEM; *n*=3; Data analysed by One-way ANOVA test followed by Dunnett Test of multiple comparisons. Level of significance: ###p<0.001, in comparison with the normal group, and *p>0.05, **p<0.01, ***p<0.001 when compared with the induction control group.

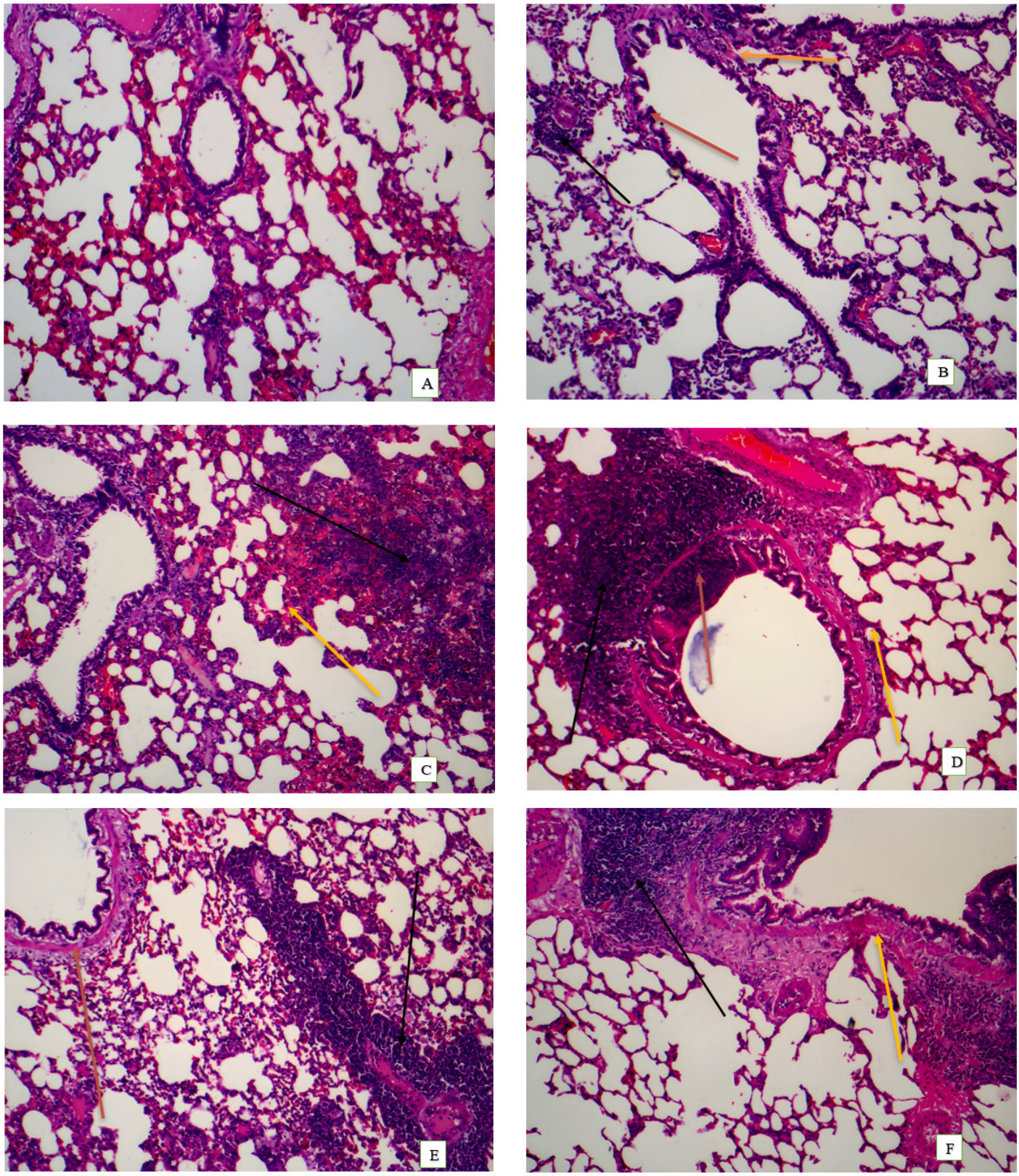

Figure 12: A: Normal control: normal architecture of alveoli, alveolar septa, and bronchioles, H&E, 100x. B: Induction control: Moderately high infiltration of inflammatory cells (black arrow), moderate peri-bronchial oedema (red arrow), Moderate alveolar septal thickening (yellow arrow). C: Standard: Mild infiltration of inflammatory cells (black arrow), Mild alveolar septal thickening (yellow arrow), H&E, 100x. D: DDZ 50 mg/kg: Moderate infiltration of inflammatory cells, Moderate peri-bronchial oedema (red arrow), Mild Alveolar septal thickening (yellow arrow), H&E, 100x. E: DDZ 100 mg/kg: Moderate infiltration of inflammatory cells, Mild peri-bronchial oedema (red arrow), H&E, 100x. F: DDZ 200 mg/kg: Mild infiltration of inflammatory cells, Mild Alveolar Septal Thickening (yellow arrow), H&E, 100x.

all dose levels, significantly prevented weight loss and decreased inflammatory cells in a dose-dependent manner, which reflects Daidzein's amelioration of cachexia associated with disease and airway inflammation. Serum and BALF levels of IL-4 and TNF-α were significantly increased in OVA-induced rats, while Daidzein treatment decreased the levels of these cytokines, reflecting its ability to reactively modulate Th2-mediated responses. In the same way, Daidzein lowered the high levels of IgE observed in the OVA group, indicating inhibition of allergic sensitisation.

Histopathological analysis proved that the exposure to OVA led to goblet cell hyperplasia, inflammatory cell infiltration, peribronchial oedema, and alveolar septal thickening. Daidzein treatment resulted in decreasing goblet cell hyperplasia and airway inflammation in a dose-dependent manner, showing structural airway protection. In addition, an OVA challenge resulted in significantly increasing NFKB1 mRNA expression in lung tissue, indicating the NF-κB pathway was activated. Daidzein treatment caused significant dose-dependent reductions in NFKB1 expression, providing some evidence of suppression of NF-κB-mediated inflammation, but more mechanistic-based research is required.

A change in cytokine response may explain the suggested protective effect of Daidzein in OVA-induced allergic asthma, reduced IgE-mediated allergic state, reduced inflammatory cell infiltration, and reduced expression of the NFKB1 gene, which ultimately resulted in less airway inflammation and remodelling.

CONCLUSION

Daidzein exhibits significant antihistaminic, anti-allergic, mast cell stabilising, anti-inflammatory, anti-anaphylactic, bronchodilatory, mucolytic, and antioxidant activities in various experimental models and effectively suppresses asthmatic exacerbations. Our data demonstrate that administration of Daidzein controls the exaggerated inflammatory response, exhibiting significant anti-inflammatory activity, possibly by preventing the secretion of selected Th2 cytokines (IL-4 and TNF-α) and inflammatory cell infiltration, as observed in this study. This effect may be attributed, at least in part, to the downregulation of the NF-κB signalling pathway, as indicated by the suppression of NFKB1 gene expression. Histological analysis further supports Daidzein's protective role in preserving lung architecture by reducing inflammatory infiltration, oedema, and goblet cell hyperplasia. Thus, our findings support the possible use of Daidzein as a safe, natural, and multi-target therapeutic candidate for the prevention and treatment of allergic asthma.

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ABBREVIATIONS

BHT: Butylated hydroxytoluene; **STAT1:** Signal Transducer and Activator of Transcription; **GI:** Gastrointestinal; **LPS:** Lipopolysaccharide; **DC:** Dendritic cells; **MHC:** Major Histocompatibility Complex; **CD83, CD80, CD86:** Cluster of Differentiation (markers); **Cxcl2:** Chemokine (C-X-C motif) ligand 2; **PARP-1:** Poly (ADP-ribose) polymerase 1; **OECD:** Organisation for Economic Co-operation and Development; **LD50:** Lethal Dose,50%; **RPM:** Revolutions per minute; **RPMI 1640:** Roswell Park Memorial Institute 1640 (medium); **DDZ:** Daidzein; **DRC:** Dose-response curve; **DEX:** Dexamethasone; **BA:** Bronchial asthma; **OVA:** Ovalbumin; **BAL:** Bronchoalveolar lavage; **IL-4:** Interleukin-4; **IL-5:** Interleukin-5; **IL-6:** Interleukin-6; **TNF-α:** Tumor necrosis factor-alpha**; IgE:** Immunoglobulin E; **NF-κB:** Nuclear factor-kappa B; **Th2:** T-helper 2; **ICS:** Inhaled corticosteroids; **DMSO:** Dimethyl sulfoxide; **IAEC:** Institutional Animal Ethical Committee; **CPCSEA:** Committee for Control and Supervision of Experiments on Animals; **DPPH:** 1,1-diphenyl-2-picryl hydrazyl; **PBS:** Phosphate-buffered saline; **NAC:** N-acetylcysteine; **ELISA:** Enzyme-linked immunosorbent assay; **qPCR:** Quantitative real-time PCR; **H&E:** Hematoxylin and Eosin; **PAS:** Periodic acid-Schiff; **SEM:** Standard Error of the Mean; **ANOVA:** Analysis of Variance; **BALF:** Bronchoalveolar lavage fluid.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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AUTHOR CONTRIBUTIONS

A.H., P.W. and N.V.: Conceptualisation, design of study. A.H and P.W: Manuscript draft. N.V and P.W: Supervised and revised the manuscript. A.H and K.U.: Animal experiments, P.W. and S.D.: Analysis of biochemical and histopathological data. A.H.: literature review, data curation, and preparation of figures and tables. All authors have read and agreed to the published version of the manuscript.

SUMMARY

This study evaluated the anti-asthmatic potential of Daidzein, a natural isoflavone, using comprehensive *in vitro* and *in vivo* experimental models of allergic asthma. Daidzein demonstrated significant antioxidant, antihistaminic, mucolytic, and mast cell stabilising activities. In the ovalbumin-induced asthma model, treatment with Daidzein markedly reduced inflammatory cell infiltration, suppressed elevated levels of IL-4, TNF-α, and IgE, and improved lung histopathological changes. Furthermore, Daidzein significantly downregulated NFKB1 gene expression, indicating inhibition of NF-κB-mediated inflammatory pathways. Overall, the findings suggest that Daidzein exerts multi-target anti-inflammatory and immunomodulatory effects and may serve as a promising natural therapeutic candidate for the management of allergic asthma.

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