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Anti-asthmatic effect of aerial parts of *Pergularia daemia* through broncho-relaxation, mast cell stabilization, and decreasing bronchial hyperreactivity in experimental animals

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ABSTRACT: *Pergularia daemia* is one of the medicinal plants traditionally used for the management of asthma. The current study evaluated the anti-asthmatic activity of aerial parts of *Pergularia daemia* in ovalbumin-induced asthma in BALB/c mice, histamine induced bronchospasm in guinea pigs, and compound 48/80 induced mast cell degranulation in rat peritoneal mast cell. Administration of ovalbumin in BALB/c mice has significantly increased total leukocyte count, infiltration of inflammatory cells, and altered the levels of *in vivo* anti-oxidant enzymes such as glutathione, catalase with the elevation of lipid peroxidation and total protein. Treatment with the methanolic extract of *Pergularia daemia* significantly reduced the total leukocyte count, elevated inflammatory cells, lipid peroxidation, and total proteins, and restored the altered levels of glutathione and catalase. The onset of Pre convulsion Dyspnea in guinea pigs was significantly increased on treatment with the methanolic extract of *Pergularia daemia*. Further, the methanolic extract of *Pergularia daemia* exhibited significant protection against compound 48/80 induced mast cell degranulation in rat peritoneal mast cells.

KEYWORDS: Guinea pig; compound 48/80; histamine; BALB/c; ovalbumin.

1. INTRODUCTION

Bronchial asthma is a chronic inflammatory disease of the airway that involves multiple components and is orchestrated by numerous cell types, particularly mast cells, eosinophils, and CD4+ lymphocytes [1]. Activation of these cells leads to the release of proinflammatory mediators which in turn causes vascular leakage, bronchial smooth muscle contraction, inflammatory cell infiltration, mucous hypersecretion, airway hyperresponsiveness, and ultimately airway remodeling, which are expressed clinically as recurrent wheezing, dyspnea, chest tightness, cough, and airway obstruction [2, 3]. Salmeterol, formoterol, fluticasone, montelukast, zafirlukast and budesonide/formoterol are commonly used synthetic anti-asthmatic drugs. However, these drugs having some adverse effects including respiratory tract infection, cough, fever and headache [4]. Many plants have long been employed in traditional medicine [5]. The *Pergularia daemia* (Forsk.) Chiov. (Asclepiadaceae) is one of the important medicinal plants used in indigenous medicine [6, 7]. It has been traditionally used to treat childhood diarrhea, asthma, and malarial irregular fever as well as acting as a laxative, anthelmintic, expectorant, and antipyretic [8-10]. The aerial parts of this plant have been shown to perform a variety of pharmacological effects, including hepatoprotection [11], antifertility [12], and antidiabetic [13]. The leaf juice is administered with lime to rheumatic swellings and utilized in asthma cases [14]. However, no *in vivo* pharmacological standardization for anti-asthmatic action has been documented, according to a study of the literature on this crude medication [15]. So, the goal of the current inquiry is to assess the *in vivo* anti-asthmatic activity of *Pergularia daemia*'s aerial parts in different animal models.

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2. RESULTS

The methanolic extractive yield obtained was 14.23 %. The preliminary phytochemical test revealed PDME contains alkaloids, flavanoids, phenolics, tannins, steroids, and saponins. The gallic acid equivalent of 4.742 mg/g was discovered to be the total phenolic content of methanolic extract of *Pergularia daemia* (PDME). The amount of total flavonoids discovered in PDME was determined to be 27.4 mg/g, which is equal to quercetin. Table 1 indicates ABTS and DPPH radical cation scavenging potential of the PDME at each concentration, in the range of 20-100 µg/mL. The PDME was shown to have an ABTS radical scavenging IC_{50} value of 11.39 µg/mL, while the reference quercetin concentration was 5.57 µg/mL. The IC_{50} values for PDME's ability to scavenge DPPH radicals were determined to be 29.12 µg/mL compared to the standard quercetin's 5.07 µg/mL.

The total antioxidant capacity of PDME was determined by FRAP assay which was found to be 75.69 µg/mL equivalent to vitamin-C. The total antioxidant capacity of PDME by phosphomolybdate assay was found to be 113 µg/mL equivalent to vitamin-C.

PDME was tested for its antiasthmatic activity in two different *in vivo* models and one ex vivo model and various parameters were checked. In the ovalbumin-induced asthma model, the smear of the bronchoalveolar lavage (BAL) fluid obtained from BALB/c mice showed different types of white blood cells (Figure 1).

The total leukocyte count, eosinophil, monocyte, and lymphocyte count revealed a change after sensitization of BALB/c mice with ovalbumin. There was a significant ($p < 0.05$) increase in the total leukocyte count, eosinophil, neutrophils, macrophage, and lymphocyte counts were considerably raised in the control group compared to the normal group (Figure 2).

The treatment with the standard and plant extract produced a significant ($p < 0.05$) decrease in the elevated total leukocyte, eosinophil, neutrophils, monocyte, and lymphocyte counts as compared to the control group respectively (Figure 2). In the lung homogenate of BALB/c mice, significant ($p < 0.05$) increase in the LPO level was seen with the control group (12.23±0.090) as compared to the normal group (7.79±0.049 Mmol MDA/mg protein). The treatment with standard produced a significant ($p < 0.05$) decrease in the elevated LPO (9.7±0.088 Mmol MDA/mg protein) as well as the plant extract that also produced a significant ($p<0.05$) decrease (low dose 12.12±0.22, high dose 9.81±0.014 Mmol MDA/mg protein) as compared to the positive control group (Table 2).

Table 1. Percentage inhibition and IC_{50} value of DPPH and ABTS radical by Quercetin and PDME.

Treatment	Concentration (µg/mL)	Percentage inhibition		IC_{50} Value (µg/mL)	
		ABTS	DPPH	DPPH	ABTS
Standard (Quercetin)	20	84.75	97.57	5.07	5.57
	40	92.96	97.87		
	60	96.98	97.94		
	80	97.03	98.20		
	100	97.12	99.13		
Sample (PDME)	20	96.11	65.68	29.12	11.39
	40	98.53	75.39		
	60	98.58	77.57		
	80	98.64	81.91		
	100	98.67	82.3		

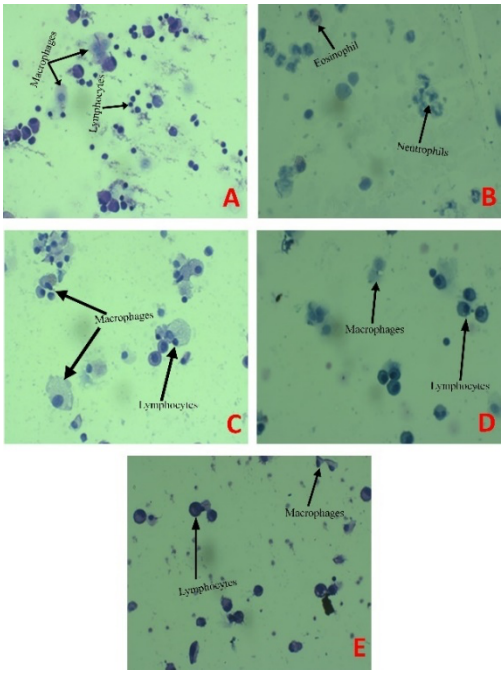

Figure 1. Differential leukocytes of the smear of Bronchoalveolar lavage fluid obtained from BALB/c mice. (A: Normal group; B: Control group; C: Standard group; D: PDME low dose and E: PDME high dose. The cells were fixed and stained using DiffQuik staining solution).

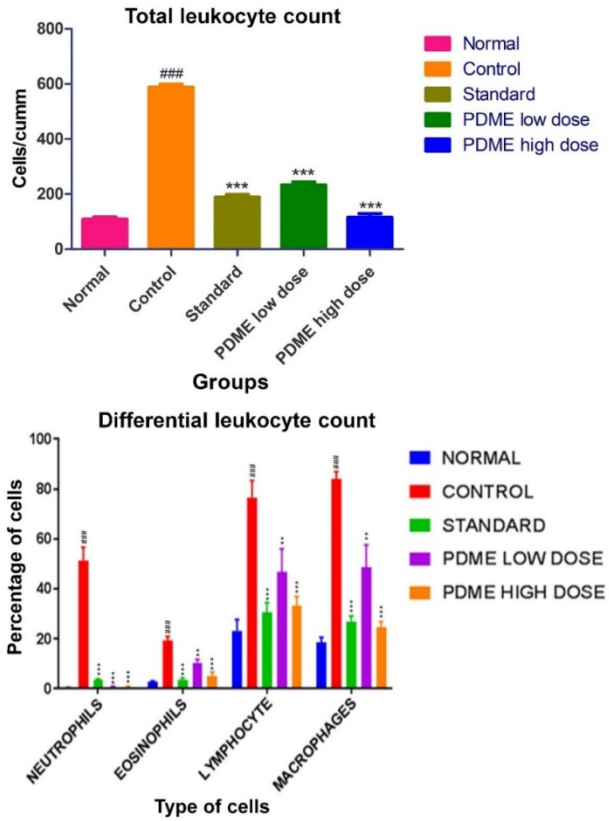

Figure 2. Total and differential leukocyte count obtained from the smear of Bronchoalveolar lavage fluid obtained from BALB/c mice. (Data is expressed as mean±SEM; n=6, One way ANOVA followed by Dunnett's test; Compared with normal: # p<0.05, ##p<0.01, ### p<0.001; Compared with control: * p<0.05, ** p<0.01, *** p<0.001).

Table 2. Effect of PDME and standard on LPO, GSH, CAT, and total protein of ovalbumin-induced asthma in BALB/c mice.

Groups	LPO (Mmol MDA / mg protein)	GSH (Mmol/mg tissue protein)	CAT (µmol of H_2O_2 consumed/ min/mg/protein)	Total protein (mg/100 mg of tissue)
Normal	7.79±0.049	9.817±0.0080	46.6±4.499	2.13± 0.39
Control	12.23±0.090###	0.7214±0.0031###	12.0±4.499##	8.52±0.54##
Standard	9.7±0.088*	9.721±0.010**	31.1±2.603***	3.32±0.38***
PDME Low dose	12.12±0.22**	7.089±0.003**	19.5±5.839*	8±0.57**
PDME High dose	9.81±0.014*	8.37±0.014*	33.2±2.971**	4.37±0.64***

Data is expressed as mean±SEM; n=6, One way ANOVA followed by Dunnett's test; Compared with normal: # p<0.05, ##p<0.01, ### p<0.001; Compared with control: * p<0.05, ** p<0.01, *** p<0.001.

Further control group showed significant (0.7214±0.0031 Mmol/mg tissue protein) ($p<0.05$) decrease in the level of glutathione which was reversed by the treatment with standard significantly (9.721±0.010 Mmol/mg tissue protein) and also PDME significantly ($p<0.05$) increased the levels of glutathione (low dose 7.089±0.003 Mmol/mg tissue protein, high dose 8.37±0.014 Mmol/mg tissue protein) (Table 2). The levels of catalase were significantly decreased in the control group (12.0±4.499) when compared with the normal group (46.6±4.499 µmol of H_2O_2 consumed/ min/mg/protein) which was significantly ($p<0.05$) elevated by the standard as well as treatment with PDME (Table 2). Moreover, elevated total protein levels in the positive control group (8.52±0.54 mg/100 mg of tissue) significantly reduced in the standard (3.32±0.38 mg/100mg of tissue) ($p<0.05$) and treatment with the plant extract low dose (8±0.57 mg/100 mg of tissue), high dose (4.37±0.64 mg/100 mg of tissue) groups. In contrast to the control group's pneumonic patch produced by mixed inflammatory cells and bronchoconstriction, the treatment group's lungs exhibited preserved normal bronchioles and alveoli structure with dilated blood vessels and very few chronic inflammatory cells (Figure 3).

In histamine-induced bronchospasm model, the PCD time of groups treated with the PDME as well as the standard has significantly increased when compared to the PCD recorded before treatment. It also offered a percentage increase in PCD time (Table 3).

Table 3. Effect of PDME and standard on pre-convulsive dyspnoea of histamine-induced bronchospasm in guinea pigs.

Groups	Pre-convulsive dyspnoea time		
	Before treatment (Seconds)	After treatment (Seconds)	Percentage increase (%)
Normal	No significant change	No significant change	No significant change
Control	82±0.577	89±1.155	7.86±0.553
Standard	29±1.856	86±2.082	66.27±1.369***
PDME low dose	57±1.817	79±5.63	33±0.750**
PDME high dose	39±3.441	106±6.05	63.20±0.386***

Data is expressed as mean±SEM; n=6, One way ANOVA followed by Dunnett's test; Compared with control: * p<0.05, ** p<0.01, *** p<0.001.

Normal rat peritoneal mast cells were oval and contained many fine granules surrounding a prominent nucleus (Figure 4).

Compound 48/80 treatment induced degranulation of RPMCs, exhibited enlargement of the cells, and ejected granules close to the cell surface (Figure 4). When compared with the control group pretreatment with standard ketotifen fumarate (10 µg/mL) and PDME (0.5, 1, and 2 mg/mL) indicated suppression of Compound 48/80-induced de-granulation ($p<0.05$) (Table 4). İt revealed that, PDME produced potential dose dependence activity.

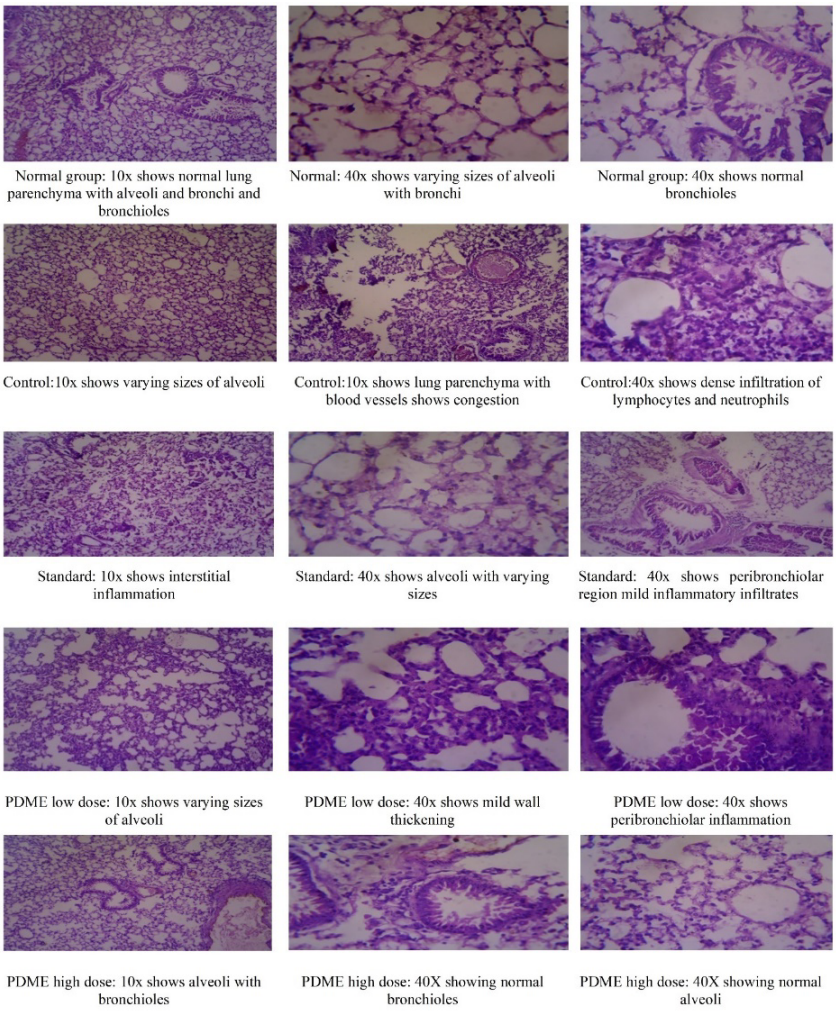

Figure 3. Histopathology of lungs of ovalbumin-induced asthma in BALB/c mice. (Specimens of lungs were stained with haematoxylin and eosin dye and magnified at 10X and 40X).

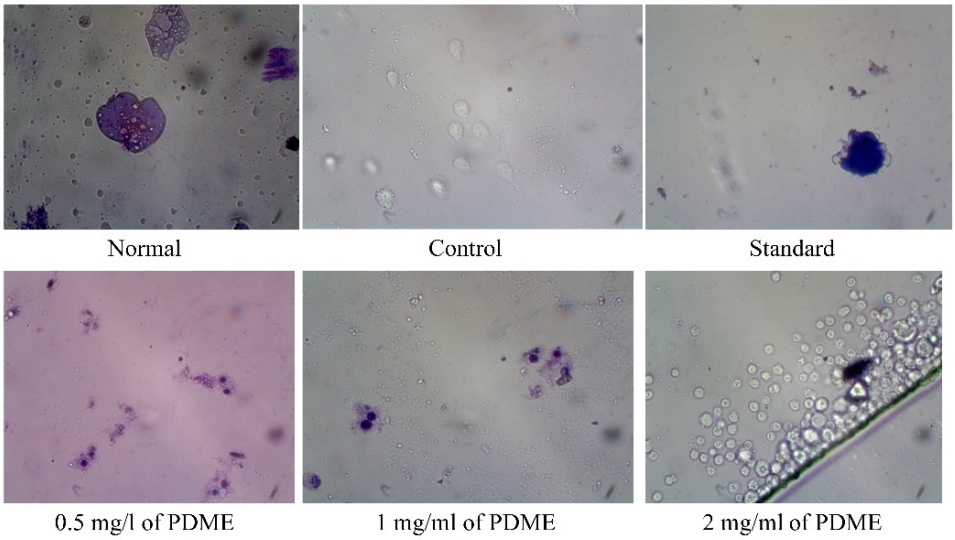

Figure 4. Compound 48/80 induced mast cell degranulation in rats. (The peritoneal fluid cells were stained with 0.1% toluidine blue solution made in distilled water and magnified at 450X)

Table 4. Effect of PDME and standard on Percentage inhibition of mast cell degranulation against Compound 48/80 induced mast cell degranulation in rats.

Groups	Percentage inhibition of mast cell degranulation
Normal	97±1.00
Control	43±0.5###
Standard	90.2±0.4***
PDME 0.5 mg/ml	66.66±0.83**
PDME 1 mg/ml	79±0.50**
PDME 2 mg/ml	86.95±0.225***

Data is expressed as mean±SEM; n=6, One way ANOVA followed by Dunnett's test, Compared with normal: # p<0.05, ##p<0.01, ### p<0.001; Compared with control: * p<0.05, ** p<0.01, *** p<0.001.

3. DISCUSSION

Flavonoids are proven to possess antioxidative properties, based on scavenging oxygen radicals and also by their hydrogen-donating capacity [16]. Flavonoids have immune-modulating, anti-inflammatory, antioxidant, and anti-allergic properties. Numerous investigations on flavonoids in asthmatic animals have demonstrated their advantageous properties [17]. It is also efficient in suppressing inflammatory mediators and inhibiting enzymes like lipoxygenase and peroxidase [18]. The anti-inflammatory and immunomodulating effects of flavonoids, which can be used to treat late-phase and late-late-phase bronchial asthma responses, are a result of all of the modes of action discussed [19, 20]. The preliminary phytochemical study and the total flavonoid content examination of PDME established flavonoid's existence and measured it.

The major causes of the phenols' antioxidant action are their redox, hydrogen-donor, and singlet oxygen quenching properties [21]. At ambient temperature, DPPH and ABTS are stable free radicals that can receive an electron or a hydrogen ion to form a stable diamagnetic molecule [22]. The quantity of accessible hydroxyl groups can be connected to the decline in the number of DPPH and ABTS molecules [23]. The inclusion of molecules with hydroxyl groups may contribute to the PDME extracts' antioxidant properties.

In the current investigation, PDME was able to decrease Fe3+ ions and, as seen, produce the Fe2+-TPTZ complex at higher concentrations. Hence, it may be said that the extract significantly exhibits antioxidant properties [24]. The bioactive chemicals contained in the PDME, notably the phenolics and flavonoids, may have contributed to the reduction of Mo (VI) to Mo (V) by transferring electrons or hydrogen ions.

The smear of the bronchoalveolar lavage (BAL) fluid showed different types of white blood cells. The total leukocyte count, eosinophil, macrophages, polymorphs, and lymphocyte count revealed a change after sensitization of BALB/c mice with ovalbumin. There was a significant increase in the total leukocyte, polymorph, eosinophil, macrophage, and lymphocyte counts in the sensitized animals. The treatment with PDME produced a significant decrease in the elevated total leukocyte, eosinophil, macrophage, and lymphocyte counts as compared to sensitized animals respectively. Particularly, the number of eosinophils, the major cells contributing to airway inflammation of allergic asthma, was significantly decreased with PDME supplement. Data suggested that PDME inhibited the infiltration of inflammatory cells further the ovalbumin administration altered the levels of antioxidant enzymes such as glutathione, and catalase with the elevation of lipid peroxidation and total protein. Treatment with the plant extract reduced the levels of LPO and total proteins and restored the altered levels of glutathione and catalase.

Mast cells can control innate immunity, host defense, and inflammation [25]. Compound 48/80 raises intracellular calcium levels, which causes granules to fuse with the plasma membrane [26]. A well-known histamine releaser is compound 48/80, therefore compound 48/80 is employed as a classic mast cell activator and induces about 90% release of histamine from the mast cells [27]. In the present study, PDME at 0.5 mg/mL, 1 mg/mL, and 2 mg/mL concentration inhibited the compound 48/80 induced mast cell degranulation.

Histamine-induced bronchoconstriction is the traditional immunological model of antigen induced airway obstruction. When histamine aerosol is breathed, it generates hypoxia [28], which results in convulsions in guinea pigs. As observed in control group, a major impact of histamine causes the guinea pigs to experience severe bronchoconstriction, which results in hypoxia and convulsions. Nevertheless, using PDME plus normal therapy enhanced PCT, indicating that the medication has potent bronchodilatory and anti-histaminic properties. Limitation of current study is only evaluated the anti-asthmatic activity using

different animal models. Relative gene expression, specific protein concentration studies are still needed to find out molecular mechanism.

4. CONCLUSION

In conclusion, our data suggest that the PDME possesses significant antioxidant and anti-asthmatic activity and has a beneficial effect on asthma by causing broncho relaxation, mast cell stabilization, and decreasing bronchial hyperreactivity.

5. MATERIALS AND METHODS

5.1. Collection and authentication of plant

The aerial parts of *Pergularia daemia* were collected from the surrounding areas of Rajapalayam, Tamil Nadu, India during the month of December and authenticated by the Botanical Survey of India (BSI) southern circle, Coimbatore, Tamil Nadu. The authentication certificate number is No. BSI/SRC/5/23/2017/Tech/1777. Soon after collection, the aerial parts were cleaned, dried in shade, and crushed to a coarse powder using mortar and pestle, stored in an airtight plastic container, until further use [29].

5.2. Extraction of plant material

Coarsely powdered aerial parts of *Pergularia daemia* were defatted by using petroleum ether (60-80 ℃) and then extracted with methanol using Soxhlet apparatus for about 72 h at 40 ℃ [30]. The extract was further concentrated under vacuum using a rotary vacuum evaporator (Buchi R-V120, Switzerland) at 40 ℃. The obtained crude extract was weighed and stored at 4 ℃ for further analysis [31].

5.3. Preliminary phytochemical analysis

The main phytochemicals found in the methanolic extract of *Pergularia daemia* (PDME) were identified qualitatively using preliminary phytochemical tests [32, 33].

5.4. Quantification of total phenolics and flavonoids

5.4.1. Estimation of total phenolics

The total phenolic content of PDME was determined by the Folin-Ciocalteau assay method [34]. To an aliquot 100 µL of extract (1 mg/mL) or standard solution of Gallic acid (10, 20, 40, 60, 80, 100 µg/mL) added 50 µL of Folin-Ciocalteau reagent followed by 860 µL of distilled water and the mixture was incubated for 5 min at room temperature. 100 µL of 20 % sodium carbonate and 890 µL of distilled water were added to make the final solution to 2 mL. It was incubated for 30 min in dark to complete the reaction. The absorbance of the mixture was measured at 765 nm against blank. Distilled water was used as a reagent blank. The tests were performed in triplicate to get mean the values. Gallic acid's calibration curve was used to determine the total phenolic content, which was calculated as milligrams of Gallic acid equivalents (GAE) per gram of dry extract [35, 36].

5.4.2. Estimation of total flavonoids

The aluminium chloride colorimetric technique was used to determine the total flavonoid content of PDME [37]. Separately, ethanol was added to an aliquot of 100 µL of the extract or standard solutions of quercetin (10, 20, 40, 60, 80, and 100 µg/mL). The resulting mixture was treated with 0.1 mL of 10 % aluminium chloride, 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water. Mixed and allowed to stay at room temperature for 30 min [38]. The absorbance was measured at 415 nm against the blank, where a solution of 2 mL ethanol, 0.1 mL potassium acetate, 2.8 mL distilled water, and 0.1 mL of aluminium chloride serve as the blank solution. The standard Quercetin calibration curve was used to calculate the total flavonoid content, which was then reported as milligrams of Quercetin equivalents (QE) per gram of dry extract [39, 40].

5.5. *In vitro* antioxidant study

5.5.1. DPPH free radical scavenging assay

The radical scavenging capacity of the extract on 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical was evaluated [41, 42]. One mL of an extract in methanol comprising 0.02-0.1 mg of the extract was mixed with one mL of a solution of 0.135 mM DPPH in methanol [43]. The mixture was incubated at room temperature

for 30 min in the dark. The absorbance of the mixture was measured spectrophotometrically at 517 nm. Quercetin was used as standard [44]. The percentage inhibition of DPPH radical scavenging activity was calculated by the following formula,

Percentage inhibition (%) = (Absorbance of Control-Absorbance of Sample)/(Absorbance of Control)×100

Different sample concentrations were used to obtain calibration curves and to calculate the IC50 values [45].

5.5.2. ABTS radical scavenging assay

To determine 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assay, the stock solutions included 7 mM ABTS solution and 2.4 mM potassium persulfate solution [46]. Both the reagents were mixed in equal quantities for preparing the working solution. Afterward, let it react for 12 hours in the dark and at room temperature. To further dilute the combination, 1.0 mL of ABTS solution was combined with 60 mL of methanol, resulting in an absorbance of 0.7060±001 units at 734 nm on a spectrophotometer. For each test, a brand-new ABTS solution was created. A 1000 µL of plant extracts and 1000 µL of the ABTS solution were allowed to react for 7 min before the absorbance was measured at 734 nm. The extract's capacity to scavenge ABTS was compared to quercetin, and the % inhibition was computed using the formula below [47].

Percentage inhibition (%) = (Absorbance of Control-Absorbance of Sample)/(Absorbance of Control) × 100

To create calibration curves and determine the IC_{50} values, several sample concentrations were used.

5.5.3. Ferric reducing antioxidant power assay (FRAP)

The typical solutions included 20 mM $FeCl_3$. 6 H_2O solution, 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) solution, and 300 mM acetate buffer pH 3.6. 2, 4, 6- Tri (2-pyridyl)-s-triazine, 2.5 ml of acetate buffer, and 2.5 mL of $FeCl_3$. 6 H_2O were combined to create the working solution [48]. Before usage, the solution's temperature was raised to 37 °C. About 30 min were given for the plant extract (100 µL) and 2.850 mL of the FRAP solution to react in the dark. At 593 nm, the yellow product's absorbance was calculated [49]. The standard curve was linear between 20 to 100 µg/mL of $FeSO_4$. Results are expressed in µg/ml of Fe (II) equivalent to quercetin [50].

5.5.4. Phosphomolybdate assay for total antioxidant activity

To get an extract concentration of 500 µg/mL, the extract was dissolved in methanol. The mixture was incubated at 95 °C for roughly 90 min in a water bath using 3 mL of extract, 0.3 mL of reagent solution (0.6 M Sulphuric Acid, 4 mM Ammonium Molybdate, and 28 mM Sodium Phosphate) in a test tube [51]. After the mixture had reached room temperature, a UV-visible spectrophotometer was used to measure each solution's absorbance at 695 nm in comparison to a blank [52]. The experiment was carried out three times, a calibration curve was drawn, and the total antioxidant activity of the extract (20-100 µg/mL) was expressed as vitamin C equivalents [47].

5.6. Evaluation of the antiasthmatic activity of *Pergularia daemia*

5.6.1. Animals and diet

The Wistar rats (170-200 g) BALB/c mice (25-30 g) guinea pigs (350-400 g) were, housed in the polypropylene cages at a temperature of 22±2 °C, with 12 h day-light cycle [53]. Animals were given a balanced diet and unlimited access to water over the course of the experiments [54]. Institutional Animal Ethics Committee gave its approval to the study's experimental protocol (Registration No. 685/PO/Re/S/2002/CPCSEA).

5.6.2. Ovalbumin-induced asthma in BALB/c mice

Mice were divided into different groups each consisting of six animals (Table 5). The toxicity study conducted by previous literature; it confirms the PDME was safer up to 2500 mg/kg. So that, doses for the animal experiment were selected as 250 mg/kg and 500 mg/kg [55].

Table 5. Experimental design of ovalbumin-induced asthma in BALB/c mice.

Groups	Sample size	Group specification	Intervention
Group I	6	Normal	1 % Tween 80

Group II	6	Control	20 µg of ovalbumin with 1mg $Al(OH)_3$, i.p
Group III	6	Standard	20 µg of ovalbumin + Dexamethasone (1 mg/kg, i.p.)
Group IV	6	PDME low dose	20 µg of ovalbumin + PDME 250 mg/kg p.o.
Group V	6	PDME high dose	20 µg of ovalbumin + PDME 500 mg/kg p.o

All animals (except group I) were sensitized by intraperitoneal injection of 20 µg of ovalbumin (Grade V Sigma) emulsified in 2 mg of aluminium hydroxide gel in 200 µL PBS buffer (pH7.4) on days 0, 7 and 14 [56, 57]. On days 21, 22, and 23 after initial sensitization, mice were challenged with ovalbumin (1 % w/v in PBS) for 20min using an ultrasonic nebulizer [58]. Animals in the nonsensitized group (group I) were exposed to aerosolized saline. Mice in the standard and plant extract groups were treated with dexamethasone and methanolic extract of *Pergularia daemia* (PDME) respectively once daily on days 18-23 [59, 60]. The extract was suspened in 1% Tween 80 for enhance the solubility of extract. Mice in the normal control were given 1% Tween 80 [61].

5.6.3. Collection of BALF (Bronchoalveolar lavage fluid)

On day 24, mice were given Pentobarbital (50 mg/kg, i.p.), and they were then killed. The trachea was cannulated and then BALF was obtained by washing the airway lumina [62]. To collect the BALF, ice-cold PBS (0.5 mL) was permeated into the lung and reserved over tracheal intubation 3 times (total volume 1.5 mL) [63].

5.6.4. Evaluation of inflammatory cells

After excluding dead cells with trypan blue staining, the overall number of inflammatory cells was measured by counting the cells in at least 5 squares of a hemocytometer [64]. A thin smear onto slides was obtained from centrifuging 100 µL of BALF using a Cytospin machine (200 g, 4 °C, 10 min) to estimate the differential cell counts [59]. The cells were secured and stained with DiffQuik staining solution (B4132-1A; IMEB Inc., Deerfield, IL) after the slides were dry [65].

5.6.5. Histological examination

The influence of the PDME on the immune-mediated chronic inflammation caused by sensitization with ovalbumin and histopathology studies of lung tissues taken from each rat was performed [66, 67].

5.6.6. Determination of antioxidant enzymes and lipid peroxidation

Determination of catalase (CAT)

The catalase activity was assayed by the method of Colorimetric assay of Lung homogenate (0.1 mL) taken, to which 1.0 mL of phosphate buffer and hydrogen peroxide were added [68]. After the application of 0.2 mL of dichromate acetic acid reagent, the reaction was stopped. Standard hydrogen peroxide in the series of 4 to 20 µL was taken and treated similarly. The tubes were heated in a boiling water bath for 10 min. The green color developed was read at 570 nm in a Double beam UV-VIS spectrophotometer (UV 1700, Shimadzu). The activity of Catalase was estimated and expressed in µmole of H_2O_2 consumed/min/mg protein [69].

Determination of reduced glutathione (GSH)

Reduced Glutathione was estimated by Ellman's procedure. To 500 µL of homogenate, 2 mL of 5 % Trichloro acetic acid was added and centrifuged at 3000 g for 10 min [70, 71]. To 250 µL of the above supernatant, 1.5 mL of 0.2 M phosphate buffer was added and mixed well. 250 µL of 0.6 mM Ellman's reagent (DTNB solution) was added to the aforementioned mixture, and within 10 min, the absorbance at 412 nm was recorded [72]. A standard graph was plotted using a standard glutathione-reduced solution (1000 µg/mL) and the GSH content present in the tissue homogenate was calculated by interpolation. Amount of glutathione expressed as mmol/mg tissue protein [73].

Determination of lipid peroxidation (LPO)

To 0.1 mL of sample, 2 mL of TBA-TCA-HCL reagent (ratio of 1:1:1) was added, mixed, and kept for 15 min in a water bath [74]. Afterward, the solution was cooled and the supernatant was removed and absorbance was measured at 535nm against a reference blank. The level of lipid peroxides was given as moles of MDA formed/mg protein [75, 76].

5.7. Determination of total proteins

0.1 mL of the lung homogenate, 0.9 mL of water, and 4.5 mL of alkaline copper sulphate reagent were added, then given a 10 min rest at the ambient temperature [77]. To this 0.5 mL of Folin's reagent was added. The blue color formed after 20 min was measured at 640 nm. The level of protein present was articulated as mg/g tissue or mg/dL [78, 79].

5.8. Compound 48/80 (n-methyl-p-methoxy phenethylamine with formaldehyde) induced Mast cell degranulation studies in rats

Normal saline containing five units/mL of Heparin was injected into the peritoneal cavity of male rats slightly anesthetized with Ketamine. The peritoneal liquid comprising mast cells was gathered after a gentle abdominal massage and then placed in a microcentrifuge tube under ice [80]. Four to five rats' peritoneal fluids were collected, and centrifuged for five min at 2000 rpm after being combined. The supernatant was rejected, and the cell pellet was collected, twice saline-washed, and then re-dissolved in 1 mL of saline [81].

Test tube no 1 : 0.1 mL peritoneal fluid (Normal)

Test tube no 2 : 0.1 mL of peritoneal fluid + Compound 48/80 (0.1 mL, 10 µg/mL) (Control)

Test tube no 3 : 0.1 mL of peritoneal fluid + Compound 48/80 + 10 µg/mL of Ketotifen fumarate (standard)

Test tube no 4 : 0.1 mL of peritoneal fluid + Compound 48/80 + PDME (0.5 mg/mL)

Test tube no 5 : 0.1 mL of peritoneal fluid + Compound 48/80 + PDME (1 mg/mL)

Test tube no 6 : 0.1 mL of peritoneal fluid + Compound 48/80 + PDME (2 mg/mL)

Compound 48/80 (0.1 mL, 10 g/mL) was then applied to all test tubes except test tube number 1 after each test tube had been incubated at 37 °C for 15 min. The cells were then stained with a 0.1 % toluidine blue solution produced in distilled water and observed under high-power objective fields (x450) after an additional 10 min of incubation at 37 °C. By measuring the number of degranulated mast cells from a total of at least 100 mast cells enumerated, the percentage protection of the mast cells in the control group and the treated groups were computed [82].

Percentage inhibition of mast cell degranulation = (1-number of degranulated mast cells/ Total number of mast cells) x 100

5.9. Histamine-induced bronchospasm in guinea pigs

Guinea pigs of either sex were used for the present study. Bronchospasm was induced in Guinea pigs by placing animals in a closed chamber and exposing them to an aerosol of 0.2 % histamine hydrochloride at a flow rate of 0.4 mL/min with the particle size of 5 µm under the average pressure of 450±50 mm Hg for 15 sec by an ultra-sound nebulizer [83, 84, 85]. The Pre convulsion Dyspnea time (PCD), i.e. It was noticed how long it took after exposure to aerosols for dyspnea to start and then turn into convulsions. Animals were taken out of the chamber and set outside to recover as soon as PCD started. For PCD, this time was used as day 0. Several groups of guinea pigs received the proper care [86, 87]. The grouping of animals are shown in Table 6.

Table 6. Experimental design of histamine-induced bronchospasm in guinea pigs.

Groups	Sample size	Group specification	Intervention
Group I	4	Normal	Only normal saline
Group II	4	Positive control	0.2 % histamine hydrochloride w/v (aerosol spray)
Group III	4	Standard	0.2 % histamine hydrochloride w/v (aerosol spray) + Chlorpheniramine maleate 2 mg/ kg, i.p.)
Group IV	4	PDME low dose	0.2 % histamine hydrochloride w/v (aerosol spray) + (PDME 250 mg/kg, p.o.)
Group V	4	PDME high dose	0.2 % histamine hydrochloride w/v (aerosol spray) + (PDME 500 mg/kg, p.o.)

The normal group was treated with saline, the standard group was treated with chlorpheniramine maleate (2 mg/kg) and for the control group, no treatment was given. Treatment groups were given PDME low dose (250 mg/kg) and high dose (500 mg/kg).

The effect of the drug was calculated by the following formula:

% increase in PCD time = [1-T1/T2] x 100

Where, T1 = time for PCD onset on day 0, T2= time for PCD onset on day 7

5.10. Statistical analysis

All the values were stated as mean±SEM of six observations. One-way ANOVA was used for the statistical analysis, then Dunnett's multiple comparison tests was used. The value of p less than 5 % ($p < 0.05$) was considered statistically significant.

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