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Azadirachta indica attenuates airway inflammation and oxidative stress in the asthmatic mice

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Abstract
Azadirachta indica Linn (*A. indica*), is a marvelous traditional Indian medicine with a key therapeutics role in health management. Studies have revealed the anti-inflammatory, immunomodulatory and antioxidative role of *A. indica*. Inflammatory immune cells generate high level of ROS for stimulated severe AHR and airway inflammation. Therefore, the present study is designed to investigate the ameliorating potentiality of the leaves extract of *A. indica* in the OVA-induced lung injury, airway inflammation and oxidative stress. BALB/c mice were sensitized with ovalbumin (OVA) to induce asthma symptoms and prednisolone was used as a standard drug for treatment. The animals were divided into six groups: Group I: Untreated control mice, Group II: Ova-induced asthmatic mice, Group III: Prednisolone treated ova-induced asthmatic mice, Group IV: Vehicle treated ova-induced asthmatic mice, Group V: *A. indica* (100mg/kgBW/day) treated ova-induced asthmatic mice, Group VI: *A. indica* (200mg/kgBW/day) treated ova-induced asthmatic mice. Mice were sacrificed by cervical dislocation 24 hrs after the last challenge. Further, broncheoalveolar lavage fluid (BALF), serum and lungs were collected to study the cell count, activity of EPO, ROS generation, antioxidative enzyme activities, and histology of lungs. The results expressed bronchoconstriction in the lungs of asthmatic group marked by elevated total and differential cell count. Further, the antioxidant enzyme activities were also altered with enhanced ROS production. The alterations observed in the asthmatic mice were significantly ameliorated following treatment with *A. indica* leaf extract as compared to the standard drug, prednisolone treated mice and control group. The findings suggest therapeutic potentiality of *A. indica* for ameliorating asthma symptoms in the future.

Keywords: *A. indica*, asthma, leaf extract, lung inflammation, oxidative stress

Introduction
Asthma is a common clinical airway inflammatory disease triggered by antigens [1], and characterized by reversible airflow obstruction, high airway reactivity, increased secretion of airway mucus and immunoglobulin E, and eosinophil infiltration [2-3]. It is mediated by T helper (Th) 2 cells and involves multiple inflammatory cells and molecules [4].
Numerous studies have shown that oxidative stress plays an important role in the pathogenesis of asthma [5-7]. When airway inflammation occurs, inflammatory mediators such as histamine and leukotriene are released. These mediators recruit and activate a variety of inflammatory cells, including eosinophils, neutrophils, lymphocytes, macrophages and platelets [8]. The activation of inflammatory cells leads to the release of various free radicals and oxidative stress. Free radicals include reactive oxygen species (ROS) and reactive nitrogen species. These free radicals can cause serious damage or apoptosis of airway epithelial cells [9-10].
However, such pharmacological interventions in the lungs may be ameliorated through medicinal plants due to their minimum or no side effects on the body or body parts. In this regard, *A. indica* is one of the plants, widely used in the traditional Indian medicine, for its pharmacological properties. *A. indica* and its ingredients have several therapeutic implications such as antifungal, antiviral, antibacterial, anticancer, antidiabetic etc. properties [11]. Earlier studies have revealed the anti-inflammatory effect of *A. indica* leaf extract [12] suggesting nimbidin, one of its ingredients, to suppresses the functions of macrophages and neutrophils relevant to inflammation [13]. Further, the antioxidative property of the leaf, root, bark and flower extract of *A. indica* has also been well established [14-15].
Despite the several studies exploring the pharmacological properties of *A. indica*, a study revealing its anti-asthmatic role is still lacking. In order to test the hypothesis that *A. indica* may exert a protective effect against asthma, the present study was designed in the mouse

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model with the objective to investigate the ameliorating potentiality of the leaves extract of *A. indica* in the OVA-induced lung injury, airway inflammation and oxidative stress.

Materials and Methods
Experimental animals: BALB/c mice were procured from Central Drug Research Institute, Lucknow, India. They were maintained in a clean animal house under standard conditions of temperature ($24 \pm 2^\circ$ C), light/dark cycle and relative humidity (60-70%) in polypropylene cages, with rice husk as the bedding material. Animals were maintained on pelleted food and water *ad libitum*.

Development of experimental mouse model of asthma: For developing allergic asthma model, mice were immunized with 0.1 ml saline suspension containing 50 µg Ovalbumin (OVA, Grade V, Sigma) emulsified in 4 mg aluminium hydroxide (Reagent Grade, Sigma) on days 0, 7 and 14. From days 19 to 33, mice were subjected to 1% OVA (prepared in normal saline) inhalation 30 minutes daily in a plexiglass chamber by

nebulizer (Omron) with an airflow rate 9 l/min. Control mice received i.p. injections of 0.2 ml saline containing 4 mg alum & ovalbumin and were challenged with saline alone.

Preparation of the herbal extract of *A. indica* (leaves): Fresh young neem leaves were used for the herbal preparation. Herbal extract was prepared with the help of soxhelet apparatus from young leaves. Young leaves were collected from the plant and washed under running water. The washed leaves were free from any visible contamination. These were further dried in incubator at 37^0C for the period of 15 days, and then leaves were crushed in grinder to powdered form which was soaked in distilled water for the extraction in soxhelet.

Experimental protocol: Mice were classified into six groups (n=5): Group I (normal), Group II (Asthmatic), Group III (Prednisolone-treated, 1mg/kg/bw, i.p.), Group IV, (Vehicle-treated: distilled water), Group V (*A. indica*-treated, 100 mg/kgbw), Group VI (*A. indica*-treated, 200 mg/kgbw).

S. No.	Group	Inducer	Treatment	Challenge
1.	Normal	Ovalbumin + Alum	------------------------	Saline
2.	Asthmatic	Ovalbumin + Alum	------------------------	1%OVA aerosol
3.	Standard drug (SD)	Ovalbumin + Alum	Prednisolone	1%OVA aerosol
4.	Vehicle	Ovalbumin + Alum	DW	1%OVA aerosol
5.	Treatment	Ovalbumin + Alum	*A. indica* (100mg/kgbw)	1% OVA aerosol
6.	Treatment	Ovalbumin + Alum	*A. indica* (200mg/kgbw)	1% OVA aerosol

Treatment with the herbal extract and standard drug: Prednisolone, used as a standard drug, was administered intraperitoneally with the dose of 1 mg/kgBW, one hour before the challenge. Likewise, two doses (100 mg/kgBW and 200 mg/kgBW) of the leaf extract of *A. india* were administered intraperitoneally one hour before the challenge. Mice were sacrificed 24 hours after the last challenge day.

Sampling: After the last OVA aerosol, mice were sacrificed by cervical dislocation. Further, broncheoalveolar lavage fluid (BALF), serum and lungs were collected for the study. BALF was collected by tracheal cannulation and obtained by washing of the airway lumen with chilled PBS. Briefly, the lungs were washed three times with 1ml of ice-cold PBS and

further centrifuged at 3000 rpm for 10 min at 4°C. Cell pellet was used to analyze inflammation by total inflammatory cell count, differential cell count (eosinophils, neutrophils and lymphocytes) and reactive oxygen species (ROS). Supernatant was stored at -80^{0}C and was used to analyze the release of inflammatory mediators as activity of inflammatory enzymes such as Eosinophil Peroxidase (EPO), Myeloperoxidase (MPO), Reactive Oxygen Species (ROS) and Nitric oxide (NO). Blood was collected by retro orbital bleeding from each mouse and centrifuged at 4500 rpm for 30 min for the detection of IgE antibody in the serum. Lung tissue was dissected out for histology to study the architectural and pathological changes occurring by H&E staining as well as to study antioxidant enzymes activities.
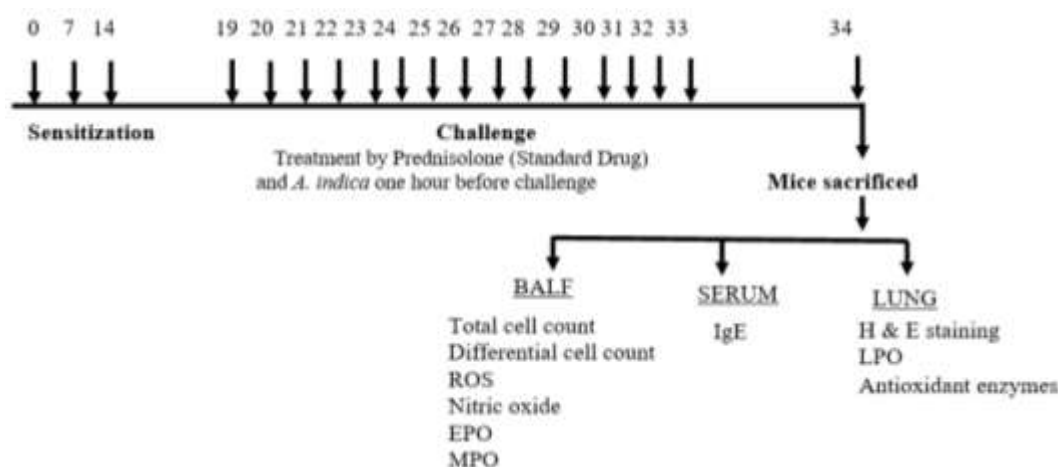
Total Cell count: Inflammation in the lungs has been studied by enumerating total cell by Trypan blue dye exclusion test in the pellet of the BALF. BALF pellet has been stained by trypan blue and cells have been counted on haemocytometer.

Differential cell count: For differential cell count the BALF pellet has been cytospun on slides and further stained with Giemsa stain. Further the differential cell type (neutrophil,

eosinophil, lymphocytes) has been confirmed on the basis of the nucleus morphology.

Lung histology: Lungs were inflated with 10% neutral buffered formalin and then removed aseptically for 24 hours. After dehydration, lung tissue were embedded in paraffin, then cut into 5 μm thickness sections and processed further for stain with haematoxylin-Eosin (H&E) to examine the general morphology and inflammation in the lungs.

Biochemical analysis
(a) In Balf
Reactive oxygen species: Intracellular ROS were measured in BALF cells by the protocol of Alaimo and co-authors [16] and was measured using DCF-DA (Sigma) as an indicator of superoxide production in OVA induced lung damage. BALF cells were washed with chilled PBS. iROS were labeled by incubating cells (1×10^5) in 100 μl of 20 mM dichlorofluorescein diacetate (DCFDA) for 45 min at 37°C temp in the dark. After incubation, fluorescence intensity was monitored by using fluorescence spectrometer (λex: 485 nm; λem: 535 nm). Values were normalized by the fluorescence intensity of the normal cells.

Nitric oxide: Nitric oxide was determined as per the protocol of Miranda *et al.* (2001) [17]. Nitric Oxide was measured by Griess reagent in BALF. Briefly 100 μl of sample was mixed with 100 μl of 8mg/ml of Vanadium chloride for the release of nitric oxide. This is rapidly followed by the addition of Griess reagent which includes 50 μl of sulphanamide (2% in Distilled water) and 50 μl of NED (0.1% in 5% HCl). Plate was incubated for 45 to 60 minutes till pink color is developed. O.D was measured at 540 nm. The content of released nitric oxide was expressed in μg/ml.

Activity of EPO: Level of EPO was measured in BALF supernatant by the protocol of Strath *et al.* (1985) [18]. 100 μl of substrate solution (0.1 mM O- phenylene-diamine dihydrochloride, 0.1% Triton-X 100, 1mM hydrogen peroxide in 0.05 M Tris HCl) was added to 100 μl of supernatant in microtiter plates and incubated for 30 min at 37ºC. The reaction was stopped by adding 50μl of 4M sulphuric acid and the optical density was read at 490 nm. The results were expressed in Optical Density (O.D) values.

Activity of MPO:
Myeloperoxidase (MPO) activity was measured as given by Bradley *et al.* (1982) [19] with slight modifications. The supernatant along with the pellet have undergone freeze and thaw cycle three times then centrifuged (12000 rpm for 15 min). MPO assay was performed in 300 μl of total volume in 96 well microplate. In brief 20μl of the supernatant was mixed with 280μl reaction mixture containing 0.167 mg/ml o-dianisidine dihydrochloride and 0.002% hydrogen peroxide in 50mM phosphate buffer. The absorbance was measured at 460nm for 20 min in a microplate reader. MPO activity was expressed as unit/mg of tissue.

In Serum
IgE estimation: OVA specific IgE was detected in the blood serum by direct Enzyme linked Immunosorbet Assay [20]. For serum preparation blood was centrifuged at 3500rpm for 30 min at 4⁰C. Microtitre plates were coated with 20 μg of OVA in 100μl coating buffer (100mM sodium carbonate and bicarbonate buffer, 9.6 pH) at 37⁰C for 2 hrs and then

incubated at 4⁰C for overnight. The wells were washed three times with PBS and then blocked with blocking buffer (1% BSA) dissolved in PBS for 2 hrs at 37⁰C. After washing three times with TBST (0.05% Tween 20 in Tris buffer) 100μl of serum (1:9 in blocking buffer) was added to the wells and kept for 2 hrs at 37⁰C. The wells were washed again three times with TBST and 100 μl of horseradish peroxidase labelled anti-IgE antibody (1:1000) was added and kept for 37⁰C for 2 hours. After washing five times with TBST, 100μl of substrate TMB (3.3'.5.5' tetramethylbenzedine) was added to the well. The reaction was stopped by adding 50 μl of 2.4M sulphuric acid (H_2SO_4) to each well. Plates were read by ELISA plate reader (Biotech, USA) at 450nm. Values are presented in term of OD (optical density).

(b) In Lungs
Antioxidant enzyme activities: The lungs were homogenized in phosphate buffer according to the method suggested by Vaithinathan *et al.* (2008) [21]. The lungs from each animal of all the groups were carefully excised, washed in ice-cold physiological saline solution, blotted dry on filter paper and weighed. 10% homogenate of the lungs were prepared in ice-cold phosphate buffer (0.05M, pH 7.0). The homogenate was centrifuged at 10,000 rpm for 20 minutes at 4⁰C. The supernatant was used for the enzyme assays and estimation of the protein. Different aliquots of the supernatant were prepared and stored in deep freezer at -80⁰C for the estimation of the enzyme activity.

Protein estimation: The protein content was determined by the method of Lowry *et al.* (1951) [22] using bovine serum albumin as a standard. Briefly 5.0 μl of supernatant of homogenized lungs was added in 995 μl of distilled water in the test tubes. For blank 1.0 ml of distilled water was taken in a test tube. 5.0 ml of reagent C (0.2 g of sodium hydroxide and 1.6 g of sodium carbonate in 50 ml of distilled water, and 0.013 g and 0.05 g of copper sulphate of sodium potassium tartarate in 1.0 ml of distilled) was added to all the tubes including the blank. All the tubes were vortexed. After 30 minutes 0.5 ml of 1N folin reagent (diluted with distilled water) was added in all the tubes and then vortexed. After 15 minutes O.D. of all the tubes was measured spectrophotometrically at 750 nm against the blank. The concentration of the sample was determined using standard curve of BSA (standard protein).

Estimation of Lipid Peroxidation (LPO): The level of LPO was measured in the tissue supernatant using MDA concentration as a surrogate measure [23]. The reaction mixture was formed by adding 100 μl supernatant, 1400 μl of 15% TCA containing TBA (0.375%) and 14 μl of BHT (20 mg/ml absolute alcohol). Samples were heated at 100⁰C for 15 minutes in water bath and the absorbance was measured at 532 nm against blank containing all the reagents except the test sample. The values were expressed as nanomoles MDA produced per milligram protein.

Estimation of Superoxide Dismutase (SOD): Superoxide dismutase (EC 1.15.1.1) was assayed by the method of Marklund and Marklund (1974) [24]. The assay mixture was prepared by adding 300 μl pyrogallol (0.2mM) and 100 μl enzyme source to 2.4 ml of Tris-HCl (50mM) containing 1 mM EDTA (pH 7.6). The increase in absorbance was measured immediately at 420 nm, against a blank containing all components except the enzyme source and pyrogallol, at

the interval of 10 seconds for 3 minutes on the spectrophotometer. The enzyme activity was expressed as units per milligram protein.

Estimation of Catalase (CAT): Catalase (EC 1.11.1.6) was assayed by the method of Claiborne (1985) [25]. The assay mixture contained 2.4 ml of phosphate buffer (50mM, pH 7.0), 10 µl of H_2O_2 (19mM) and 50 µl enzyme source. The decrease in absorbance was measured immediately at 240 nm, against a blank containing all the components except the enzyme source, at the interval of 10 seconds for 3 minutes on a spectrophotometer. The enzyme activity was expressed as micromoles of H_2O_2 consumed per min per milligram protein.

Estimation of Glutathione Peroxidase (GPx): Glutathione peroxidase was assayed by the method of Flohe and Gunzler (1984) [26]. GPx assay mixture consisted of potassium phosphate buffer (0.1M, pH 7.0), 2.25mM NADPH in 0.1% $NaHCO_3$, GR (7.1µl/ml), GSH (11.52mg/ml) and 1.5mM of H_2O_2. Enzyme assay was carried out by pipetting out 750 µl of potassium phosphate buffer, 60 µl of NADPH, 15 µl of GR and 25 µl of reduced glutathione in 1.0 ml cuvette. Enzymatic reaction was started by adding 50 µl of sample (supernatant) and 100 µl of H_2O_2. The extinction coefficient of the sample was registered at 340 nm every minute for a period of 120 seconds in the spectrophotometer. The GPx activity was expressed in units per milligram of protein. One unit of enzyme activity was reported as one µmole of NADPH oxidized per minute assuming 6.22×10^3 to be the molar absorbance of NADPH at 340 nm.

Estimation of Glutathione Reductase (GR): Glutathione reductase was assayed by the method of Carlberg and Mannervick (1955) [27]. GR assay mixture consisted of potassium phosphate buffer (0.2M, pH 7.0), 2mM NADPH in 0.1% $NaHCO_3$ and GSSG (20mM). Enzyme assay was carried out by pipetting 600 µl of potassium phosphate buffer, 250 µl triple distilled water, 50 µl GSSG, 50 µl NADPH and 50 µl of sample (supernatant) in 1.0 ml cuvette. The extinction of the sample was recorded at 340 nm every minute for a period of 120 seconds in the spectrophotometer. The GR activity was expressed in units per milligram protein. One unit of enzyme activity was reported as one µmole of NADPH oxidized per minute assuming 6.22×10^{-3} to be the molar absorbance of NADPH at 340 nm.

Statistical Analysis: Results are expressed as mean ± SEM and analyzed by one-way ANOVA followed by post hoc test. Statistical significance at $p<0.05$ were considered significant.

Results
In BALF
Total cell count: Total cell count was performed as a marker of inflammatory cells recruited to the lungs. Total cell count was performed by dissolving the BALF pellet in 100µl of PBS which was further diluted with Trypan blue dye and cell were counted in haemocytometer as a dye exclusion test. Total cell count was higher in the asthmatic mice as compared to the normal mice which marks inflammation in the lungs. The total cell recruited in the lungs was further, declined as a result of prednisolone treatment, used as a standard drug, and *A. indica* treatments, thus, indicating an anti-inflammatory property of the drugs (Graph A).

Differential cell count: Differential cell count was performed by Giemsa staining and the cells were identified on the basis of nucleus morphology. Mainly neutrophils, eosinophils and lymphocytes were identified. The number of eosinophils which was low in normal mice. However, it was elevated in asthmatic mice. Even the number of neutrophils was elevated in asthmatic mice as compared to low number in normal mice. Similarly, lymphocytes were also elevated in asthmatic mice but the number of lymphocytes was less as compared to neutrophils. This suggests that the inflammation in asthmatic mice was mainly due to the large number of neutrophils cells. Eosinophils, neutrophils and lymphocytes all were suppressed in prednisolone and *A. indica* treated mice which suggest the protective anti-inflammatory role of these treatments (Graph B).

Reactive oxygen species: Reactive oxygen species (ROS) including superoxide anion and hydrogen peroxide (H_2O_2), byproducts of aerobic respiration leads to oxidative stress damaging DNA, protein, and lipid. ROS production was enhanced in asthmatic mice which were controlled by the treatment of prednisolone. *A. indica* also suppressed the production of ROS in dose dependent manner (Graph J).

Nitric oxide: The content of nitric oxide has been evaluated as a marker of oxidative stress. Nitric oxide concentration was higher in the asthmatic mice as compared to the normal mice which marks the increased stress in asthmatic mice. However, the treatments of prednisolone and *A. indica* (200mg/kgBW) declined the increased nitric oxide concentration, indicating the anti-stress activity of the treatments (Graph C).

EPO activity: EPO, a marker of eosinophils recruitment and activation, activity was higher in the asthmatic mice as compared to the normal mice. Treatment with prednisolone inhibited the activity of EPO. Treatment with *A. indica* was also found to be effective in suppressing the activity of EPO. The higher dose 200mg/kgbw was more effective. This result also supports the result of differential cell count where the no. of eosinophils were found to be more in asthmatic mice and the number was suppressed with the treatments (Graph I).

MPO activity: MPO activity, a marker of neutrophils infiltration into tissue, was higher in the asthmatic mice as compared to the normal mice. Treatment with prednisolone inhibited the activity of MPO. Treatment with *A. indica* was also effective in suppressing the activity of MPO with the higher dose 200mg/kgbw exhibiting more efficacy. This result also supports the result of differential cell count where the no. of neutrophils were more in the asthmatic mice and its suppression following treatments (Graph K).

In Serum
IgE level: IgE level was higher in the asthmatic mice as compared to the normal mice which was recovered following treatment with the standard drug, prednisolone. Treatment with *A. indica* also effectively ameliorated the level of IgE, however, the higher dose 200mg/kgbw was more potent. (Graph L).

In Lungs
Histopathological study: The architectural changes were observed in H&E stained slides. The alveolar spaces were observed for inflammation. The stained sections of the lungs indicates no inflammation neither bronchoconstriction in the

normal lung section (A) while asthmatic mice showed bronchoconstriction and inflammation in the alveolar space of lungs (B) similar to the vehicle group (D). Prednisolone treated mice (C) and *A. indica* treated mice (E) showed no bronchoconstriction neither any inflammation in the alveolar space when compared with asthamatic mice which suggest its anti inflammatory role.
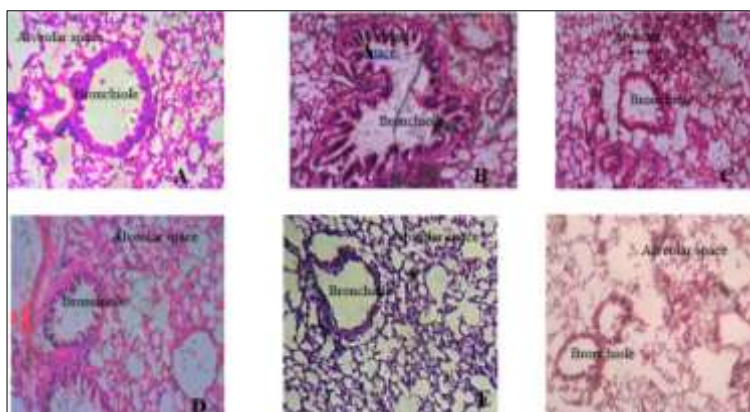
Fig 1: Lung sections of (A) normal mice showing no bronchoconstriction, (B) asthmatic mice showing bronchoconstriction and inflammation in the alveolar space, (C) prednisolone (SD)-treated mice showing no bronchoconstriction, (D) vehicle-treated mice showing bronchoconstriction bronchoconstriction and inflammation in the alveolar space, (E) *A. indica* (AI, 100mg/kgBW)-treated mice showing reduced bronchoconstriction, and (F) *A. indica* (AI, 200mg/kgBW)-treated mice showing no bronchoconstriction.

Lipid peroxidation (LPO) level: The level of LPO product is widely used as an index of oxidative stress. LPO level, as indicated by the concentration of MDA, was significantly higher in asthmatic group as compared to the normal mice. Treatment of prednisolone and *A. indica* significantly declined the increased LPO level as compared to the asthmatic group. This marks the ameliorating potentiality of *A. indica* against increased LPO level in asthmatic mice (Graph D).

Superoxide dismutase (SOD) activity: SOD, a superoxide free radical scavenging enzyme, is considered as the first line of defense against the deleterious effect of oxygen radicals in the cells which scavenges reactive oxygen radical species by catalyzing the dismutation of O^{2-} radical to H_2O_2 and O_2. The activity of this enzyme was significantly declined in the asthmatic group as compared to the normal mice. However, treatment of prednisolone and *A. indica* significantly increased the declined activity as compared to the asthmatic group. This marks the ameliorating potentiality of *A. indica* against decreased activity of SOD in asthmatic mice, attributing its antioxidative property (Graph E).

Catalase (CAT) activity: CAT is as an antioxidant enzyme, which removes H_2O_2 generated by SOD, to extremely reactive molecule such as OH, thereby converting it into H_2O and O_2. The activity of this enzyme was declined in the asthmatic group as compared to the normal mice. However, treatment of prednisolone and *A. indica* increased the declined activity as compared to the asthmatic group. This marks the ameliorating potentiality of *A. indica* against decreased activity of CAT in asthmatic mice, attributing its antioxidative property (Graph F).

Glutathione peroxidase (GPx) activity: GPx is considered to be the major enzyme that removes peroxides and, hence, protects the cell against damage, caused by free radicals and product of lipid peroxidation. The activity of this enzyme was declined significantly in the asthmatic group as compared to the normal mice. However, treatment of prednisolone and *A. indica* significantly increased the declined activity as compared to the asthmatic group. This marks the protective approach of the treatments against increased free radicals in the asthmatic mice (Graph G).

Glutathione reductase (GR) activity: GR is an antioxidative enzyme, which catalyzes the reduction of oxidized glutathione (GSSG) to GSH and is essential for the glutathione redox cycle maintaining an adequate level of cellular GSH. The activity of this enzyme was declined significantly in the asthmatic group as compared to the normal mice. However, treatment of prednisolone and *A. indica* (200mg/kgBW) significantly increased the declined activity as compared to the asthmatic group. This marks the protective approach of *A. indica* against imbalanced glutathione redox cycle in asthmatic mice, thus, attributing its antioxidative property (Graph H).
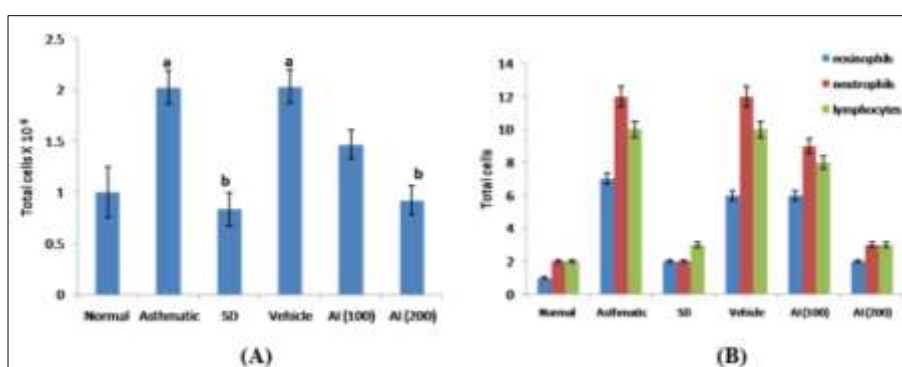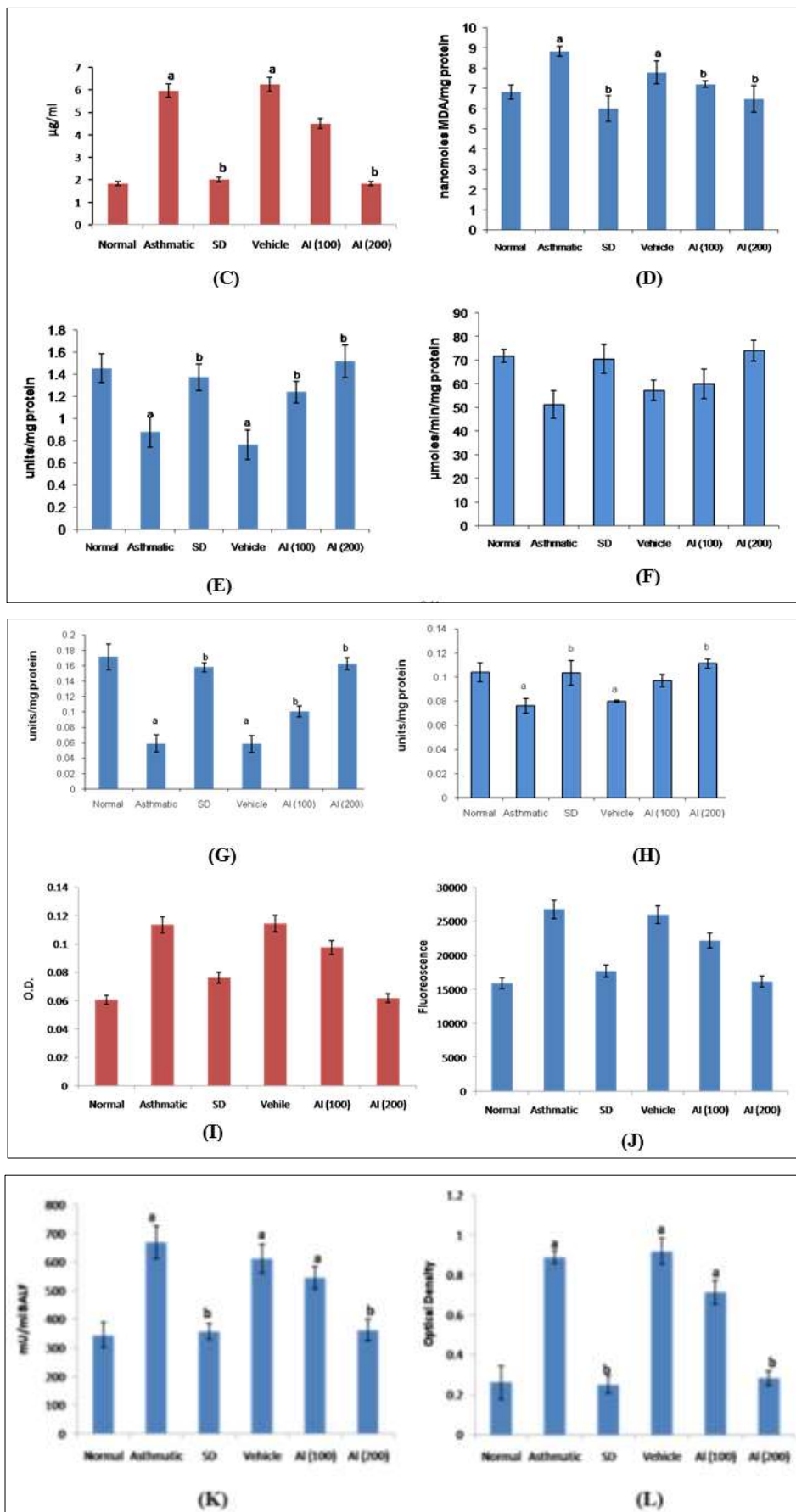
Values are considered significant by ANOVA followed by Newman- Keul's multiple range test at
$p < 0.05$. a: Significantly different from normal b: Significantly different from asthmatic group

Graphs (A-L): Effects on the (A) Total cell count, (B) Differential cell count, (C) Nitric oxide content, (D) Lipid peroxidation level, (E) Activity of superoxide dismutase, (F) Activity of catalase, (G) Activity of glutathione peroxidase and, (H) Activity of glutathione reductase (I) EPO (J) Reactive oxygen species (K) MPO, and (L) IgE levels in asthmatic mouse treated with the leaf extract of *A. indica*, (AI) and its comparision with the standard drug (SD), prednisolone. (Values are mean ± SE of five animals).

Discussion

Chronic airway inflammatory diseases are characterized by airflow obstruction and bronchial hyper responsiveness. Such respiratory diseases are global with over 200 million people suffering worldwide. However, herbal remedies over the chemical drugs are the first and safer choice of people nowadays, due to their minimum or no side effects. In the present study an attempt has been made to explore the potentiality of *A. indica* and find out its therapeutic implications against OVA-induced mouse model of asthma. The impact of *A. indica* in lung disease has not been studied extensively inspite its excess availability to the general public. Inflammation and oxidative stress being cross linked plays a pivot role in the pathophysiology of chronic asthma and is directly responsible for the severity of disease. The present study shows inflammation in lungs including alveolar and peribronchiolar spaces in asthmatic mice as a consequence of recruitment of inflammatory cells as eosinophils and neutrophils. Further, the increased eosinophils and neutrophils recruited to the lungs were confirmed by increased activity of EPO and MPO as these are considered as biochemical markers of airway inflammation representing the recruitment and activation of eosinophil and neutrophil respectively. Raisan and Sadeq [28] have established the importance and correlation of EPO and MPO with asthma pathogenesis. However, administration of *A. indica* reduced the recruited eosinophils and neutrophils in dose dependent manner and hence, EPO and MPO activities were also inhibited. Anti-inflammatory effects of *A. indica* leaf extract are also earlier reported suggesting nimbidin, one of its constituent, suppressing the functions of macrophages and neutrophils [13]. Immunomodulatory and the anti-inflammatory effects of the bark and leave extracts as well as oil seeds are also reported by Arora and co-authors [29] and Biswas and co-authors [30].

Inflammation is also noticed in the lung sections were alveolar space and peribronchiolar region were inflamed with number of cells. These bronchoconstriction's and structural changes as noticed in the histopathological study were reversed with the treatment of *A. indica.* Apart of this, increased level of IgE has also been detected in serum of asthmatic mice. It is reported that the increased level of IgE is positively correlated with airway inflammation and severity of asthma [31]. It is also well known that IgE-driven amplification of the mast cell population and activation state might provide an increased tissue reservoir of both mediators of immediate hypersensitivity and of the cytokines and chemokine's capable of initiating the recruitment of inflammatory cells in chronic pathogenesis. Thus, in the present study the recruitment of inflammatory cells may be attributed to the increased IgE level. Further, the inflammatory cells which are recruited to the airways in asthma are reported to be the major source of reactive oxygen and nitrogen species which have an important role in the pathogenesis of asthma [32-34]. Activated macrophages, neutrophils and lymphocytes are reported to release ROS [35] and asthma is closely related with increased ROS production in the airways [36-38]. Therefore, increased ROS and NO level observed in the asthmatic group may be attributed to the elevated neutrophils and eosinophils level as observed in the study. EPO and MPO react with H_2O_2 generated during respiratory burst and form hypochlorous acid (HOCl) and similar other compounds which can injure the surrounding tissue during inflammatory process [39]. The increased activity of EPO and MPO in the asthmatic group may act as a powerful oxidant and damage the lungs if they are produced

as a part of the inflammatory response in asthma [40]. Further, MDA, the end product of lipid peroxidation, is estimated as an indicator of membrane lipid damage and its measurement provides the estimate of free radical activity [41]. Hence, increased LPO level of asthmatic group supports the increased ROS level as noticed in the study.

A prominent role of oxidative stress and decreased antioxidant defence in asthma pathophysiology is evident from several studies [42]. SOD is the key enzyme of the antioxidant defense system of the body, which plays an important role in scavenging superoxide radicals during oxidative stress. The inactivation of this enzyme serves as a sensitive and quantitative functional measure of global oxidative stress in asthma [43]. CAT catalyzes the decomposition of H2O2, produced by the action of SOD, to water, thus, protecting the cell from deleterious effects of H2O2. Under prolonged oxidative stress, NADPH binds to this enzyme and stabilizes its structure and protects catalase from inactivation. This leads to the decrease in catalase activity [44]. Reports suggest that lower catalase activity is due to nitration and oxidation, which identifies oxidative inactivation as the mechanism of activity loss [45]. GPx catalyzes the reduction of H2O2 and lipidhydroperoxides by glutathione and is, therefore, essential for maintaining GSH/GSSG redox balance. Its decrease activity is well reported in the platelets of aspirin induced asthma [46-47] and in asthmatic individuals [48-49]. Glutathione reductase is a flavoprotein that catalyzes the reduction of GSSG to GSH. Few studies have been assessed with glutathione reductase activity in asthma. Some reports suggests decreased glutathione reductase activity in erythrocytes from asymptomatic asthmatics [50-51]. Therefore, from the above based evidences and reports, it can be concluded that deficiencies in the antioxidant defenses ie., SOD, CAT, GPX and GR, as noticed in the study, have lead to an imbalance which may be associated for exacerbation of chronic and lung tissue damage, as supported by the histological analysis evidenced in the lung sections.

Till date chronic disease like asthma is not completely cured, but can be controlled through anti-inflammatory therapies. For anti-inflammatory therapy, corticosteroid remains the mainstay for treatment which is emphasized in all guidelines[52-53] but use of corticosteroid has certain limitations and side effects. Corticosteroids exert direct effects on oxidative stress by decreasing the number and/or activity of cells involved in ROS [54]. Therefore, they have a beneficial effect on antioxidants [55]. One of the corticosteroid, prednisolone, effectively reduces the symptoms of asthma [56], however, its antioxidative role in the chronic diseases is still not elucidated. In the present study, treatment with prednisolone effectively inhibited OVA-induced lung tissue damage as evidenced through histological analysis and oxidative stress. The treatment restored the antioxidative enzyme activities in the lungs and serum IgE levels. The number of eosinophils, netrophils and basophils were also reduced with a decrease in the EPO and MPO levels.

The present study illustrates the potentiality of leaf extract of *A. indica* in alleviating the inflammatory and stress markers. The higher dose of the extract significantly inhibited the OVA-induced oxidative stress in the lungs by increasing the antioxidant defenses i.e., the activities of SOD, CAT, GPX and GR. The extract inhibited the ROS and NO formation, thus, reducing asthma associated bronchial constriction in lungs, recruitment of inflammatory cells and remodeling process. The extract reduced the cellular infiltration by

inhibiting the eosinophil and neutrophil recruitment responsible for chronic airway inflammation. Further, the levels of EPO and MPO, which are the biochemical markers of airway inflammation, were also restored with the administration of higher dose of *A. indica* leaf extract in asthmatic mouse. The results also revealed significantly decreased IgE level following intraperitoneal administration of higher dose of *A. indica* leaf extract as compared with the increased level observed in the asthmatic group, hence, indicating its therapeutic efficacy against asthma. Inactivating IgE by antibodies is reported a potent therapeutic effect against asthma in both animal and clinical studies[57]. Therefore, the potential role of the leaf extract in recovering the lung tissue damage and reducing the symptoms of asthma may be attributed to the reduction in oxidative stress. These attenuated changes were, further, successfully established when compared with the prednisolone treated group.

Conclusion
Intraperitoneal administration of the higher dose of *A. indica* leaf extract effectively bears the anti-inflammatory and antioxidative property against OVA-induced lung tissue damage. Our findings may be validated in further research by determining its effect on genetic level in order to elaborate its mechanism and enhance its use as a therapeutic agent. The study also supports the concept that oxidant/ antioxidant equilibrium is disturbed in asthma.

Conflict of Interest
The authors declare no conflict of interest.

Acknowledgements

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