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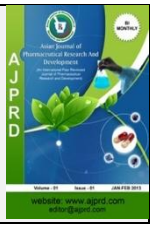

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Research Article

Evaluation of Cellular and Molecular Mechanism of Anti-Asthmatic Effects of A Traditional Herbal Drug In Rats

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A B S T R A C T

The present study was performed to ascertain the therapeutic effects/aspects of *Lychnis coronaria* in ovalbumin (OVA) induced model of bronchial asthma in rats. Wistar rats were immunized with ovalbumin adsorbed on toaluminium hydroxide on day 0 followed by challenge with OVA on day 14 to experimentally induce/simulate bronchial asthma. *Lychnis coronaria* (1.5 mg/kg, 3 mg/kg and 6 mg/kg) was administered (orally) daily for 14 days and its effects on airway hyper responsiveness to spasmogen and airway inflammation were assessed. *Enhanced pause* (*p-enh*), a marker of airway hyper responsiveness, was measured in response to different doses of inhaled methacholine using whole body plethysmography, following which rats were anaesthetized and blood and bronchoalveolar lavage fluid (BALF) were collected and analyzed for OVA specific IgE, pro-inflammatory cytokine (TNF-α) and the Th-2 cytokine (IL-4). Results showed that OVA sensitization and challenge rats increased *enhanced pause* (*P-enh*) in response to different doses of methacholine as measured by whole body plethysmography. Assay of blood and BALF showed increased IgE, TNF-αand IL-4 levels as well as in OVA immunized + challenged rats compared to that of normal control rats thus validating the experimental model. Administration of *Lychnis coronaria*for 14 days induced dose-dependent reductions in *p-enh* values, as well as levels of OVA specific IgE, TNF-α and IL-4 as compared to that in the vehicle treated Disease control group. The result with prednisolone (10 mg/kg, orally) were comparable with *Lychnis coronaria* on all the parameters tested. The results showed anti-asthmatic effects of *Lychnis coronaria* via reducing bronchial hyper responsiveness as well as cellular and molecular markers of airway inflammation and immunity and thus, validating the therapeutic benefits of this Indian traditional medicinal plant for bronchial asthma

Keywords: Bronchial asthma, Ovalbumin, *Lychnis coronaria*, Prednisolone, Methachloine, Enhanced Pause

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INTRODUCTION

Bronchial asthma is a chronic inflammatory airway disease which is manly characterized by complex interactions between various inflammatory cells, mediators, and cytokines that result in airway obstruction, airway inflammation as well as airway hyperresponsiveness[1-2]. Various factors aggravating leading to asthma involve obesity, severe and repeated infections, dietary factors, environmental exposure and increased allergen exposure. Pharmacotherapy of bronchial asthma includes use of β-2 sympathomimetics, oral corticosteroids, anti-histamines, anti-cholinergics etc. Pharmacotherapy of bronchial asthma aims at providing relief by producing bronchodilation and reducing inflammation by mast cell stabilization, immunomodulation, and inhibition of inflammatory mediators such as phosphodiesterase, cyclooxygenase, lipoxygenase, leukotrienes. However, studies have reported various adverse effects of synthetic drugs on humans. Corticosteroids have been shown to increase risk of osteoporosis, osteonecrosis, adrenal suppression, cushingoid appearance, weight gain, glaucoma and cataract[3]. Use of Beta-agonist also involves side effects such as tremors, skin rash, headache, nausea, dizziness, diarrhea, anxiety etc. Moreover, there lies significant inter-individual variability in responses of asthmatic patients to

synthetic drugs for example only few individual gets benefitted from leukotrienes antagonists whereas many others are resistant to these drugs. Similarly, there are reports of development of steroid resistant and those individuals are less benefitted from corticosteroid therapy. Therefore, there has been a shift in focus towards herbal drug research. The World Health Organization (WHO) also promotes use of traditional medicines due to safety, low cost, easy availability and faith of people in such remedies. Under such changed world health scenario, it is quite reasonable to explore use of less known plants as potential sources of medicines and also to determine the underlying molecular and cellular mechanism involved in their therapeutic effects.

Lychniscoronaria, commonly known as "Rose Campion" or "Mullein pink", has been reported to treat, lung and liver ailments, leprosy, diarrhoea, heal cuts & inflamed wounds [4,5].*Lychnis coronaria*has also been reported to havehepatoprotective and anti-inflammatory properties [6,7]. In Northern India, the crushed roots of *Lychnis coronaria*are macerated in water and administered orally for chronic cough [8].Therefore, the plant was selectedto ascertain its therapeutic potentials in ovalbumin induced model of asthma in experimental animals by utilizing modern scientific methodology to validate the traditionally described effects. The study was conducted to evaluate the efficacy of standardized extract of whole plant of *Lychnis coronaria* (LC) against hyperresponsiveness to spasmogen (methacholine) and airway inflammation in experimental model of bronchial asthma.The validation of anti-asthmatic effects of the herbal extract is of considerable translational value as this can provide good herballeads for drug development for the treatment of bronchial asthma.

MATERIALS AND METHODS

Animals: Wistar rats (either sex), weighing180-220 g, were used in the present study (n=6/group). They were housed in polyacrylic cages under standard laboratory conditions (25 ± 2°C, 12 hours light and dark cycle). The rats were fed with standard food pellets and water ad libitum.Care of the animals was done according to the guidelines of Committee for the purpose of control and supervision of experiments on animals (CPCSEA),New Delhi, India. The study protocol was approved by Institutional Animal Ethics Committee (IAEC).

Drugs and Chemicals: The standardized aqueous extract of *Lychnis coronaria* (whole plant) was procured from National Innovation Foundation (NIF), Ahmedabad, India.Various chemicals such as Ovalbumin, Prednisolone and Methacholine were procured from Sigma Aldrich-USA. Cytokine assay kits Ovalbumin specific IgE, TNF–α and IL-4 were procured from Weldon Biotech, New Delhi. All other routine chemicals were procured from Sisco Research Laboratories (SRL, New Delhi).

OVA-induced model of airway inflammation/bronchial asthma:

As described by Kwasnieski et al., [9] rats were sensitized with intraperitoneal administration of Ovalbumin (10 mg/rat) adsorbed on to 10 µg of aluminium hydroxide on day 0. On 14th day the animals were challenged with Ovalbumin (1 mg per rat) in 0.5 ml of isotonic saline.

Experimental protocol

Rats were divided into 6 groups (n=6/group) viz., (1) Normal control [immunized with OVA on day 0 and treated with vehicle (distilled water, orally) for 14 days]; (2) Disease control [immunized with OVA followed by OVA challenge on day, treated with vehicle for 14 days]; (3) Positive control [immunized with OVA on day 0 treated orally with prednisolone (10 mg/kg) from day 1 to 14, followed by OVA challenge on day 14]; (4-6) LC1. LC2 and LC3 [immunized with OVA on day 0 treated orally with *Lychnis coronaria* at the dose of 1.5 mg/kg, 3 mg/kg, or 6 mg/kg in respective groups from day 1 to 14, followed by OVA challenge on day 14. The dose of *Lychnis coronaria* was calculated from human dose used in Traditional system of medicine.

After 24 hour of ovalbumin challenge on 14th day, *enhanced pause* (*P-enh*), an index of bronchial hyperresponsiveness and airway resistance was assessed in response to different doses of inhaled methacholine using whole body plethysmography[10]. Briefly, rats were placed in a whole-body chamber and basal readings for Penh were obtained and averaged for a 3 min period. Subsequently, rats were exposed to methacholine aerosol (2.5 mg/ml, 5 mg/ml, 10 mg/ml and 20 mg/mL) for 3 minutes and readings were taken and averaged for 3 min after each dose of methacholine nebulization [11].

After evaluating airway hyperresponsiveness, rats were anesthetized with ketamine (24 mg/kg, i.p.) and blood was collected by cardiac puncture and centrifuged at 3000 rpm for 10 minutes at 4ºC and the serum was separated and stored at -80ºC for biomarker analysis. After blood collection, BAL fluid was collected by lavaging the airways through a tracheal cannula with 0.9% sodium chloride solution and centrifuged at 1500 rpm for 10 minutes at 4ºC and supernatant was recovered and stored at -80ºC for assay of various biomarkers [12].

Assay for TNF-α and IL-4

The TNF-α and IL-4 in blood and BAL fluid were analyzed using solid phase sandwich ELISA method by using commercially available enzyme linked immunosorbent assay (ELISA) kits. Antigen and biotinylated polyclonal antibody specific for TNF-α and IL-4 were added to the microtitre plates whose wells were already precoated with polyclonal antibody specific for TNF-α and IL-4 and incubated for specific period. HRP conjugate streptavidin was added and incubated followed by addition of TMB substrate to induce a colored reaction product. The plate was then incubated in the dark for 10-20 min at room temperature to avoid direct exposure to light. The enzyme-subsrate reaction was stopped by adding sulphuric acid. The absorbance of the colored end product was measured at a wavelength of 450 nm using ELISA plate reader and values were expressed in pg/ml.

Assay for OVA specific IgE

Assay for OVA specific IgE in blood and BAL fluid samples was analysed by double antibody sandwich ELISA method by using commercially available enzyme linked immunosorbent assay (ELISA) kits.Briefly, the samples were added into the microtiter plate which was pre-coated

with an antibody specific to OVA specific IgE and is incubated. After incubation, secondary OVA specific IgE antibody labeled with biotin was added. Streptavidin-HRP conjugate was added in order to form antibody-antigen-antibody immune complex and incubated. Chromogenic solutions were added to develop color and the reaction was stopped by adding stop solution. The absorbance of colored microtiter plate was measured using software based microtiter plate reader at 450 nm and values are expressed in ng/ml

Statistical analysis

All data are expressed as Mean ± S.E.M and analyzed by one way ANOVA followed by Dunnett's test. A p value < 0.05 was used as a level of significance in all statistical tests.

RESULTS

Effect of *Lychnis coronaria*on Bronchial Hyperresponsiveness

Enhanced Pause (*P-enh*), marker of airway resistance and airway hyperresponsiveness was assessed in response to inhaled methacholine (2.5 mg/kg, 5 mg/kg, 10 mg/kg and 20 mg/kg) using whole body plethysmography. OVA immunization followed by sensitization in Disease control rats significantly accentuated Penh versus that of Normal control rats, indicating increased degree of hyperresponsiveness tospamogen i.e. different doses of inhaled methacholine (2.5 mg/kg, 5 mg/kg, 10 mg/kg and 20 mg/kg). Treatment with *Lychnis coronaria* (1.5 mg/kg, 3 mg/kg and 6 mg/kg) for 14 days, induced significant attenuations in*P-enh* values as compared with Disease control group ($p < 0.05$). Amongst different treatment groups, maximum reduction in *P-enh* values was observed at highest dose level of *Lychnis coronaria*, i.e. 6 mg/kg when compared with Disease control group ($p < 0.05$). Prednisolone treated group also showed significant reduction in *P-enh* values as compared to that of Disease control group ($p < 0.05$). These results are summarized in Figure 1.

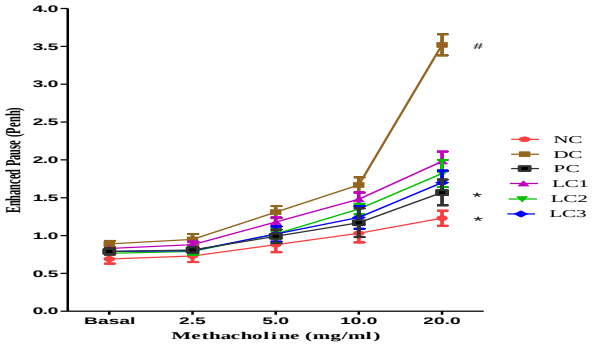

Figure 1: Effects of *Lychnis coronaria* on enhanced pause (*p-enh*) as measured by whole body plethysmography. (NC) Normal control: OVA sensitized; (DC) Disease control: OVA sensitized+challenged rats treated with vehicle; (PC) Positive Control: OVA sensitized and challenged rats treated with Prednisolone (10 mg/kg); LC1, LC2 and LC3: OVA sensitized and challenged rats treated with standardized extract of*Lychnis coronaria* at doses of 1.5 mg/kg, 3mg/kg and 6 mg/kg respectively orally. Data are expressed as Mean ± SEM. #p < 0.05 vs Normal control (NC) group;*p < 0.05 versus Disease control (DC) group

Effect of *Lychnis coronaria* on OVA specific IgE levels in blood and BALF

Assay of OVA specific IgE levels showed significant increase in IgE levels in both blood and BAL fluid of Disease control group i.e. OVA sensitized and challenged rats when compared to that in the normal control group ($p < 0.05$).Pretreatment with aqueous extract of *Lychnis coronaria* for 14 days induced dose-dependent attenuation in IgE levels in both blood and BAL fluid samples.*Lychnis coronaria* at the dose of 6 mg/kg induced significant attenuation in IgE levels in blood (approximately 46%) and BAL fluid (approximately 18% and 25% by 3 mg/kg and 6 mg/kg)as compared to that of Disease control group ($p < 0.05$). Pretreatment with *Lychnis coronaria* at lower doses although reduced the OVA specific IgE levels in both blood and BAL fluid but did not reach the level of significance ($p > 0.05$). Pre-treatment with prednisolone also induced significant attenuations in OVA-specific IgE levels by 55% in blood and by 22% in BALF as compared to that of Disease control group ($p < 0.05$). These results are summarized in Figure 2.

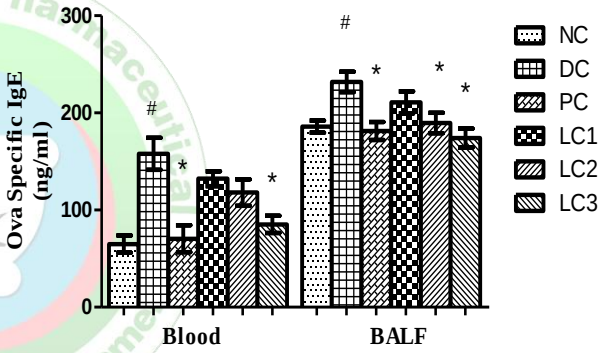

Figure 2: Effects of *Lychnis coronaria* extract on OVA specific IgE levels in blood and BAL fluid of OVA sensitized+challenged rats. (NC) Normal control: OVA sensitized; (DC) Disease control: OVA sensitized+challenged rats treated with vehicle; (PC) Positive Control: OVA sensitized and challenged rats treated with Prednisolone (10 mg/kg); LC1, LC2 and LC3: OVA sensitized and challenged rats treated with standardized extract of *Lychnis coronaria* at doses of 1.5 mg/kg, 3 mg/kg and 6 mg/kg respectively orally. Data are expressed as Mean ± SEM. #p < 0.05 vs Normal control (NC) group;*p < 0.05 versus Disease control (DC) group.

Effect of *Lychnis coronaria* on TNF-α in blood and BALF

Immunization of rats followed by challenge treatment with OVA (on day 14) resulted in significant increase in levels of TNF-α in both blood and BAL fluid as compared to that of normal control rats ($p < 0.05$). Administration of standardized extract *Lychnis coronaria* for 14 days induced attenuation in TNF-α level at all doses but significant attenuations were observed at 6 mg/kg dose in both blood and BALFas compared to that in Disease control rats ($p < 0.05$).*Lychnis coronaria*in the doses of 1.5 mg/kg, 3 mg/kg or 6 mg/kg in separate groups resulted in dose dependent attenuation in TNF-α level in blood by 19%, 27% and 37%, respectively, while in BAL fluid, TNF-α level were suppressed by 5%, 15% and 25% respectively. Treatment with prednisolone (10 mg/kg) also resulted in significant

attenuations of TNF-α level by 42% in blood and by 27% in BAL fluid as compared to Disease control group ($p < 0.05$).These results are summarized in Figure 3.

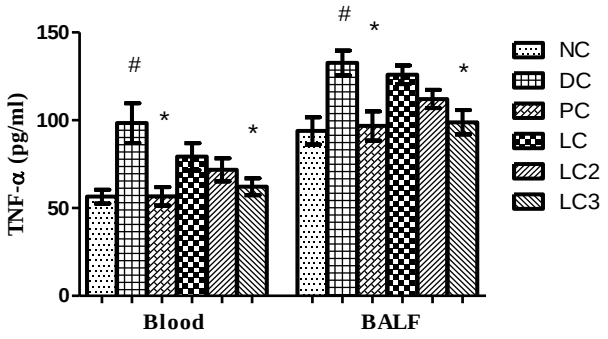

Figure 3: Effects of *Lychnis coronaria* on TNF-α level in blood and BAL fluid of OVA sensitized and challenged rats. (NC) Normal control: OVA sensitized; (DC) Disease control: OVA sensitized+challenged rats treated with vehicle; (PC) Positive Control: OVA sensitized and challenged rats treated with Prednisolone (10 mg/kg); LC1, LC2 and LC3: OVA sensitized and challenged rats treated with standardized extract of *Lychnis coronaria* at doses of 1.5 mg/kg, 3 mg/kg and 6 mg/kg respectively orally. Data are expressed as Mean ± SEM. #p < 0.05 vs Normal control (NC) group;*p < 0.05 versus Disease control (DC) group

Effect of *Lychnis coronaria* on IL-4 levels in Blood and BAL fluid

Immunization and challenge with OVA in Disease control rats resulted in significant increase in IL-4 levels in both blood and BALF as compared to that of normal control rats ($p < 0.05$). Administration of aqueous extract of *Lychnis coronaria* for 14 days resulted in dose-dependent attenuation of IL-4 levels in both blood and BALF as compared to that of Disease control rats ($p < 0.05$). Treatment with *Lychnis coronaria*at the dose of 1.5 mg/kg, 3 mg/kg and 6 mg/kg, showed suppression in IL-4 levels by 54%, 57% and 80% in blood, while in BAL fluid, IL-4 levels were suppressed by 27%, 40% and 44%, respectively at the three dose levels. *Lychnis coronaria* induced significant attenuation in IL-4 levels at all the three doses in blood while in BAL fluid, significant attenuations in IL-4 levels were observed at 3 mg/kg and 6 mg/kg doses. Pre-treatment with Prednisolone (10 mg/kg) showed significant attenuation in IL-4 levels inblood and BAL fluid as compared to Disease control groups ($p < 0.05$). These results are summarized in Figure 4.

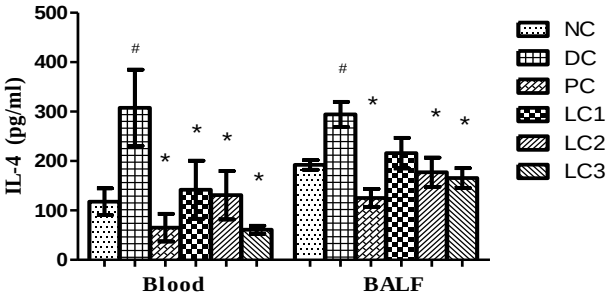

Figure 4: Effects of *Lychnis coronaria* on IL-4 levels in blood and BAL fluid of OVA sensitized and challenged rats. (NC) Normal control: OVA sensitized; (DC) Disease control: OVA sensitized+challenged rats treated with vehicle; (PC) Positive Control: OVA sensitized and challenged rats treated with Prednisolone (10 mg/kg); LC1, LC2 and LC3: OVA sensitized and challenged rats treated with standardized extract of *Lychnis coronaria* at doses of 1.5 mg/kg, 3 mg/kg and 6 mg/kg respectively orally. Data are expressed as Mean ± SEM. #p < 0.05 vs Normal control (NC) group;*p < 0.05 versus Disease control (DC) group

DISCUSSION

Bronchial asthma is a chronic inflammatory airway disease characterized by airway inflammation and obstructionas well as bronchial hyperresponsiveness. It involves a cascade of events leading predominantlyto Th-2 mediated immune response which orchestrate release of cytokines (1L-4, IL5as well as IL-13), inflammatory cells (eosinophils and neutrophils) and chemokines which maintain the disease pathology. Pharmacotherapy of bronchial asthma aims at providing symptomatic relief with the use of either controllers (corticosteroids) as well as relievers (beta agonists). Short term use of anti-asthmatic drugs are relatively safe, however long term use may lead to various adverse effects as well as refractoriness (13-15), which inturn compromise safety, compliance as well as halt the patient's faith towards the use of these drugs. Therefore, in order to combat all these adverse effects, search for more viable alternatives which can be used primarily or as an adjunct therapy in the management of bronchial asthma is the need of the hour. As a result, focus has been shifted towards traditional systems of medicine and especially medicinal plants and their derived products to explore and validate their therapeutic values using modern methodology so as to establish them as pharmacoeconomically viable alternatives or as complementary therapy. *Lychnis coronaria*, commonly known as "Rose Campion" or "Mullein pink", is used to treat, lung and liver ailments, leprosy, diarrhoea, heal cuts & inflamed wounds (4,5).*Lychnis coronaria* possess hepatoprotective, anti-inflammatory properties and administered orally as a medication for chronic cough (6-8).Therefore, the present study was performed to validate the therapeutic effects of *Lychnis coronaria* in OVA induced animal model of bronchial asthma. Rats were immunized and challenged with OVA on to experimentally induce an inflammatory response, marked by mobilization of mediators such as IgE and bronchial hyperresponsiveness/airflow restriction to simulate bronchial asthma.

In our study, enhanced pause (*P-enh*), a marker of airway hyperresponsiveness was measured in response to aerosolized spasmogen (methacholine) exposure for 3 minutes at different doses of 0 (basal), 2.5 mg/ml, 5 mg/ml, 10 mg/ml and 20 mg/ml using whole body plethysmography. Enhanced Pause (*P-enh*) is positively correlated with airway resistance (16, 17) and airway responsiveness in several asthma studies (11, 18). Higher *P-enh*values are considered as an indicator of higher airway resistance as well as airway hyperresponsiveness. In our study, OVA immunizationfollowed by challenge induced increased *P-enh*values indicating increased airway resistance which may be due to bronchospasm following antigenic challenge.Interestingly, treatment with *Lychnis coronaria* for 14 days resulted in a dose-dependentreduction airway resistance and hyper-responsiveness as evident from reduction in *P-enh* valuesin response to methacoline challenge and the results were

comparable with that observed after Prednisolone treatment.

IgE is a reaginic antibody which plays a vital role in airway inflammation and other allied allergic reactions. High IgE levels are observed in adults and children suffering with asthma which is correlated with airway hyperresponsiveness, lower baseline lung function as well as asthma severity (19, 20). In our present study, OVA immunized + challenged rats (Disease control) induced increased IgE levels in both blood and BALF as compared to that of normal control rats thus indicating increased airway inflammation in experimental rats.Treatment with *Lychnis coronaria*reduced the levels of IgE in both blood and BAL fluid as compared to OVA-induced sensitized and challenged group (Disease controls)in a dose dependent mannerthus confirming the pharmacological relevance of the herbal agent in this experimental model of asthmain rats.

TNF-α is a pro-inflammatory cytokine released by T cells, monocytes/ macrophages, mast cells, eosinophils, and epithelial cells. Release of TNF-α resulted in increased expression of adhesion molecules which triggers the release of cytotoxic mediators and toxic products of reactive oxygen and nitrogen, further damaging the airways (21). TNF-alpha also contributes to airway remodeling by inducing activation and proliferation of fibroblasts, subepithelial fibrosis, production of extracellular matrix glycoproteins, and goblet cell metaplasia (22). TNF-alpha also has direct effects on airway reactivity to methacholine or allergen, as shown in isolated tracheal ring preparations (23). In our study, immunization of rats followed by sensitization with OVA i.e. Disease control rats, induced increased TNF-α levels in both blood and BALF as compared to that in normal control rats.Administration of *Lychnis coronaria*for 14 days induced dose-dependent attenuations in TNF-α level in both blood and BALF compared to that in disease control rats thus, indicating anti-inflammatory activity of *Lychnis coronaria*.

IL-4 is a Th-2 cell specific cytokine and actively participates in allergic airway inflammation and in the pathophysiology of bronchial asthma. Activation of transcription factor Stat6 due to IL-4 signaling results in up-regulated expression of Th2 lineage–specific transcription factor GATA-binding protein 3 (GATA-3) which further promotes expression of Th2 cytokine such as IL-4, IL-5, IL-9, and IL-13[24,25]. IL-4 along with IL-13 results in over expression of endothelial vascular cell adhesion molecule-1 (VCAM-1) which in turn induces inflammatory response in the airways by facilitating migration of basophils, eosinophils, monocytes, T-lymphocytes. In the present study, Disease control rats (OVA immunized + challenged) showed increased IL-4 levels in both blood and BALF indicating increased inflammation in airways. Interestingly, treatment with *Lychnis coronaria* resulted in dose-dependent attenuation in IL-4 levels in both blood and BALF as compared to that of Disease control rats, corroborating to the above finding of anti-inflammatory of the herbal extract.Since IL-4 is a Th2 cytokine that plays a crucial role in allergic responses, *Lychnis coronaria* could be beneficial in asthma of allergic origin. The present results showed that extract of *Lychnis coronaria*lowered levels of IgE, TNF-α and IL-4 (markers of inflammation and immunity) in both blood and BALF which was supported by attenuation of enhanced pause (marker of bronchial hyperresponsiveness) thus validating the reported beneficial effects of this herbal agent in bronchial asthma.

CONCLUSION:

The present study evaluated anti-asthmatic effects of *Lychnis coronaria* in standardized OVA-induced model of bronchial asthma and it was found that the herb reduced the cellular markers of inflammation and immunity (IgE, TNF-αand IL-4) airway hyperresponsiveness to spasmogens in the OVA sensitized and challenged rats. Taken together, it can be concluded from our study that the*Lychnis coronaria*has anti-inflammatory, immunomodulatory and anti-spasmogenic activity and this might be contributing to its therapeutic benefit in bronchial asthma. The study can be an important step to integrate traditional and modern medicine systems for rationalizing drug therapy in respiratory diseases like bronchial asthma.

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CONFLICTS OF INTEREST

Authors have no personal or financial conflicts of interest in relation to the publication of this manuscript.

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