

ORIGINAL ARTICLE

An anti-asthmatic activity of natural Toll-like receptor-4 antagonist in OVA-LPS-induced asthmatic rats

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Funding information

This research was financially partial supported by Gujarat Council on Science & Technology (GUJCOST).

Summary

Toll-like receptor-4 (TLR4) is a key component of the innate immune system and activation of TLR4 signaling has a significant role in the pathogenesis of asthma. Therefore, our objective was to identify the natural TLR4 antagonist and evaluate its activity in experimentally induced asthma. Soya lecithin origin phosphatidylcholine (soya PC) was identified as a natural TLR4 antagonist by computational study. Based on the computational study, TLR4 antagonist activity of soya PC was confirmed in *in vitro* lipopolysaccharide (LPS)-induced neutrophil adhesion assay. In the *in vivo* study, rats were sensitized with ovalbumin (OVA) (100 µg/kg, *i.p.*) on the 7th, 14th and 21st days and challenged intranasally with OVA (100 µg/100 µL) and LPS (10 ng/100 µL), 4 days/wk for 3 weeks. At the end of the experiment, we performed lung function parameters (respiratory rate, tidal volume, airflow rate), inflammatory cytokines (interleukin [IL]-4, IL-5, IL-13), total and differential leukocytes in blood as well as bronchoalveolar lavage fluid (BALf) and histological examinations. The computational study indicated that TLR4 antagonist activity of soya PC is due to linoleic acid (18:2) fatty acid chain. Soya PC significantly suppressed the LPS-induced neutrophil adhesion in a concentration-dependent manner to 1 µg/mL. The treatment of soya PC (5 and 10 mg/kg, 18 days, *i.p.*) significantly improved the lung function parameters, total and differential leukocyte counts in blood and BALf in asthmatic rats. This efficacy of soya PC was in extent similar to dexamethasone (2.5 mg/kg, 18 days, *i.p.*). However, soya PC was superior to dexamethasone in terms of benefits. The protective action of soya PC may be due to TLR4 antagonist activity and linoleic acid composition.

KEYWORDS

asthma, inflammation, lipopolysaccharide, phosphatidylcholine, TLR4

1 | INTRODUCTION

Asthma is a chronic inflammatory disease which is characterized by recurrent episodes of wheezing, breathlessness, chest tightness and coughing. Bronchoconstriction and airway inflammation are the key features of asthma, triggered by exposure to an allergen.¹ Inflammation in bronchial asthma is associated with Th2 (T-helper type-2 cells) responses such as eosinophil, neutrophil

and macrophage infiltration which further leads to release of inflammatory mediators and cytokines (interleukin [IL]-4, IL-5, IL-9, IL-13 and tumor necrosis factor [TNF]-α), mast cell degranulation and goblet cells hyperplasia.²⁻⁵ These pathological changes cause airway remodeling (thickening of basement membrane, epithelial shedding, airway smooth muscle hypertrophy and hyperplasia), airway hyper-responsiveness (AHR) and mucus hyper-production.⁶ The allergic responses of Th2 cells to allergens and endotoxins

are regulated by dendritic cells (DC). Hamida et al reported that allergic response to the allergens initiated by triggering Toll-like receptor-4 (TLR4) of lung structural cells drives the activation of mucosal DC cascades.⁷

TLR4 is a key component of innate and adaptive immunity via recognition of bacterial endotoxins. TLR4 is a transmembrane protein, which belongs to the pattern recognition receptor family. Activation of the myeloid differentiation primary response gene 88 (MyD88)-dependent intracellular signaling pathway of TLR4 leads to activation of nuclear factor- κ B (NF- κ B) and increases the gene expression of inflammatory cytokines.^{8,9} A previous study reported that messenger RNA expression levels of TLR4 and NF- κ B increased in mononuclear cells of blood in ovalbumin (OVA)-induced asthmatic mice.¹⁰ Other studies reported that challenge with lower doses of lipopolysaccharide (LPS) (0.1 μ g) to OVA-sensitized mice induces Th2 associated response, resulting in increased cytokine (IL-4 and IL-5) levels, histamine release airway inflammation and asthma exacerbation through the TLR4 signaling pathway. However, high doses of LPS (100 μ g) induce Th1-associated response in mice.^{11,12} Steroid therapy is well documented in the treatment of asthma, although, many studies have reported emerging evidence on steroid-insensitive asthma and resistance to steroid therapy on prolonged usage. Several studies indicate resistance to steroids is connected to neutrophilic inflammation in lungs.¹³ Moreover, Yang et al demonstrated the role of interferon (INF)- γ and TLR4/MyD88 signaling in macrophage-dependent steroid-resistant AHR.¹⁴ Additionally, Dawson et al suggested an increase in transcription of inflammatory cytokines as a result of increased NF- κ B activity in steroid-resistant patients.¹⁵ These pieces of evidence suggest the significant role of TLR4 signaling in the pathogenesis of initial stage asthma as well as in later stage steroid-resistant asthma. Therefore, we have aimed to identify the novel natural TLR4 antagonist by computational study and to assess its therapeutic potency in experimentally induced asthma in rats.

2 | RESULTS

2.1 | Computational study

In the computational study, LPS and eritoran showed the same binding site on the MD2 domain of TLR4 and MD2 complex at 6th and 10th number conformations, respectively (Figure 1D,E). LPS and eritoran were bound on Lys 91 amino acid of MD2 whereas the binding of LPS and eritoran did not show such selectivity on the TLR4

domain. The docking of three types of soya phosphatidylcholine (PC) showed the variation in their binding to a pocket on 3FXI according to the difference in their fatty acid compositions. Among three soya PC, PC(16(0):18(2)) and PC(18(2):18(2)) showed the same binding site on the MD2 domain at the 3rd, and 3rd and 4th conformations, respectively (Figure 1F,G), as LPS and eritoran, while PC(16(0):16(0)) did not show binding on the MD2 domain (Figure 1H). The comparison of binding energy (Table 1) and binding site of all five docked ligands suggested the following order of relative bindings: LPS < eritoran < PC(16(0):18(2)) < PC(18(2):18(2)).

2.2 | In vitro study

2.2.1 | Percentage neutrophil adhesion assay

Lipopolysaccharide significantly ($P < 0.05$) increased the neutrophil adhesion in blood as compared to the control group. LPS-induced neutrophil adhesion was significantly ($P < 0.01$ and $P < 0.001$) decreased in the presence of soya PC 0.1 and PC 1 μ g/mL, respectively, as compared to the soya PC untreated group (Figure 2).

2.3 | In vivo study

2.3.1 | Effect of treatments on lung function parameters

The sensitization and challenge of OVA and LPS significantly increased the respiration rate ($P < 0.001$) while tidal volume ($P < 0.001$) and airflow rate ($P < 0.01$) were significantly decreased. The treatment of soya PC (5 and 10 mg/kg) significantly reduced the respiration rate ($P < 0.01$, $P < 0.001$, respectively) whereas tidal volume ($P < 0.01$, $P < 0.01$, respectively) and airflow rate ($P < 0.05$, $P < 0.01$, respectively) were found to be significantly increased compared to asthmatic rats. Treatment of dexamethasone significantly increased tidal volume ($P < 0.01$) and airflow rate ($P < 0.01$) whereas respiration rate was significantly ($P < 0.001$) decreased (Figure 3A-C).

2.3.2 | Effect of treatments on total and differential leukocytes count in blood

The total leukocytes ($P < 0.001$) and differential leukocytes count (neutrophils $P < 0.001$, eosinophils $P < 0.001$, lymphocytes $P < 0.001$ and monocytes $P < 0.01$) were significantly increased in blood of asthmatic rats as compared to normal rats. The treatment of soya PC (5 and 10 mg/kg) significantly decreased the total

FIGURE 1 Structure and binding characteristics. (A) Dimeric structure of Toll-like receptor-4 (TLR4) and MD2 complex (PDB No.3FXI). It is presented by ribbon model. Blue and green ribbons indicate TLR4, while yellow and red ribbons indicate MD2. The grid region for docking is marked by a square box. (B) Molecular structure of lipopolysaccharide (LPS) (TLR4 agonist) and eritoran (TLR4 antagonist). (C) Molecular structure of phosphatidylcholine (PC) such as PC18(2)18(2)/PC (not present in humans), PC16(0)16(0)/PC (present in lung) and PC16(0)18(2)/PC (present in plasma). Sugar, phosphate and fatty acyl moieties of these compounds are shown by black, light blue and red color circles, respectively. Preferably docked confirmation no. 6, 10, 3, 10 and 3 or 4 of ligands such as (D) binding characteristics of LPS, (E) binding characteristics of eritoran, (F) binding characteristics of PC16(0)18(2), (G) binding characteristics of PC18(2)18(2) and (H) binding characteristics of PC16(0)16(0) on dimeric TLR4-MD2 complex (3FXI), respectively. Black circles indicate the binding site of ligands on 3FXI

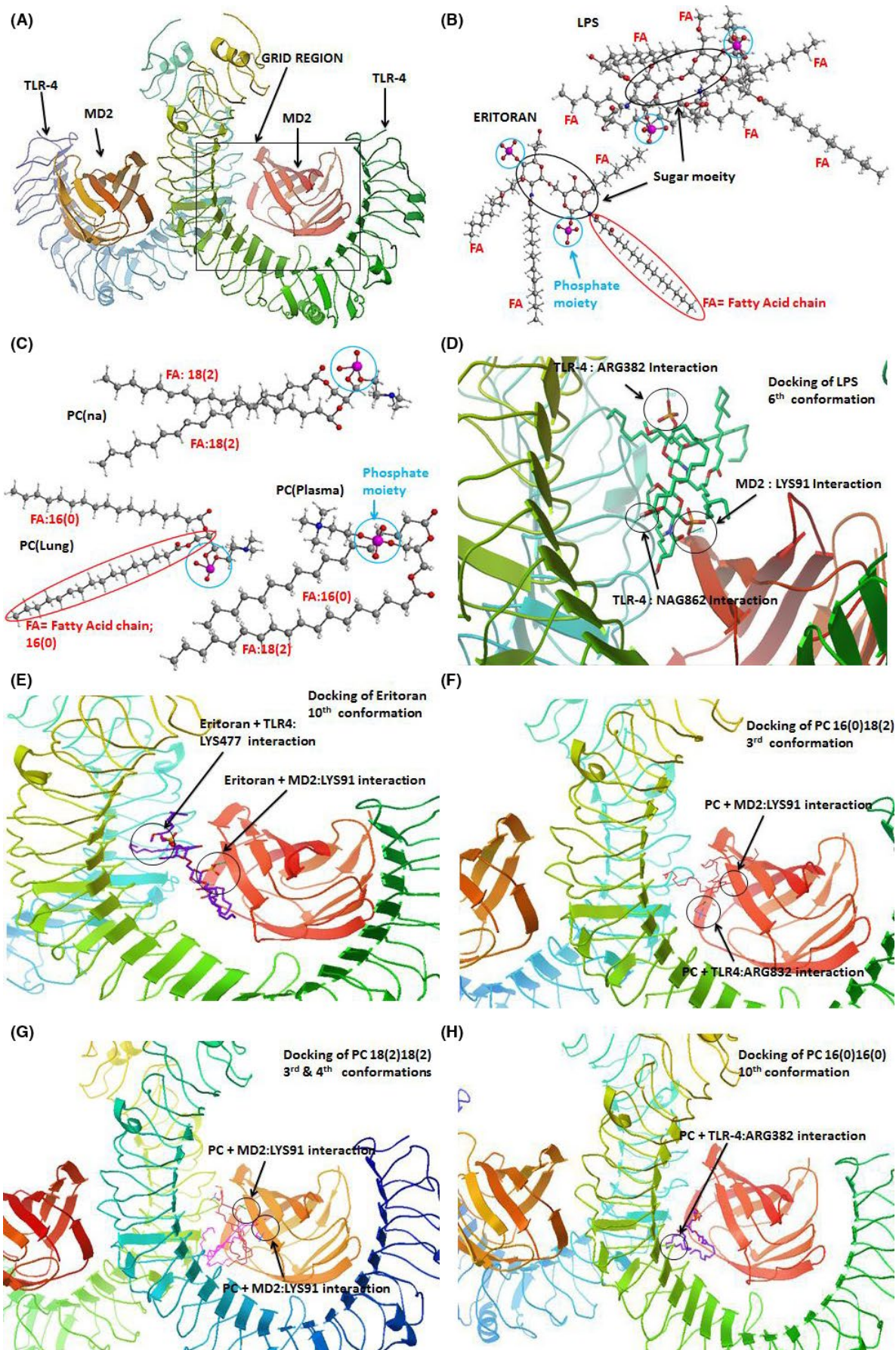


TABLE 1 Binding energy of preferably docked conformation (presented in Figure 1D-H) of lipopolysaccharide (LPS), eritoran, soya phosphatidylcholine (PC18(2)18(2), PC16(0)16(0) and PC16(0)18(2)) on 3FXI (dimeric Toll-like receptor-4-MD2 complex) using Autodock 4.2.6

Sr. No.	Ligands	Binding energy
1	LPS	18.89
2	Eritoran	7.58
3	PC16(0)18(2)/PC(Plasma)	1.89
4	PC18(2)18(2)/PC(na)	-7.1
5	PC16(0)16(0)/PC(Lung)	0.22

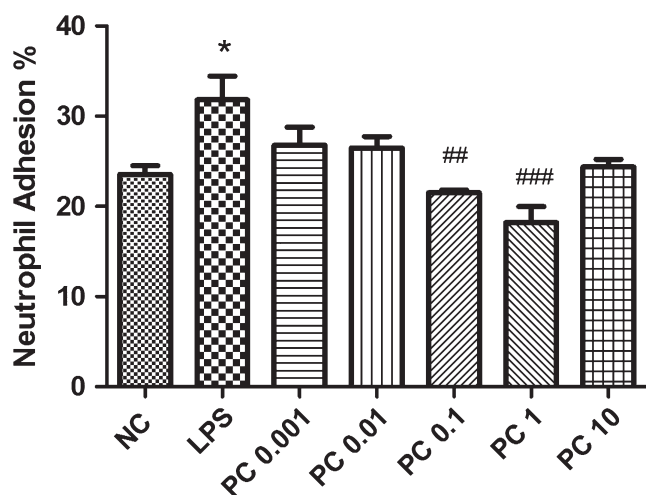


FIGURE 2 Concentration-dependent action of soya phosphatidylcholine (PC) on lipopolysaccharide (LPS) (100 ng/mL) induced rat neutrophil adhesion. NC = vehicle-treated, LPS = LPS-treated, PC 0.001 = PC (0.001 μg/mL) + LPS-treated, PC 0.01 = PC (0.01 μg/mL) + LPS-treated, PC 0.1 = PC (0.1 μg/mL) + LPS-treated, PC 1 = PC (1 μg/mL) + LPS-treated and PC 10 = PC (10 μg/mL) + LPS-treated neutrophils. * $P < 0.05$ vs NC. ## $P < 0.01$ and ### $P < 0.001$ vs LPS

leukocytes ($P < 0.01$, $P < 0.01$, respectively) and differential leukocytes count (neutrophils [$P < 0.001$, $P < 0.001$, respectively], eosinophils [$P < 0.001$, $P < 0.001$, respectively], lymphocytes [$P < 0.001$, $P < 0.001$, respectively] and monocytes [$P < 0.01$, $P < 0.001$, respectively]) in the blood of asthmatic rats. Treatment of dexamethasone significantly decreased the total ($P < 0.01$) and other differential leukocytes count ($P < 0.001$) in asthmatic rats (Figure 4A-E).

2.3.3 | Effect of treatments on total and differential leukocytes count in bronchoalveolar lavage fluid

Total leukocytes count ($P < 0.001$) and differential leukocytes count (neutrophils $P < 0.01$, eosinophils $P < 0.001$, lymphocytes $P < 0.001$ and monocytes $P < 0.01$) were significantly increased in bronchoalveolar lavage fluid (BALF) of asthmatic rats as compared to normal rats. Conversely, treatment with soya PC 5 mg/kg, soya PC 10 mg/kg and dexamethasone significantly reduced total leukocytes count

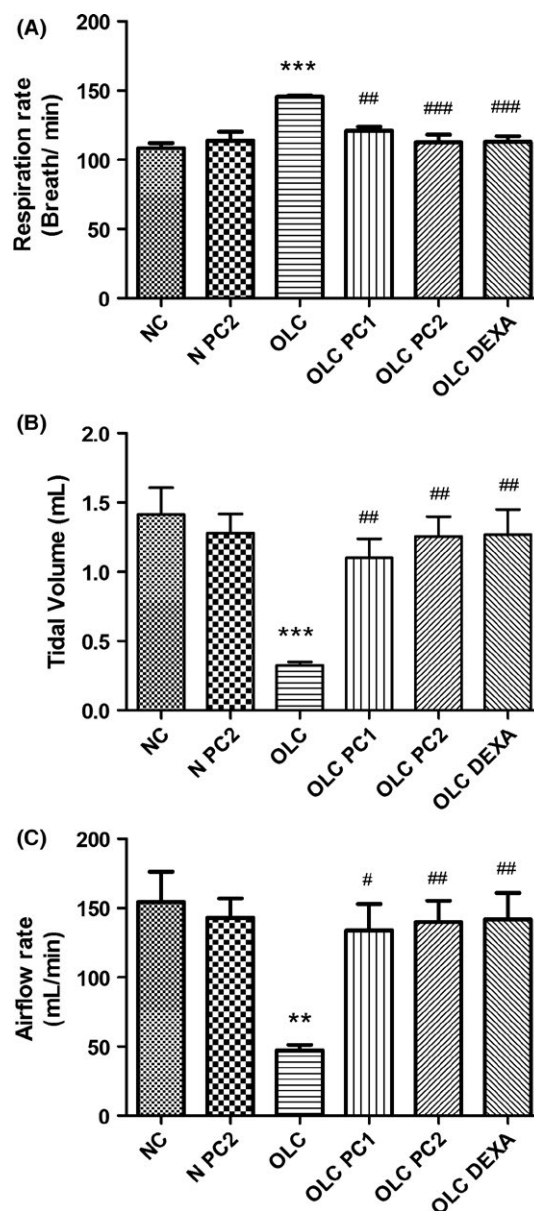


FIGURE 3 Effect of soya phosphatidylcholine (PC) and dexamethasone on (A) respiration rate, (B) tidal volume and (C) airflow rate in normal and asthmatic rats. NC = vehicle (10 mL/kg, i.p.)-treated normal rats, N PC2 = PC (10 mg/kg, i.p.)-treated normal rats, OLC = ovalbumin (OVA)-lipopolysaccharide (LPS)-challenged asthmatic rats, OLC PC1 = PC (5 mg/kg, i.p.) treated OVA-LPS-challenged asthmatic rats, OLC PC2 = PC (10 mg/kg, i.p.)-treated OVA-LPS challenged asthmatic rats and OLC DEXA = dexamethasone (2.5 mg/kg, i.p.)-treated OVA-LPS challenged asthmatic rats. ** $P < 0.01$ and *** $P < 0.001$ vs NC. # $P < 0.05$, ## $P < 0.01$ and ### $P < 0.001$ vs OLC

($P < 0.01$, $P < 0.001$, $P < 0.001$, respectively) and other differential leukocytes count (neutrophils $P < 0.01$, $P < 0.001$, $P < 0.001$, respectively), eosinophils ($P < 0.05$, $P < 0.001$, $P < 0.001$, respectively), lymphocytes ($P < 0.01$, $P < 0.01$, $P < 0.01$, respectively) and monocytes ($P < 0.01$, $P < 0.001$, $P < 0.001$, respectively) in asthmatic rats (Figure 5A-E).

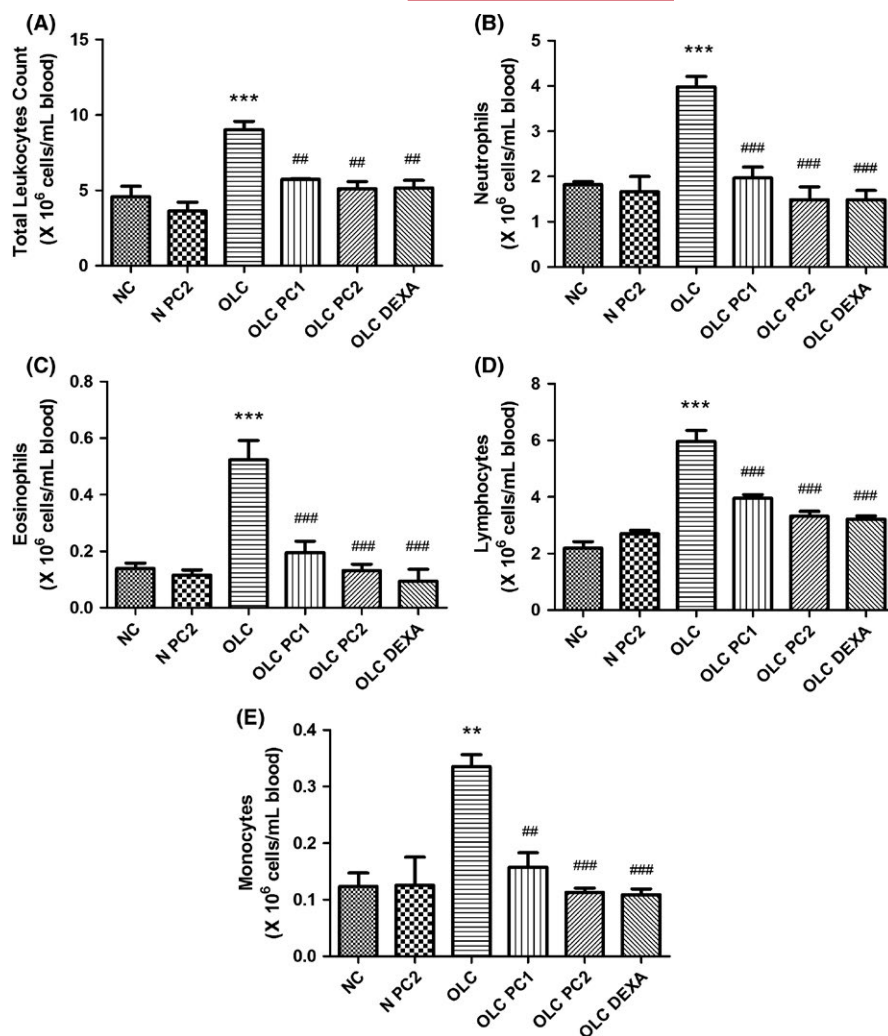


FIGURE 4 Effect of soya phosphatidylcholine (PC) and dexamethasone on blood; (A) total leukocytes count, (B) neutrophils, (C) eosinophils, (D) lymphocytes and (E) monocytes in normal and asthmatic rats. NC = vehicle (10 mL/kg, i.p.)-treated normal rats, N PC2 = PC (10 mg/kg, i.p.)-treated normal rats, OLC = ovalbumin (OVA)-lipopolysaccharide (LPS)-challenged asthmatic rats, OLC PC1 = PC (5 mg/kg, i.p.)-treated OVA-LPS-challenged asthmatic rats, OLC PC2 = PC (10 mg/kg, i.p.)-treated OVA-LPS-challenged asthmatic rats and OLC DEXA = dexamethasone (2.5 mg/kg, i.p.)-treated OVA-LPS-challenged asthmatic rats. ** $P < 0.01$ and *** $P < 0.001$ vs NC. ## $P < 0.01$ and ### $P < 0.001$ vs OLC

2.3.4 | Effect of treatments on inflammatory cytokines in serum

The inflammatory cytokines IL-4 ($P < 0.001$), IL-5 ($P < 0.001$) and IL-13 ($P < 0.001$) were significantly increased in serum of asthmatic rats as compared to normal rats. The treatment of soya PC (5 and 10 mg/kg) and dexamethasone significantly decreased IL-4 ($P < 0.001$), IL-5 ($P < 0.01$, $P < 0.001$, $P < 0.01$, respectively) and IL-13 ($P < 0.001$) in the serum of asthmatic rats (Figure 6A-C).

2.3.5 | Effect of treatments on biochemical parameters in serum

Treatment of dexamethasone significantly increased serum glucose ($P < 0.05$) level in asthmatic rats. However, serum cholesterol and triglyceride levels did not alter by dexamethasone treatment in asthmatic rats. Soya PC (5 and 10 mg/kg) did not show modulation in serum glucose and cholesterol levels. However, serum triglyceride ($P < 0.05$) level was significantly decreased in soya PC (5 and 10 mg/kg) treated asthmatic rats. (Figure 7A-C).

2.3.6 | Effect of treatments on histopathology of lung tissue

Histological examination of lung tissue showed increase in neutrophil infiltration and vacuolization in asthmatic rats as compared to normal rats. Such pathological changes were reversed by the dexamethasone and soya PC treatment in asthmatic rats. (Figure 8A-F).

3 | DISCUSSION

The present study is the first to identify soya PC as a natural TLR4 antagonist and reveals its therapeutic potential in OVA-LPS-induced asthmatic rats. It is well known that MD2 dependent TLR4 signaling cascade increases transcription of inflammatory cytokines through activated NF- κ B.¹⁶ Therefore, we targeted the MD2 site in the computational study. The previous study also showed that LPS increased the NF- κ B expression and transcription of inflammatory cytokines.¹⁷ Eritoran has previously been reported as a TLR4 antagonist, used as a therapy for sepsis. However, it failed in a phase III clinical trial due to its lower efficacy in severe sepsis compared to placebo.¹⁸ In our

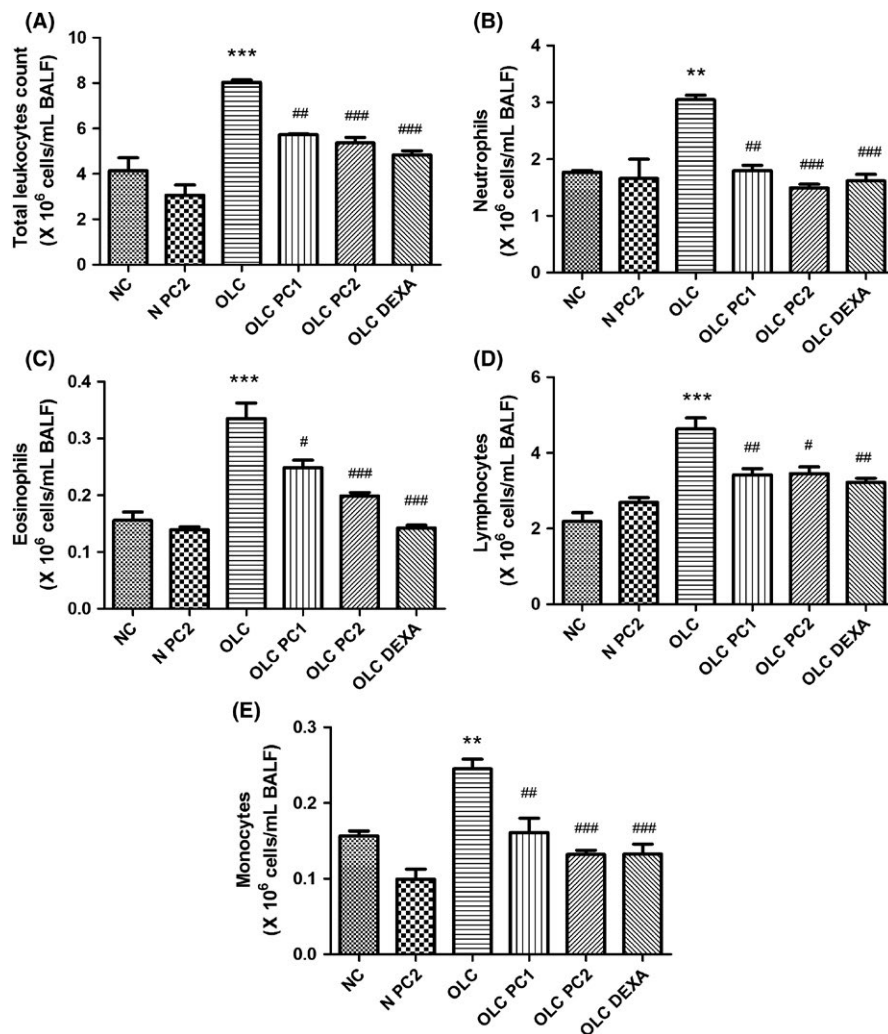


FIGURE 5 Effect of soy phosphatidylcholine (PC) and dexamethasone on bronchoalveolar lavage fluid (BALf); (A) total leukocytes count, (B) neutrophils, (C) eosinophils, (D) lymphocytes and (E) monocytes in normal and asthmatic rats. NC = vehicle (10 mL/kg, i.p.)-treated normal rats, N PC2 = PC (10 mg/kg, i.p.)-treated normal rats, OLC = ovalbumin (OVA)-lipopolysaccharide (LPS)-challenged asthmatic rats, OLC PC1 = PC (5 mg/kg, i.p.)-treated OVA-LPS-challenged asthmatic rats, OLC PC2 = PC (10 mg/kg, i.p.)-treated OVA-LPS-challenged asthmatic rats and OLC DEXA = dexamethasone (2.5 mg/kg, i.p.)-treated OVA-LPS-challenged asthmatic rats. ** $P < 0.01$ and *** $P < 0.001$ vs NC. # $P < 0.05$, ## $P < 0.01$ and ### $P < 0.001$ vs OLC

computational study, eritoran, LPS and soya PC with fatty acid composition PC(16(0):18(2)) and PC(18(2):18(2)) showed the common binding site on the MD2 domain. The free binding energy indicated that soya PC with the fatty acids composition PC(18(2):18(2)) has more binding affinity as compared to PC(16(0):18(2)), eritoran and LPS. Ultimately, fatty acid chain and its conformation present on the ligand have a significant role in binding with TLR4 and MD2 complex.

In the current study, TLR4 antagonist activity of soya PC was evaluated by LPS (TLR4 agonist)-induced in vitro neutrophil adhesion assay. Soya PC attenuated the LPS-induced neutrophil adhesion in a concentration-dependent manner to 1 μ g/mL. Consistent with our findings, a previously reported in vitro study indicated that TLR4 agonist (LPS) increased neutrophil migration and motility in human blood.¹⁹

Ovalbumin-induced asthma is well reported for induction of experimental asthma in rats. However, in the present study, experimental asthma in rats was induced by sensitization with OVA and subsequent challenge with OVA and LPS to exacerbate asthma. Inflammatory responses of LPS are governed by neutrophil activation and its cascades,²⁰ while OVA inflammatory responses are governed by eosinophil activation and mast cell degranulation.²¹ A previous study reported co-administration of LPS and OVA results

in prolonged histamine-induced bronchoconstriction mediated by mast cells degranulation in response to LPS stimuli via TLR4.²²

In the present study, there were increases in respiratory rate, inflammatory cytokine levels (IL-4, IL-5 and IL-13), total and differential leukocytes count in blood and BALf, whereas there were decreases in tidal volume and airflow rate, indicating the development of asthma in rats. A previously reported study suggested that challenge with LPS (lower dose) aerosol to OVA-sensitized mice has shown increase in cytokine (IL-4 and IL-5) levels, histamine release and airway inflammation.²³ Treatment with soya PC and dexamethasone has improved the modulation in lung function parameters such as respiratory rate, tidal volume, air flow rate and inflammatory cytokine (IL-4, IL-5 and IL-13) levels in asthmatic rats. Furthermore, treatment with soya PC and dexamethasone decreased total and differential leukocyte counts in blood as well as BALf. A previously reported study indicated that increases in IL-4 and IL-13 levels significantly induced chronic airway inflammation and remodeling in asthmatic mice.²⁴

Treatment with soya PC and dexamethasone showed a decrease in neutrophil infiltration and vacuolization in histological examination of lung tissue. This beneficial activity of soya PC may be due to inhibition of TLR4 signaling-mediated inflammatory responses and

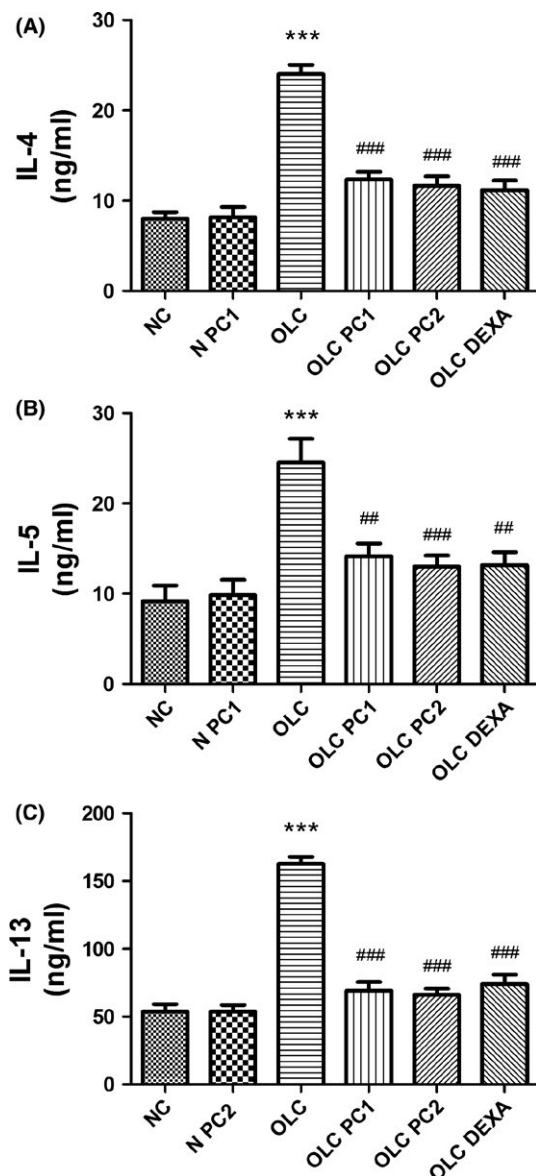


FIGURE 6 Effect of soya phosphatidylcholine (PC) and dexamethasone on serum (A) interleukin (IL)-4, (B) IL-5 and (C) IL-13 in normal and asthmatic rats. NC = vehicle (10 mL/kg, i.p.)-treated normal rats, NPC2 = PC (10 mg/kg, i.p.)-treated normal rats, OLC = ovalbumin (OVA)-lipopolysaccharide (LPS)-challenged asthmatic rats, OLC PC1 = PC (5 mg/kg, i.p.)-treated OVA-LPS-challenged asthmatic rats, OLC PC2 = PC (10 mg/kg, i.p.)-treated OVA-LPS-challenged asthmatic rats and OLC DEXA = dexamethasone (2.5 mg/kg, i.p.)-treated OVA-LPS-challenged asthmatic rats. *** $P < 0.001$ vs NC. ### $P < 0.01$ and ### $P < 0.001$ vs OLC

airway remodeling. It is well reported that activation of the MyD88-dependent intracellular signaling pathway of TLR4 leads to activation of NF- κ B and increases the gene expression of inflammatory cytokines which drive the activation of mucosal DC cascades and airway remodeling.²⁵

In the present study, we assessed additional benefits data of soya PC as compared to dexamethasone. Soya PC showed a beneficial

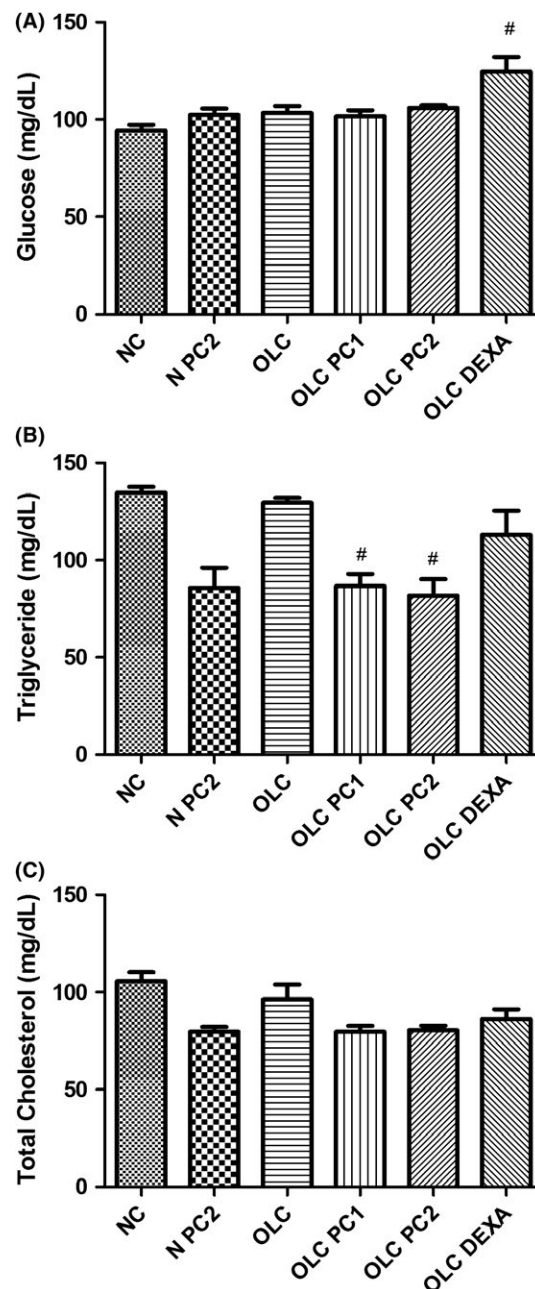


FIGURE 7 Effect of soya phosphatidylcholine (PC) and dexamethasone on serum (A) glucose, (B) triglycerides and (C) total cholesterol in normal and asthmatic rats. NC = vehicle (10 mL/kg, i.p.)-treated normal rats, NPC2 = PC (10 mg/kg, i.p.)-treated normal rats, OLC = ovalbumin (OVA)-lipopolysaccharide (LPS)-challenged asthmatic rats, OLC PC1 = PC (5 mg/kg, i.p.)-treated OVA-LPS-challenged asthmatic rats, OLC PC2 = PC (10 mg/kg, i.p.)-treated OVA-LPS-challenged asthmatic rats and OLC DEXA = dexamethasone (2.5 mg/kg, i.p.)-treated OVA-LPS-challenged asthmatic rats. # $P < 0.05$ vs OLC

effect on lipid profile as compared to dexamethasone without modulating the serum glucose level. In contrast to soya PC, dexamethasone has shown an increase in glucose levels without modulating the serum lipid profile. The previously reported study indicated that treatment of dexamethasone (1 mg/kg) increased serum glucose

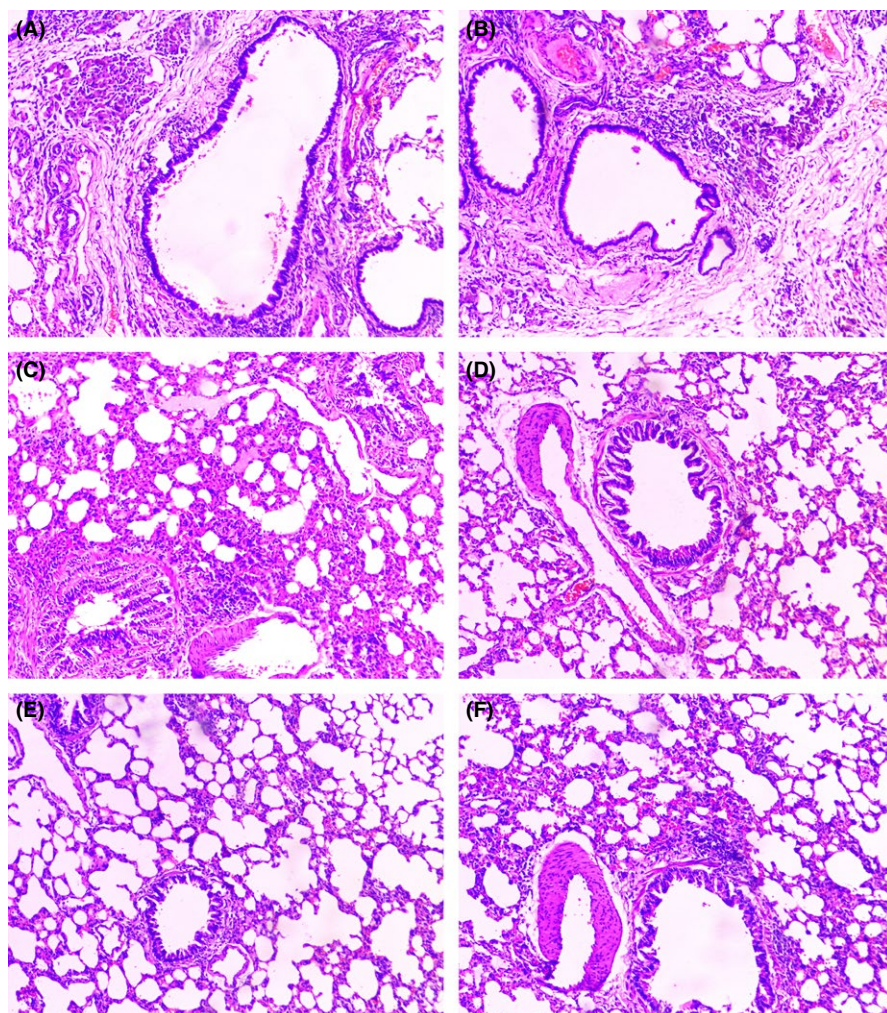


FIGURE 8 Microscopic structure of lung (transverse section) in normal and asthmatic rats. A, vehicle (10 mL/kg, i.p.)-treated normal rats, B, soya phosphatidylcholine (PC) (10 mg/kg, i.p.)-treated normal rats, C, ovalbumin (OVA)-lipopolysaccharide (LPS)-challenged asthmatic rats, D, soya PC (5 mg/kg, i.p.)-treated OVA-LPS-challenged asthmatic rats, E, soya PC (10 mg/kg, i.p.) treated OVA-LPS-challenged asthmatic rats and F, dexamethasone (2.5 mg/kg, i.p.)-treated OVA-LPS-challenged asthmatic rats

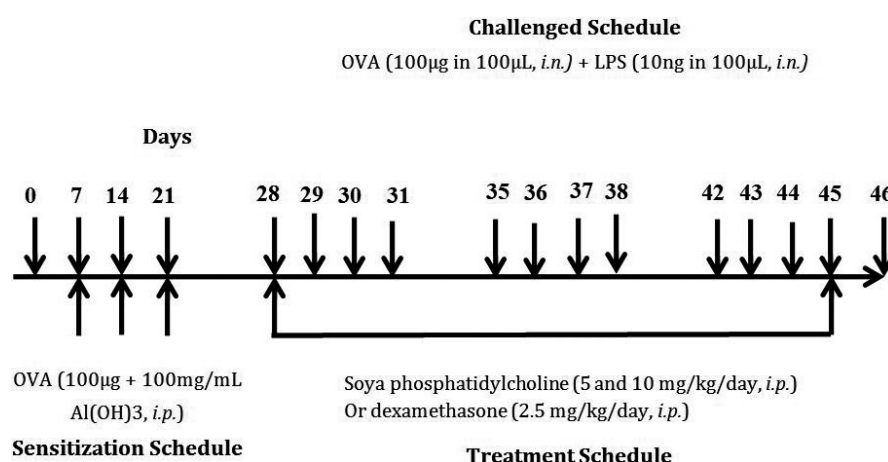


FIGURE 9 Schedule for sensitization, challenged and treatment of rats during the study. Ovalbumin (OVA), lipopolysaccharide (LPS) and soya phosphatidylcholine (PC)

levels and caused insulin resistance in normal rats and increased the risks of cardiovascular complications.²⁶

Ultimately, treatment with soya PC improved lung function parameters, inflammatory cytokine levels, circulating leukocytes count in blood as well as BALf in asthmatic rats. Consequently, this protective action of soya PC may be due to TLR4 antagonist

activity which inhibited TLR4-mediated inflammatory responses and reversed the modulation in lung function parameters, inflammatory cytokine levels and circulating blood cells in asthmatic rats.

In conclusion, treatment with soya PC showed improvement in lung functions parameters, inflammatory cytokine levels and

leukocyte count in both blood as well as BALf in OVA-LPS-induced asthmatic rats. This protective action of soya PC may be due to TLR4 antagonist activity and linoleic acid composition. However, assessment of soya PC effect on TLR4 gene expression and downstream targeting of TLR4 (NF- κ B) is of great interest for future studies.

4 | MATERIALS AND METHODS

4.1 | Animals

The protocol (LMCP/COLOGY/16/01) of the experiment was approved by the institutional animal ethical committee as per the guidance of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Animal Welfare Division, New Delhi, India. Male Wistar albino rats (200–250 g) were procured (Zydus Research Center [ZRC], Ahmedabad, India) for the experiment. All rats were housed at ambient temperature ($22 \pm 1^\circ\text{C}$), relative humidity ($55 \pm 5\%$) and 12/12 hour light/dark cycle. Animals had access to a standard pellet diet and water was given ad libitum.

4.2 | Chemicals

Soya PC (Leciva-S90, polyunsaturated phosphatidylcholine from soya bean source) was given as a gift sample by Vav Life Sciences Pvt. Ltd. Mumbai, India. OVA was purchased from Sigma Chemicals, St Louis, MO. LPS (*Escherichia coli* origin) was given by TRC, Ahmedabad India as a gift sample. Aluminum hydroxide gel (S.D. Fine-Chemical Pvt. Ltd, Mumbai, India), dexamethasone (Dexona injection; Zydus Alidac Ahmedabad, India), heparin (Troyhep; Troikaa Pharma Ltd. Ahmedabad, India), ketamine (Themis Medicare Ltd, Mumbai, India), and kits for estimation of serum glucose, triglyceride and total cholesterol, were purchased from Span Diagnostics Ltd. (Surat, India).

4.3 | Computational study

The 3FXI was selected from a protein databank for the study of the TLR4-MD2 complex. The known ligands such as LPS (*E. coli* origin, TLR4 agonist) and eritoran (TLR4 antagonist) were selected for validation of specific binding on the TLR4-MD2 complex. Based on structural characteristics of LPS and eritoran, three soya PC with different fatty acid chain conformations (16(0)16(0), 16(0)18(2) and 18(2)18(2)) were selected for evaluation of the TLR4-MD2 complex binding activity. Autodock 4, Genetic Algorithm, Lamarckian Analysis were performed for each ligand on 3FXI to characterize their site-specific binding.

4.4 | In vitro neutrophil adhesion assay

The blood was collected from the retro-orbital plexus of normal rats into heparinized tube. Blood samples were treated with LPS (100 ng/mL) in presence and absence of soya PC in doses (10^{-4} – 10^1 $\mu\text{g/mL}$) followed by incubation with 80 mg/mL of nylon thread for 15 minutes at 37°C . One negative LPS control group was kept.

The differences in the neutrophil count before and after the incubation of blood samples were determined. From that, the neutrophil index was calculated. The % neutrophil adhesion was calculated using the following equation.²⁷

$$\text{Neutrophil adhesion (\%)} = (\text{Nlu} - \text{Nlt}) / \text{Nlu} \times 100$$

where, Nlu = neutrophil index of the untreated blood sample and Nlt = neutrophil index of the treated blood sample.

Blood samples were divided into following groups:

1. NC = vehicle treated.
2. LPS = LPS (100 ng/mL) treated.
3. PC 0.001 = PC (0.001 $\mu\text{g/mL}$) + LPS treated.
4. PC 0.01 = PC (0.01 $\mu\text{g/mL}$) + LPS treated.
5. PC 0.1 = PC (0.1 $\mu\text{g/mL}$) + LPS treated.
6. PC 1 = PC (1 $\mu\text{g/mL}$) + LPS treated.
7. PC 10 = PC (10 $\mu\text{g/mL}$) + LPS treated.

4.5 | Induction of experimental asthma in rats

Rats were divided into six groups.

- Group-1: normal rats.
- Group-2: soya PC (10 mg/kg) treated normal rats.
- Group-3: disease control (asthmatic rats).
- Group-4: soya PC (5 mg/kg) treated asthmatic rats.
- Group-5: soya PC (10 mg/kg) treated asthmatic rats.
- Group-6: dexamethasone (2.5 mg/kg) treated asthmatic rats.

Asthma was induced by sensitizing the rats with OVA (100 $\mu\text{g/kg}$, i.p., adsorbed in 100 mg/mL aluminum hydroxide) on the 7th, 14th and 21st days. After sensitization, rats were challenged intranasally for the first 4 days of the week with 100 μL of OVA (1 $\mu\text{g}/\mu\text{L}$) and 100 μL of LPS (0.1 ng/ μL) for 3 weeks. The rats were anesthetized under light diethyl ether and 100 μL of OVA solution was instilled (50 μL in each nostril) with the help of a micropipette, followed by 100 μL of LPS (50 μL in each nostril) at 15-minute time intervals.

Treatment with soya PC (5 and 10 mg/kg/d, i.p.) and dexamethasone (2.5 mg/kg/d, i.p.) were started from the 28th day of the study till the end of the study in respective groups (Figure 9). At the end of the experiment, lung function parameters, total and differential leukocytes count in blood and BALf, inflammatory cytokines (IL-4, IL-5 and IL-13) and metabolic parameters (glucose, cholesterol and triglycerides) in serum were measured in all the rats. Histopathological examination of lungs was performed in all rats.

4.6 | Measurement of lung function parameters

Lung function parameters (respiration rate, tidal volume, and airflow rate) were measured using Respiromax, Columbus Instruments.²⁸ Rats were acclimatized for 15 days before the measurement of lung function parameters.

4.7 | Total and differential leukocytes count

Total and differential leukocytes were measured in blood as well as BALf.

4.8 | Collection of BALf

Rats were anesthetized by subcutaneous administration of ketamine (24 mg/kg, i.p.). A polypropylene cannula was then inserted into the trachea and 0.9% (w/v) phosphate-buffered saline (10 mL) was introduced subsequently into the lungs by means of a 10 mL syringe at 37°C and recovered 5 minutes later. The recovered lavage fluid (5 mL) was centrifuged at 2,016 g for 10 minutes at 4°C. The resultant supernatant was discarded and cells deposited at the bottom of the centrifuge tube were recovered. These cells in the pellet were washed in 0.5 mL saline and sent to the pathological lab for total and differential leukocytes count.²⁹

4.9 | Isolation of serum

Blood was withdrawn from retro-orbital plexus of anesthetized rats (under light diethyl ether anesthesia), then kept at room temperature for 30 minutes, centrifuged at 1000 g for 15 minutes, and serum was isolated by aspiration.

4.10 | Inflammatory cytokines

The inflammatory parameters (IL-4, IL-5 and IL-13) were measured in serum using spectrophotometry based kits (IL-4 RayBio; USA) (IL-5 and IL-13 Bioassay Technology Laboratory, Korain Biotech Co. LTD Shanghai, China).

4.11 | Metabolic parameters

The metabolic parameters (serum glucose, cholesterol and triglycerides) were estimated using spectrophotometry based kits (Span Diagnostic Ltd.).³⁰

4.12 | Histological examination

Dissected lung tissues were washed with normal saline and then placed in 10% (v/v) formaldehyde solution. After the tissues were fixed, lung specimens were cut and stained with hematoxylin and eosin dye. Images of selected sections were captured at 10× magnifications using a zoom digital camera (MLX; Magnus, New Delhi, India).

4.13 | Statistical analysis

All the data obtained from the in vitro and in vivo experiments were expressed as mean ± SEM. Statistical analysis was performed by one-way analysis of variance (ANOVA), followed by Tukey's test using GraphPad Prism 5.0 software (GraphPad, San Diego, CA). $P < 0.05$ was considered statistically significant.

CONFLICTS OF INTEREST

There is no conflict of interest.

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How to cite this article: Thakur VR, Beladiya JV, Chaudagar KK, Mehta AA. An anti-asthmatic activity of natural Toll-like receptor-4 antagonist in OVA-LPS-induced asthmatic rats. *Clin Exp Pharmacol Physiol*. 2018;45:1187-1197. <https://doi.org/10.1111/1440-1681.13002>