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Original article

Carica papaya ameliorates allergic asthma via down regulation of IL-4, IL-5, eotaxin, TNF-α, NF-κB, and iNOS levels

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ABSTRACT

Background: Natural products have a prime importance as an essential source for new drug discovery. *Carica papaya* leaves (CPL) have been used to treat inflammation in traditional system of medicine.
Aim/hypothesis: Current study evaluates the anti-inflammatory and immunomodulatory effects of CPL extract using mouse model of ovalbumin- (OVA) induced allergic asthma.
Methods: All the mice were intraperitoneally sensitized and subsequently given intranasal challenge with OVA except the control group. Group-III and -IV were treated for seven consecutive days with CPL extract and methylprednisolone (MP), respectively. At the end of study, histopathological examination of the lungs was performed and inflammatory cell counts were done in blood as well as bronchoalveolar lavage fluid (BALF). The mRNA expression levels of IL-4, IL-5, eotaxin, TNF-α, NF-κB, and iNOS were measured using reverse transcription polymerase chain reaction (RT-PCR).
Results: Results showed significant attenuation of lung infiltration of inflammatory cells, alveolar thickening, and goblet cell hyperplasia after treatment with CPL extract. We also found significant suppression of total and differential leukocyte counts in both blood and BALF samples of CPL extract treated group. CPL extract also alleviated the expression levels of IL-4, IL-5, eotaxin, TNF-α, NF-κB, and iNOS. Similarly, treatment with MP, used as a reference drug, also significantly ameliorated all the pro-inflammatory markers.
Conclusion: Current study shows that CPL extract possesses anti-inflammatory effect in mouse model of allergic airway inflammation by down-regulating IL-4, IL-5, eotaxin, TNF-α, NF-κB, and iNOS expression levels.

Introduction

Allergic asthma is characterized by mucus metaplasia, bronchoconstriction, chronic inflammation, increased IgE levels, Th2 phenotype skewing, and airway hyperresponsiveness (Fanta, 2009; Willart and Lambrecht, 2009). Despite the fact that pathogenesis and treatment of asthma has been improved, its prevalence is increasing (Mahajan and Mehta, 2006). It affects approximately 300 million people throughout the world and its incidence is increasing in industrialized countries (Taur and Patil, 2011).

Extensive studies on the pathogenic mechanisms have shown that the allergic asthma is generally a T-helper (Th) type 2 mediated disease that leads to an imbalance between Th1 and Th2 cells and their cytokines (Kon and Kay, 1999). Exposure of allergen to the airways enhances the concentration of IL-4 and IL-5 by activating the inflammatory cells. IL-4 is key factor in the differentiation of T cells into Th2 type which generates a Th2 dominant immune response (Deckers et al., 2013; Schuijs et al., 2013). IL-5 plays a critical role in the enhanced generation of eotaxin and eosinophilia in the lung (Zhang-Hoover et al., 2005). Presence of eosinophils in high concentrations produces some of the characteristic features of asthma and their recruitment is linked with the increased presence of eotaxin which serves as a potent chemoattractant for eosinophils (Conroy and Williams, 2001).

Upregulation of adhesion molecules causes the recruitment of eosinophils to the airways. TNF-α is known to increase the expression of

Abbreviations: CPL, *Carica papaya* leaves; IL-4, Interleukin-4; IL-5, Interleukin-5; TNF-α, Tumor necrosis factor-α; NF-κB, Nuclear factor-kappaB; iNOS, inducible nitric oxide synthase; TLC, Total leukocyte count; DLC, Differential leukocyte count; BALF, Bronchoaveolar lavage fluid; Th, T helper cells; OVA, Ovalbumin

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adhesion molecules in the airway epithelium. The role of TNF-α in asthmatic airway inflammation has been established by various *in vivo* and *in vitro* studies (Babu et al., 2004). Strong and extended activation of NF-κB has also been observed in the asthmatic patients. The activation causes the promotion of allergic sensitization to inhaled allergen (Ather et al., 2011; Kato and Schleimer, 2007). Inducible nitric oxide synthase (iNOS) drives the production of nitric oxide (NO) which affects many features of asthma pathophysiology. NO can be favorable in asthma as it may cause relaxation of airway smooth muscles. However, high concentration of NO produced by iNOS can result in mucus secretion in large quantity, vasodilation, edema, and in direct stimulation of Th2 immune response by recruiting eosinophils (Prado et al., 2011).

Inhaled corticosteroids are considered as first-line therapy of persistent asthma. The use of corticosteroids alone in high dose or in combination with long acting β_2 agonists and/or leukotriene modifiers has not been successful to achieve asthma control in all the patients. In addition to the inadequate asthma control associated with current anti-asthma therapy, there are concerns regarding efficacy and drug related adverse effects (Hanania, 2008). Plant extracts are vastly being considered as a source of alternative medicine by both physicians and patients (Markham and Wilkinson, 2004).

Carica papaya L. (family: Caricaceae), commonly known as papaya, is an ancient herbal medicinal plant. Leaves of the plant have been used to relieve asthma in traditional system of medicine (Kovendan et al., 2012). Previously, *C. papaya* leaves have been reported to possess anti-inflammatory properties, and it was proposed that disorders having inflammatory conditions, such as, asthma and arthritis can be treated using *C. papaya* leaves extract (Owoyele et al., 2008). Present study investigates the anti-inflammatory and immunomodulatory effects of *C. papaya* leaves extract using ovalbumin-induced allergic asthma model in mice.

Materials and methods

Preparation of the plant extract

C. papaya L. leaves were collected from the botanical garden of University of the Punjab, Lahore, and were authenticated by Dr. Rasool Bakhsh Tareen, a botanist at University of Quetta. The leaves were cleaned from adulterants and shade-dried for 24 h The dried leaves were ground to powdered form (500 g) and were macerated in absolute ethanol (2 l) for 7 days with occasional shaking daily. The material was successively filtered through muslin cloth and Whatman No. 1 filter paper. The filtrate was evaporated in water bath (maintained at 40 °C) to obtain semi solid extract with approximate yield of 5%. The extract was then stored in a refrigerator at 4 °C for further use (Janbaz et al., 2013, 2014).

Experimental animals

Male *BALB/c* mice (20–25 g) were purchased from National Institute of Health, Islamabad. All the animals were kept in Experimental Research Laboratory, University of Health Sciences, Lahore, at controlled room temperature (22–24 °C), humidity (45–65%), and 12 h natural light/dark cycle. The mice were fed with standard pallet diet and water *ad libitum*. A prior approval of all the experiments was attained from the Ethical Review Committee, University of Health Sciences, Lahore.

Treatment protocol and induction of allergic airway inflammation

Mice were divided into four groups *i.e.* Group-I served as a control group; group-II as diseased group; while group-III and -IV served as treatment groups. Mice in group-III were treated with CPL extract orally (100 mg/kg b.w.) (Owoyele et al., 2008). Group-IV was intraperitoneally treated with methylprednisolone (MP) used as a reference drug (15 mg/kg b.w.).

20 µg of ovalbumin (OVA) (Sigma Aldrich, USA) was dissolved in 2 mg of aluminum hydroxide in a volume of 0.1 ml phosphate buffer saline (PBS). All the mice were intraperitoneally sensitized with OVA at day 0 and day 14 except the control group, which was sham-sensitized with PBS only. One week after the sensitization, the mice were given intranasal challenge with OVA (1 mg/ml PBS) once daily for seven consecutive days *i.e.,* from day 21 to day 27. The mice in the control group were challenged intranasally with PBS only. Treatment with CPL extract and MP was also started from day 21 and continued for 7 consecutive days. Twenty four hours after the last challenge and respective treatment (on day 28), all the mice were sacrificed (Khan et al., 2015).

Inflammatory cells in bronchoalveolar lavage fluid (BALF) and blood

At day 28, blood was collected in EDTA vacutainers using cardiac puncture technique. After taking trachea out with lungs intact, BALF was collected in 1.5 ml tubes by gradual instillation and withdrawal of 1 ml of ice cold PBS through trachea and lungs. Automated hemocytometer (Sysmex XT-1800i) was used to determine total leukocyte count (TLC) and differential leukocyte count (DLC) in BALF and blood (Shabbir et al., 2014).

Histopathological evaluation of lung tissue

Briefly, one lobe of the each mouse lung was fixed with 10% buffered formalin. The tissues were dehydrated with series of graded ethanol, and then embedded in paraffin wax. The blocks were made using paraffin and sections of 5 µm thickness were cut for staining. Tissue sections were stained with Hematoxylin and Eosin (H & E) to observe infiltration of inflammatory cells and vascular congestion, while Periodic Acid Schiff (PAS) staining was used to evaluate goblet cell hyperplasia. According to following semi-quantitative scale, inflammatory score was graded: 0, none; 1, mild; 2, moderate; and 3, severe. Goblet cell hyperplasia was scored according to the percentage of PAS-positive cells among all bronchial epithelial cells: 0, none; 1, $<25\%$; 2, 25 to $<50\%$; 3, 50 to $<75\%$ (Quan et al., 2010).

Determination of mRNA expression levels of IL-4, IL-5, eotaxin, iNOS, TNF-α, and NF-κB

Total RNA from the lung tissue was extracted by using standard TRIzol method (Ashraf et al., 2015). Nanodrop spectrophotometer was used to quantify the total RNA in each sample. cDNA was synthesized using 1000 ng total RNA/sample and kit manufacturer's protocol was followed (ThermoFisher Scientific, America). The concentrations of all the agents were according to the specifications mentioned in our previous publication (Shabbir et al., 2014). Amplification of the cDNA was performed using polymerase chain reaction. Briefly, cDNA template and PCR (2X) master mix (ThermoFisher Scientific, America) were mixed with each other. Sequences of IL-4, IL-5, and TNF-α primers were picked from our previous publications (Ashraf et al., 2015; Rana et al., 2016), while rest of the primers were designed manually or by using Primer3 Input (v. 0.4.0) software (Table 1). 1.0 µl of forward and reverse primers (10 µM) were added in the mixture and the volume was made up to 20 µl with nuclease free water. Thermal cycler was programmed for 35 cycles of denaturation, annealing, and extension. The following cycle profile was used: 95 °C for 10 s, 58-62 °C for 20 s, and 72 °C for 30 s. The annealing temperatures for different genes were set as following: 58 °C for IL-4, IL-5, and TNF-α; 60 °C for eotaxin and NF-κB; and 62 °C for iNOS. PCR product was visualized using 2% agarose gel electrophoresis. ImageJ software was used for densitometry and semi-quantification.