

# Safranal of *Crocus sativus* L. Inhibits Inducible Nitric Oxide Synthase and Attenuates Asthma in a Mouse Model of Asthma

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**The present study involves evaluation of antioxidant potential of *Crocus sativus* and its main constituents, safranal (SFN) and crocin (CRO), in bronchial epithelial cells, followed antiinflammatory potential of the active constituent safranal, in a murine model of asthma. To investigate the antioxidizing potential of *Crocus sativus* and its main constituents in bronchial epithelial cells, the stress was induced in these cells by a combination of different cytokines that resulted in an increase in nitric oxide production (NO), induced nitric oxide synthase (iNOS) levels, peroxynitrite ion generation, and cytochrome c release. Treatment with saffron and its constituents safranal and crocin resulted in a decrease of NO, iNOS levels, peroxynitrite ion generation, and prevented cytochrome c release. However, safranal significantly reduced oxidative stress in bronchial epithelial cells via iNOS reduction besides preventing apoptosis in these cells. In the murine model of asthma study, antiinflammatory role of safranal was characterized by increased airway hyper-responsiveness, airway cellular infiltration, and epithelial cell injury. Safranal pretreatment to these allergically inflamed mice lead to a significant decrease in airway hyper-responsiveness and airway cellular infiltration to the lungs. It also reduced iNOS production, bronchial epithelial cell apoptosis, and Th2 type cytokine production in the lungs. Copyright © 2015 John Wiley & Sons, Ltd.**

**Keywords:** *Crocus sativus*; nitric oxide synthase; cytochrome c; apoptosis; AHR; cytokine.

**Abbreviations:** AHR, airway hyper-responsiveness; iNOS, induced nitric oxide synthase; NO, nitric oxide; NHBE, normal human bronchial epithelial cells; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end; ELISA, enzyme linked immunosorbent assay; TNF  $\alpha$ , tumor necrosis factor alpha; IFN, interferon; DHR, dihydrorhodamine; HPLC, high performance liquid chromatography; BRGM, bronchial epithelial cell growth medium.

## INTRODUCTION

Asthma is a chronic disorder, characterized by airway cellular infiltration, mucus hyper secretion, and airway hyper-responsiveness (AHR; Nakagome and Nagata, 2011, Rayees *et al.*, 2014a). Asthmatic airways are associated with the release of a variety of inflammatory mediators like free oxygen species, chemokines, cytokines, and proteases (Barnes, 2004; Barnes *et al.*, 1998). The release of these mediators recruits different inflammatory cells to the lungs, which in turn further perpetuate the asthmatic condition. Increased nitric oxide (NO) production is another important characteristic feature of asthma (Ten *et al.*, 2006). NO produced during asthma causes bronchorelaxation. However, under certain circumstances, it may have deleterious effects, particularly in conditions where superoxide ion is produced in close vicinity (Vaali *et al.*, 1996). NO may thus react with the superoxide ions and hence leads to the generation of a more toxic molecule, peroxynitrite. Peroxynitrite ion leads to protein degradation, mitochondrial dysfunction, and epithelial cell damage (Mabalirajan and Ghosh, 2013), which is a well

documented phenomenon during asthma (Prado *et al.*, 2011). During asthma, increased expression of iNOS has been shown unequivocally (Riku *et al.*, 2005). Thus, iNOS inhibition is being considered as an important therapeutic intervention for the treatment of asthma (Barnes, 2003).

*Crocus sativus* L. is a flowering plant that belongs to Iridaceae family, and its dried stigma is commonly known as saffron (Hosseinzadeh and Nassiri-Asl, 2013). The constituents of saffron responsible for its color, taste, and aroma are crocins, picrocrocin, and safranal, respectively. All these three components along with other constituents contribute to the pharmacological values of this herb (Tarantilis *et al.*, 1995). Pharmacological studies proved that saffron extract and its components have free radical scavenging and antioxidant properties (Assimopoulou *et al.*, 2005). There is enough evidence from the last 2 decades that have confirmed the antioxidant activity of saffron and its components and suggested saffron to be one of the potent antioxidant agents (Magesh *et al.*, 2006). Saffron extracts have capacity of reducing levels of Reactive oxygen species (ROS) production as effectively as the phenolic antioxidants (Ordoudi *et al.*, 2009). Hosseinzadeh *et al.* (2009) reported the free radical scavenging activity of ethanol solution extract of saffron, safranal, and crocin. Chatterjee *et al.* (2005) reported crocin to be a stronger antioxidant agent than  $\alpha$  tocopherol. Crocetin, the other

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component of saffron, has also been found to be responsible for inhibition of proliferation of lung cancer cells accounting for antitumor activity (Magesh *et al.*, 2009). Recently, it has been demonstrated that saffron shows a neuroprotective effect over oxidative damage induced by quinolinic acid in rat hippocampus (Sadeghnia *et al.*, 2013). Several *in vivo* studies have also confirmed antiinflammatory and antioxidant effects of aqueous and ethanol solution extracts of saffron, safranal and crocin (Hosseinzadeh and Younesi, 2002; Hosseinzadeh and Ghenaati, 2006).

Several other studies have been performed on *C. sativus* or its main constituent safranal, using guinea-pig models, with main focus on lung inflammation and lung pathology. These studies demonstrated that *C. sativus* or safranal has significant therapeutic effects on lung pathology (Boskabady *et al.*, 2012), tracheal hyper-responsiveness and have an inhibitory effect on inflammatory cytokine release, NO, and nitrite production (Boskabady *et al.*, 2014), endotheline-1, and total protein secretion (Gholamnezhad *et al.*, 2013) and recruitment of inflammatory cells to the lungs in sensitized guinea-pigs (Mahmoudabady *et al.*, 2013). Further, *C. sativus* has been demonstrated to increase IFN- $\gamma$  to IL-4 ratio in human lymphocytes and hence affect Th1 and Th2 balance in them (Boskabady *et al.*, 2011). The effect of *C. sativus* extract and its constituent, safranal, has been examined on the inflammatory changes of sensitized guinea-pigs. This study showed preventive effect of the *C. sativus* extract and safranal on total and differential count of White blood cells (WBC) in blood of sensitized guinea-pigs and also suggested safranal to be the main component responsible for these effects (Bayrami and Boskabady, 2012). Byrami *et al.* (2013) also demonstrated the preventive effects of the *C. sativus* extract on tracheal responsiveness and plasma levels of IL-4, IFN- $\gamma$ , total NO, and nitrite in sensitized guinea-pigs. *C. sativus* extract was reported to show preventive effect on tracheal responses, serum levels of inflammatory mediators and also showed increased Th1/Th2 balance. Although, the antioxidant and antiinflammatory activities of saffron and its constituents are well known, we are still far from elucidating the molecular mechanism and the signaling pathway by which saffron mediates its activities during allergic asthma. The earlier models used to evaluate such activities are standard guinea-pig models or human lymphocytes and have been able to successfully explore its pharmacological actions. In this study, we evaluated the antioxidant potential of *C. sativus* and its constituents in normal human bronchial epithelial cells (NHBE) and its antiasthma potential in a murine model of asthma. Here, we demonstrated that safranal, one of the important constituents of saffron, mediates its antioxidant and antiinflammatory activity by decreasing iNOS levels. We have further shown that safranal mediated iNOS inhibition, attenuates asthmatic features in mouse model of allergic asthma.

## METHODS

**Plant material.** The saffron (dried stigmas of *C. sativus*) was collected from the Pampore area of Kashmir province in October and was sun dried followed by grinding.

The identity of *C. sativus* was authenticated by a botanist, and a voucher specimen of the plant (14,988) was deposited in the herbarium of the University of Jammu.

**Preparation of plant extract.** Methanol water extract of saffron stigmas was performed according to Lozano *et al.* (1999) with some modifications. Fifty milligrams of saffron stigmas were suspended in 10 mL methanol–water (50:50, v/v) and stirred for 24 h at 4 °C in the dark. After extraction, samples were centrifuged at 20,000 g for 45 min to eliminate plant residues, and the supernatant was collected and filtered through a 0.45  $\mu$ m nylon membrane (Millipore, USA). The samples were stored in dark till analyzed. The yield of crocin and safranal in the methanol water extract of saffron was 77.46  $\pm$  0.31 and 0.5  $\pm$  0.067 mg/g (dry weight of the tissue), respectively.

**High performance liquid chromatography profiling.** High performance liquid chromatography (HPLC) was used for the detection of the saffron components, analysis was performed with a Waters (Williamsburg, USA) HPLC system equipped with 515 quaternary gradient pump, 717 Rheodyne injector, 2996 PDA detector, and Empower software (version 3.0). The column used for separation was RP-18 (4.6  $\times$  250 mm, 5  $\mu$ m; Merck) column. The mobile phase consisted of methanol–water (50:50, v/v) delivered at a flow rate of 0.8 mL/min. Crocin and safranal (purity > 88%) used in the study were purchased from Sigma Aldrich.

**Cell culture and test drug treatment.** Normal human bronchial epithelial cells (NHBE), used for the study were purchased from Lonza (Lonza Walkersville, Inc. USA) and maintained as recommended. Briefly, NHBE cells ( $5 \times 10^5$ ) were grown in the recommended media (Bronchial Epithelial Cell Growth Medium (BEGM) Lonza) and were maintained at 37 °C in a 5% CO<sub>2</sub> incubator. TNF- $\alpha$ , IL1- $\beta$ , and IFN- $\gamma$  were purchased from BD Biosciences and used at 2 ng/mL (IL1- $\beta$ ), 1 ng/mL (IFN- $\gamma$ ), and 10 ng/mL (TNF- $\alpha$ ) as described previously (Bruewer *et al.*, 2005; Kuruvilla and Kartha, 2009; Verma *et al.*, 2012). Safranal and crocin (Sigma) were used as per the concentrations mentioned within the results.

**Measurement of nitric oxide and peroxynitrite.** Normal human bronchial epithelial cells ( $1 \times 10^5$ ) were grown in six well plates, incubated with different concentrations of saffron, and were induced stress with respective inducers for 1 h. NO was measured by a fluorescent dye, DAF-FM (Life technologies, USA). After the experiment, cells were incubated with the dye (5  $\mu$ m) for 30 min, washed once with Phosphate-buffered saline (PBS) buffer and then the fluorescence was measured by flow cytometry (BD, FACS Caliber, New Jersey, USA). Fluorescence intensity of NO was quantified, and data was shown as mean of three different experiments.

For peroxynitrite ion measurement, 123 dihydro-rhodamine (DHR; Life technologies, Carlsbad, USA) was used. NHBE cells were incubated with saffron and

stimulated with cytokine combination for 1 h and then with 123 DHR for 20 min and after that analyzed by flow cytometry. Apoptosis was measured in cells by flow cytometry after staining cells with Annexin V (BD Biosciences New Jersey, USA) as per the protocol. Briefly, after the induction, cells were incubated with Annexin V for 15 min, and fluorescence was measured by flow cytometry.

**Measurement of induced nitric oxide synthase.** Normal human bronchial epithelial cells ( $1 \times 10^5$ ) were incubated with SAF, SFN, and CRO and stimulated with TNF- $\alpha$ , IL1- $\beta$  and IFN- $\gamma$ . Twenty-four hours after the stimulation of these cells, flow cytometry was used to measure iNOS levels. Briefly, the cells were trypsinized, washed with PBS, fixed with 2% paraformaldehyde for 15 min and then permeabilized with 0.1% triton X-100 for another 15 min. Blocking was performed with 10% Fetal Bovine Serum (FBS) and then cells were incubated with iNOS primary antibody (Abcam) for 3 h. After the primary antibody incubation, cells were washed with PBS (three times) and stained with anti-rabbit antibody conjugated with Alexa Fluor 488 (Life technologies, Carlsbad, USA). After 45 min of incubation with the secondary antibody, cells were washed (three times) and resuspended in PBS. Fluorescence was measured by flow cytometry using Fluorescein isothiocyanate (FITC) filter.

**Measurement of cytochrome c release.** Normal human bronchial epithelial cells ( $1 \times 10^5$ ) were incubated with SAF and stimulated with the earlier mentioned cytokine combination, which was followed by mitochondrial and cytosolic protein extraction. The total cytosolic protein was used for measurement of cytochrome c release (R&D Systems, Minneapolis, USA) as described earlier (Rahman and MacNee, 2000). Briefly, 5  $\mu$ g of cytosolic protein was used in duplicates followed by 2 h incubation at room temperature, followed by washing. After washing with wash buffer, 200  $\mu$ L of cytochrome c conjugate was added and further incubated for 2 h. Two hundred microliter of substrate solution was added after appropriate washing and 50  $\mu$ L of stop solution was used to stop the reaction. The micro plate reader set at 450 nm was used within 30 min of adding the stop solution.

**Animal experiments.** Balb/c mice (7–10 weeks old) were obtained from Indian Institute of Integrative Medicine-CSIR, Jammu, India, and acclimatized for 1–2 weeks before starting the experiments. Mice and all procedures were reviewed and approved by the Institutional Animal Care and Ethics Committee under approval number: 67/CPCSEA/99.

**Mouse model of asthma.** Mice were segregated into five groups and each ( $n=6$ ) was named according to sensitization/challenge/treatment: SHAM/PBS/VEH (normal controls, PBS used as vehicle-VEH), ovalbumin/OVA/VEH (allergic controls, OVA, chicken egg ovalbumin, Grade V, Sigma), OVA/OVA/SFN (safranal, 1 mg/kg and 10 mg/kg), and OVA/OVA/DEX (Dexamethasone, 1 mg/kg). Dexamethasone was dissolved in double

distilled water, while as final dilution of safranal was made in ethanol and 10% ethanol was used as vehicle.

**Development of mouse model of allergic asthma and drug treatment protocol.** Mice were sensitized and challenged as described by Mabalirajan *et al.*, 2008, Rayees *et al.*, 2014b. Figure 1 shows the schematic protocol for inducing asthma in mice. Briefly, the mice were injected with 50  $\mu$ g OVA in 4 mg aluminum hydroxide or only 4 mg aluminum hydroxide by intraperitoneal route on days 0, 7, and 14. Mice were then challenged with 3% OVA (in PBS or PBS alone) through inhalation from Day 21 to Day 27 (30 min/day) using a nebulizer with a flow rate of 9 L/min (OMRON CX3 Model). Vehicle, safranal, or dexamethasone was given through oral route from Day 20 to 27 once a day.

**Measurement of airway hyper-responsiveness and airway cellular infiltration.** Airway hyper-responsiveness to methacholine (MCh, Sigma, St. Louis, USA) was determined in mice by invasive measurements on anesthetized mice using flexiVent system (Scireq, Montréal, Canada), which integrates the computer controlled mouse ventilator with the measurements of respiratory mechanics as described earlier (Ahmad *et al.*, 2011).

Formalin fixed, paraffin embedded, lung tissue sections were stained with hematoxylin and eosin (H & E) (Sigma, St. Louis, USA), to assess the airway inflammation. Stained sections were observed, and microphotographs were taken with Nikon microscope with camera (Ti-90) (Nikon, Tokyo, Japan). All the images were taken with 10 $\times$  magnification. Inflammation scoring was performed as described earlier (Mabalirajan *et al.*, 2008).

**Measurements of IL-5 and IL-13 in the lungs of mouse model of asthma.** Lung tissue homogenates in triplicates were used for ELISA of IL-5 (BD Pharmingen, San Diego, CA, USA) and IL-13 ELISA (R&D systems, Minneapolis, MN, USA). Results were expressed in picograms and normalized by protein concentrations, as described earlier (Ahmad *et al.*, 2011).

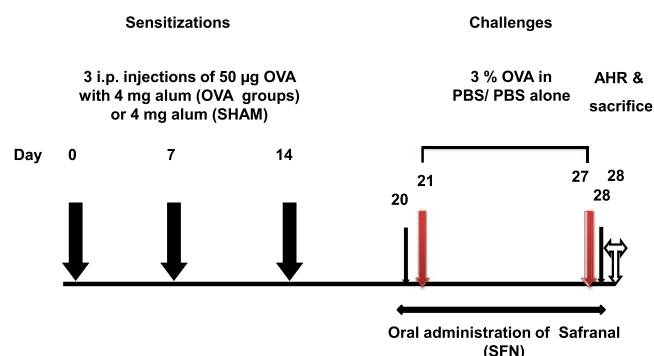


**Figure 1.** Protocol for induction of allergic asthma in mice. The schematic diagram shows the protocol for inducing allergic asthma in mice. Mice were sensitized and challenged using ovalbumin (OVA) as allergen, Alum was used as the adjuvant. After sensitization and 1 day before the challenge, mice were administered safranal (SFN) for eight consecutive days. Safran administration was performed 1 h before every challenge. The mice were sacrificed on day 28th, and airway hyper-responsiveness (AHR) was measured. Ten percent ethanol in which final dilution of safranal was made was used as vehicle.

**Immunohistochemistry.** Immunohistochemistry was performed in the paraffin embedded formalin fixed lung sections. Lung sections were dehydrated, and antigen retrieval was performed with citrate buffer. Blocking was performed with 1.5% goat serum, and after proper washing, sections were incubated with iNOS antibody (Abcam) at 4 °C overnight. After primary antibody incubation, sections were incubated with anti-rabbit secondary antibody for 1 h, washed with PBS and developed with DAB, as described earlier (Ahmad *et al.*, 2011). Images were taken with Nikon (Nikon, Tokyo, Japan) Ti with same magnification (10×). Semi-quantitative analysis was performed using Image J, as described earlier (Ahmad *et al.*, 2011).

**Terminal deoxynucleotidyl transferase dUTP nick end assay for apoptosis.** Terminal deoxynucleotidyl transferase mediated dUTP nick end (TUNEL) assay for apoptosis was performed on lung tissue sections using an apoptosis detection kit (Dead End Calorimetric TUNEL system, Promega, Madison, WI, United States of America), as per the manufacturer's instructions. Briefly, formalin-fixed paraffin embedded lung tissue sections were used for the assay. Quantification of apoptosis was performed by counting the individual number of bronchial epithelial cells per slide per lung section, and the numbers of apoptotic cells were expressed as a percentage of the whole epithelial cell population (apoptotic index) as described earlier (Ahmad *et al.*, 2011).

**Statistics.** Data is shown as mean  $\pm$  SEM and was statistically analyzed using one way analysis of variance followed by *t*-test. Also, the data is a representative of three experiments in case of cell culture experiments and two experiments for *in vivo* studies. The  $P < 0.05$  was considered to be statistically significant.

## RESULTS

### Saffron reduced iNOS levels in primary airway epithelial cells

A combination of cytokines (TNF- $\alpha$ , IL-1 $\beta$ , and IFN- $\gamma$ ) leads to increased NO production in NHBE, while individually they were less effective (Fig. 2(a)). Effect of saffron on NO production in these cells was evaluated by treating the cells with the earlier combination of cytokines and saffron. Saffron was found to show significant suppression of NO production (Fig. 2(b)). NHBE cells treated with saffron alone did not show any cytotoxicity, which was evaluated by flow cytometry using acridine orange and propidium iodide stained cells (data not shown). In order to find the source of NO, we measured the levels of iNOS by flow cytometry. Induction of cells with TNF- $\alpha$  (20 ng/ml), IL-1 $\beta$  (1 ng/ml), and IFN- $\gamma$  (1 ng/ml) significantly increased iNOS levels after 24 h. Saffron, pretreatment (1 h before induction with TNF-

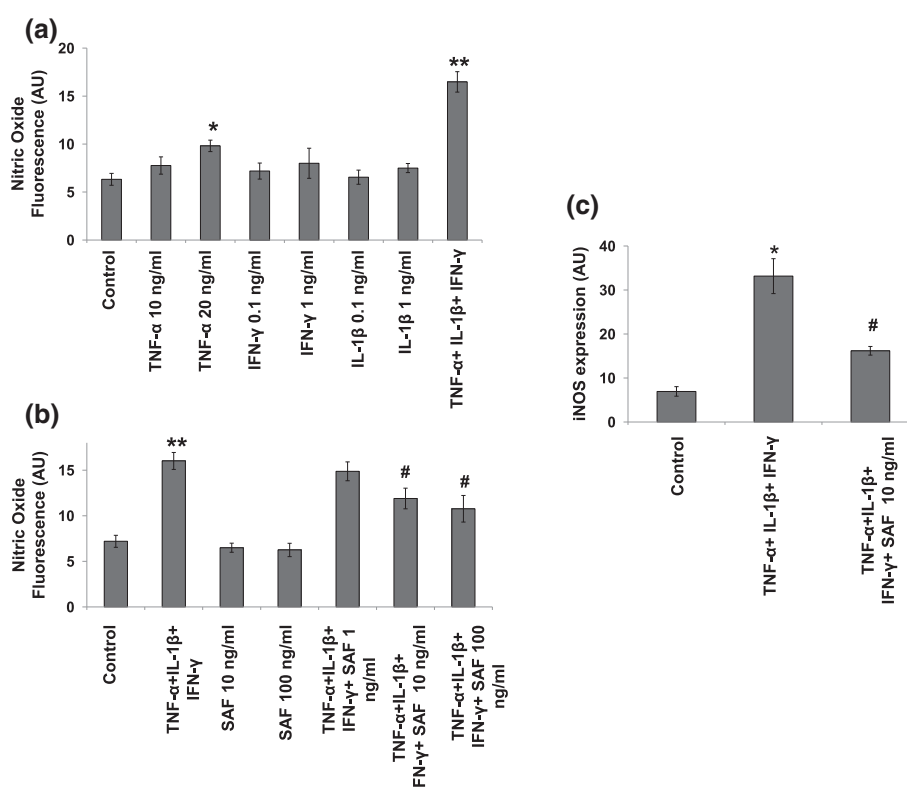


**Figure 2.** Saffron treatment reduces induced nitric oxide synthase (iNOS) levels in epithelial cells. Different concentrations of TNF- $\alpha$ , IL-1 $\beta$  and IFN- $\gamma$  were used to induce nitric oxide (NO) production as measured by a fluorescent nitric oxide detection probe using flow cytometry. (a) A combination of TNF- $\alpha$  (20 ng/mL), IL-1 $\beta$  (1 ng/mL), and IFN- $\gamma$  (1 ng/mL) induced a significant amount of NO production. (b) Saffron (SAF) significantly reduced the NO production in cytokine-induced epithelial cells, while it had no effect on uninduced cells. \* denotes  $P < 0.05$ , \*\* denotes  $P < 0.01$  versus control and # denotes  $P < 0.05$  versus TNF- $\alpha$ , IL-1 $\beta$ , and IFN- $\gamma$  induced cells. (c) FACS histogram shows the iNOS levels, which was significantly induced by a combination of TNF- $\alpha$  (20 ng/ml), IL-1 $\beta$  (1 ng/ml), and IFN- $\gamma$  (1 ng/ml), saffron (SAF) and its constituents safranin and crocin treatment significantly reduced the iNOS levels.

$\alpha$ , IL-1 $\beta$ , and IFN- $\gamma$ ) significantly reduced iNOS levels, as measured by flow cytometry, and data was expressed as mean fluorescence intensity of iNOS (Fig. 2(c)).

### Saffron treatment attenuated bronchial epithelial cell apoptosis

As NO is an important constituent for peroxynitrite ion production, we next measured peroxynitrite ion levels in the TNF- $\alpha$ , IL-1 $\beta$ , and IFN- $\gamma$  treated cells by 123 DHR fluorescence (Fig. 3(a)). Cells incubated with this cytokine combination resulted in significant increase in peroxynitrite ion formation, which was attenuated by saffron treatment (1h before induction with TNF- $\alpha$ , IL-1 $\beta$ , and IFN- $\gamma$ ; Fig. 3(a)). Increased peroxynitrite ion levels were also associated with increased cytochrome c release and epithelial cell apoptosis (Fig. 3(b) and (c)). Treatment of cells with saffron prevented both cytochrome c release and epithelial cell apoptosis shown by annexin V staining using flow cytometry (Fig. 3(b) and (c)). From the earlier results, it became evident that saffron has a potential to reduce iNOS levels and hence NO production. We next determined the role of its individual constituents (saffronal and crocin) on NO production. Saffronal treatment leads to a concentration dependent decrease in NO levels while as crocin was not much effective (Fig. 3(d)). Furthermore, the expression of iNOS was highly reduced by saffronal, although crocin also showed significant reduction in iNOS levels, but the effect of saffronal was more compared with crocin (Figs. 4(a1) and (a2)). All these effects were well reflected by decrease in mitochondrial ROS (mtROS) by saffronal treatment (Fig. 4(b)). From these results, it becomes clear that saffronal is an important constituent in the saffron associated with decreased NO production.

### Saffronal administration attenuates asthmatic features in mouse model of asthma

An ovalbumin-induced mouse model of asthma was developed (as described in materials and methods and Fig. 1), which was characterized by increased AHR measured in terms of change in airway resistance with methacholine treatment (Fig. 5(a)). Ovalbumin treatment also leads to increased airway cellular infiltration into the lungs, increased epithelial cell thickening, bronchial contraction, and mucus hypersecretion. Saffronal treatment significantly reduced both AHR (Fig. 5(a)), airway cellular infiltration measured by inflammation score, epithelial cell thickening (Fig. 5(b1) and (b2)), bronchial contraction, and mucus hypersecretion (data not shown). Hematoxylin and eosin staining was performed and the slides were evaluated by two different investigators blindly. Total inflammation score was calculated by addition of both peribronchial and perivascular inflammation scores and number of cells. Inflammation scoring was performed as described earlier (Mabalarajan *et al.*, 2008). Briefly, a grade of 0 or 1 was assigned with no or for occasional inflammatory cells, and grades 2 with 1–2 cell layers, grade 3 with 2–3 cell layers, and grade 4 with 4–5 cells, respectively.

### Saffronal treatment reduces iNOS levels and epithelial cell injury during asthma

As shown under culture conditions, saffronal leads to a significant decrease in NO levels; here, we show that asthma-induced increase in iNOS is significantly reduced in the lungs of allergically inflamed mice (Fig. 6(a) and (b)). Reduction in iNOS levels was also associated with decreased airway cellular infiltration and Th2 type of cytokine production (Fig. 6(c) and (d)). As epithelial cell injury is an important feature of asthma and increased peroxynitrite ion production is associated with epithelial cell apoptosis, we next determined the role of saffronal on epithelial cell injury. Epithelial cell injury measured in terms of TUNEL positive cells was increased during asthma and saffronal treatment prevented the epithelial cell injury in mouse model of asthma (Fig. 6(e)). These results indicate an important antiinflammatory role of saffronal by reducing iNOS levels and hence preventing damage to epithelial cells during asthma.

## DISCUSSION

Allergic asthma is one of the most common and miserable respiratory disorder, characterized by cellular infiltration, mucus hypersecretion, and AHR. During allergic asthma, infiltrated immune cells secrete various cytokines, free radicals, and proteases in order to get rid of the allergens/antigens entering the airways, but under conditions of the persistent allergen/antigen attack, these secretory products also damage the normal lung structural cells, particularly the epithelial cells (Andrew and Teresa, 2007). The lung epithelial cells play a critical role in perpetuating allergic asthma. Damaged epithelial cells elicit immune response, secrete chemokines and cytokines, produce ROS, and release NO (Boskabady *et al.*, 2014). Reactive oxygen species are mostly produced by mitochondria, and any damage to mitochondria enhances its ROS production, thereby causing epithelial cell damage, which further aggravates allergic asthma (Mabalarajan *et al.*, 2008). Mitochondrial dysfunction is emerging as one of the prominent causes of epithelial cell damage, which aids in the perpetuation of allergic airway inflammation. A number of studies have shown that epithelial cell specific mitochondria damage elicits a number of respiratory disorders, while as restoring mitochondrial dysfunction attenuates acute lung injury and allergic asthma (Mabalarajan *et al.*, 2008; Ahmad *et al.*, 2014; Islam *et al.*, 2012). Thus, a drug, which can restore mitochondrial dysfunction and prevent epithelial cell damage, will be highly promising in attenuating allergic asthma.

Another important aspect of damaged bronchial epithelial cells and macrophages during allergic airway inflammation is the expression of iNOS. Increased iNOS levels highly correlate with increased NO production during asthma. Besides NO production, damaged epithelial cells or activated macrophages also produce superoxide ions, which then react with the NO leading to the formation of highly reactive peroxynitrite ions (Barnes, 2004). Evidences suggest that mitochondria can also produce NO within or near its membrane and thus the ROS produced in close vicinity to NO leads to the production of peroxynitrite (Ricciardolo *et al.*,

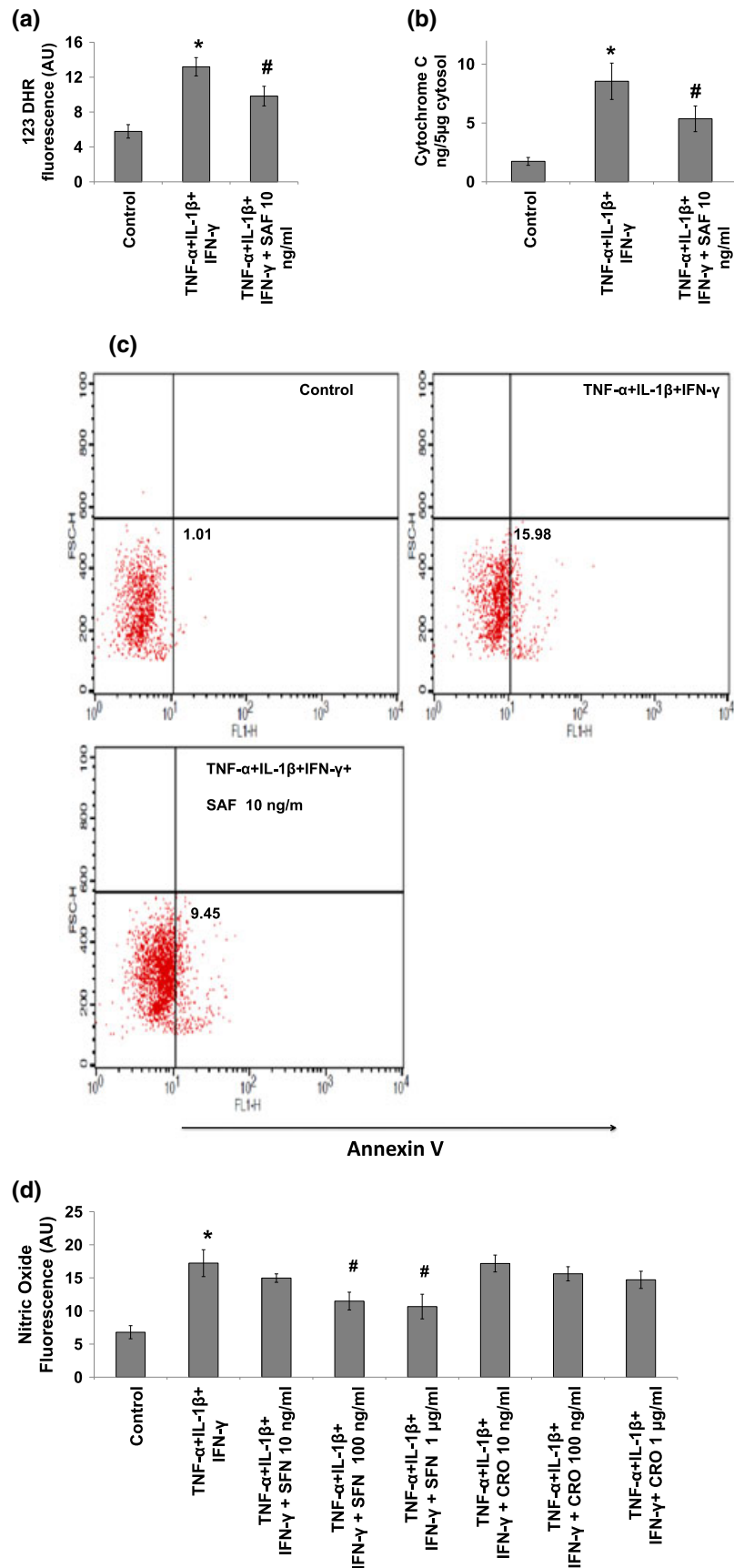
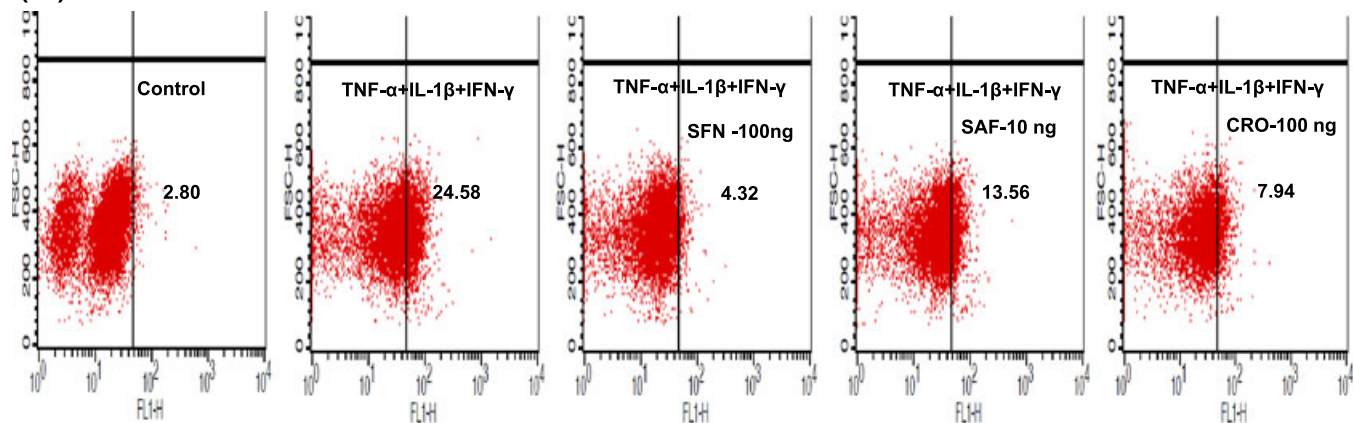


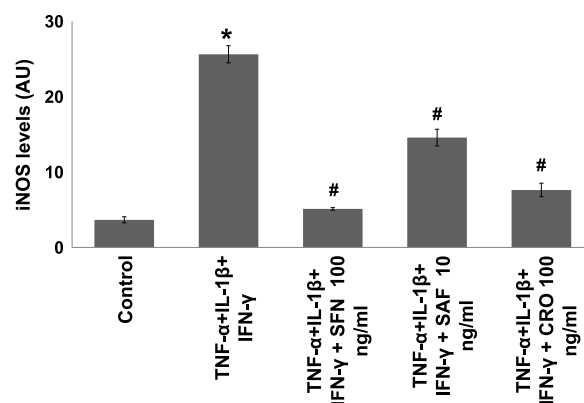
**Figure 3.** Saffron treatment reduces peroxynitrite, cytochrome c release and epithelial cell apoptosis. (a) TNF- $\alpha$ , IL-1 $\beta$ , and IFN- $\gamma$  induced peroxynitrite ion formation as measured by 123 DHR (dihydrorhodamine), by flow cytometry. Saffron treatment reduced peroxynitrite ion formation. (b) Shows mitochondrial dysfunction, measured in terms of cytochrome c release in the cytosolic fractions by ELISA. Saffron treatment reduced cytochrome c release from mitochondria into the cytosol. (c) Saffron treatment significantly reduced the epithelial cell apoptosis as measured by flow cytometry, using an *in vitro* apoptosis detection reagent, by Annexin v (FITC conjugated). (d) Constituents of saffron (safranal (SFN) and crocin (CRO)) were used to measure the effect on NO production. Safranal treatment dose dependently reduced the NO production, while as crocin does not have any effect on NO production. \* denotes  $P < 0.05$  versus control and # denotes  $P < 0.05$  versus TNF- $\alpha$ , IL-1 $\beta$  and IFN- $\gamma$  induced cells.

(a1)

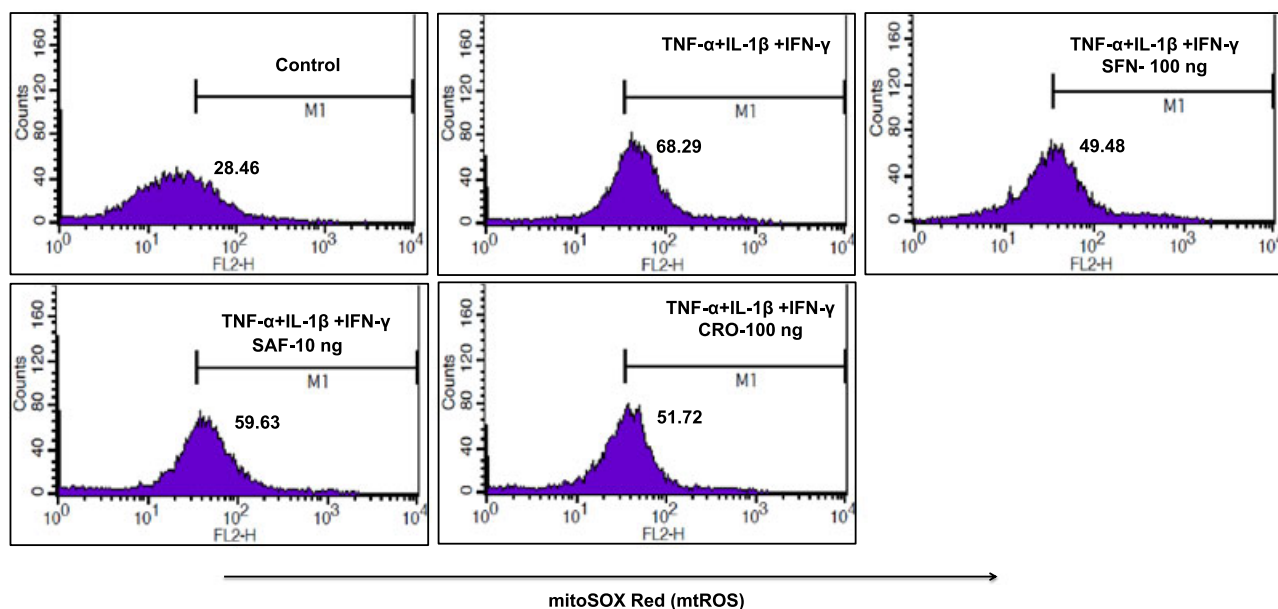


iNOS

(a2)



(b)



mitoSOX Red (mtROS)

**Figure 4.** Safranal treatment attenuated iNOS and mitochondrial ROS. (a1) Safranal treatment reduced the induced nitric oxide synthase (iNOS) levels as shown by dot plot using FACS. (a2) Quantitation of iNOS from the data obtained by FACS. (b) Safranal treatment reduced mitochondrial ROS (mtROS) production, as shown by histograms using FACS. \* denotes  $P < 0.05$  versus control and # denotes  $P < 0.05$  versus TNF- $\alpha$ , IL-1 $\beta$  and IFN- $\gamma$  induced cells.

2004). Elevated levels of peroxynitrite ions cause lipid oxidation, protein degradation, mitochondrial damage, and DNA fragmentation followed by cell death (Barnes, 2004; Bruewer *et al.*, 2005). Here, we have shown that safranal, an important constituent of saffron,

inhibits iNOS, reduces peroxynitrite levels, restores mitochondrial function, and hence prevents epithelial cell damage.

Saffron has been well documented for its antioxidant and antiinflammatory activity during various disease

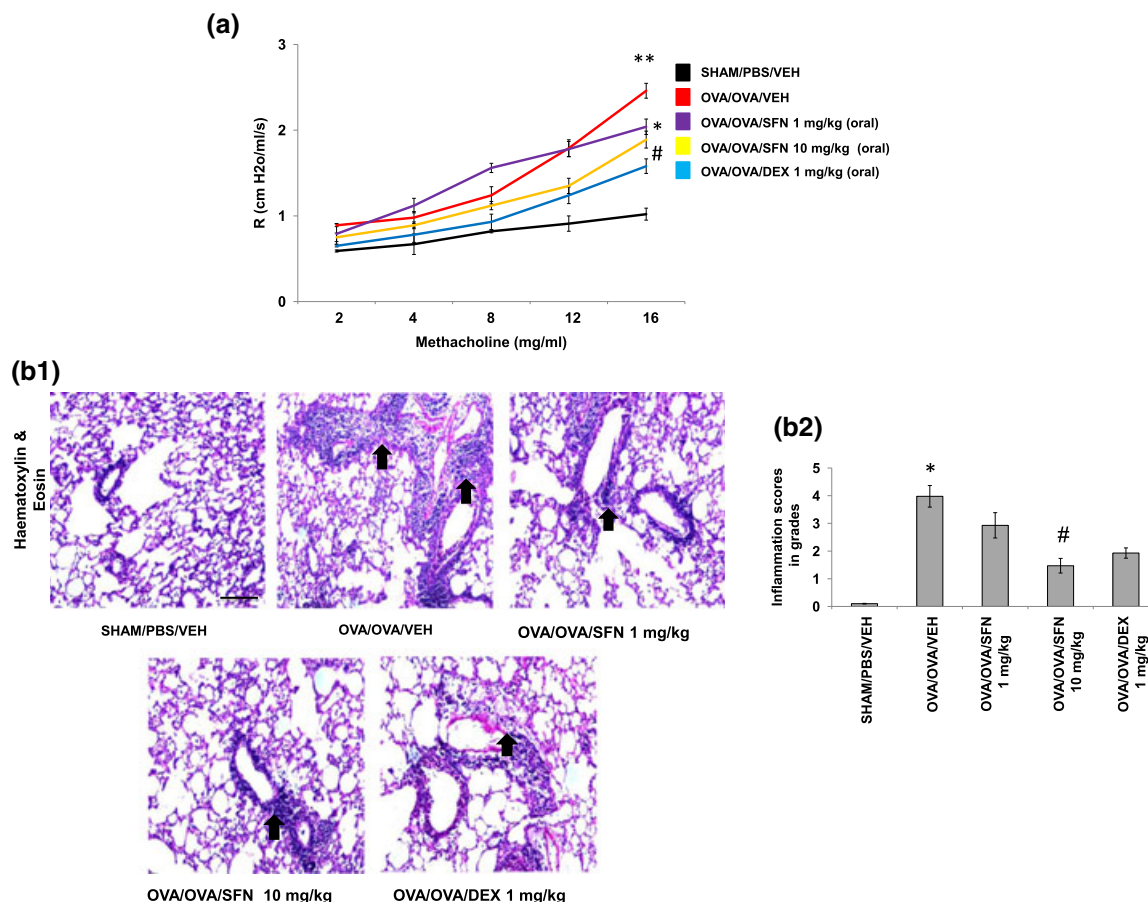


**Figure 5.** Safranal attenuates airway hyper-responsiveness and airway structural changes during allergic asthma. Safranal (SFN) administration reduces airway hyper-responsiveness (AHR) in mice as measured by a methacholine dose responsive cure for airway resistance, denoted by R (a). Safranal treatment significantly reduced airway cellular infiltration as detected by hematoxylin and eosin staining of lung sections (b1). Inflammation scoring was performed to give a quantitative measure (b2). \* denotes  $P < 0.05$  versus SHAM and # denotes  $P < 0.05$  versus OVA.

conditions and has also been shown to lower the cell apoptosis and DNA damage (Aung *et al.*, 2007; Papandreou *et al.*, 2006; Soeda *et al.*, 2001). Several other preclinical studies have demonstrated the efficacy of saffron and safranal in treating various disease conditions, especially related to respiratory system; however, most of them were performed in guinea-pigs or lymphocytes. Taking a lead from these studies, we tested the hypothesis that saffron may interfere with the peroxynitrite ion production in human bronchial epithelial cells and hence prevent the epithelial cell damage, which in turn will hamper the inflammatory cell infiltration into the lungs, during asthma using a murine model. We first used a cell culture approach where we showed that treatment of human bronchial epithelial cells with a cytokine combination induced iNOS expression and increased NO production (Fig. 2(a)). A combination of TNF- $\alpha$ , IL-1 $\beta$  and IFN- $\gamma$  was used to induce robust iNOS expression and hence NO production as described earlier (Bruewer *et al.*, 2005; Kuruvilla and Kartha, 2009; Verma *et al.*, 2012). Furthermore, we found increased peroxynitrite levels with cytokine combination treatment compared with the level generated by these cytokine when used individually. The increased induction of iNOS expression and NO production may be attributed to the synergistic effect of all the three cytokines to generate the higher levels of NO and peroxynitrite. One of the intriguing roles of peroxynitrite is to cause mitochondrial dysfunction and hence cell death. In order

to elucidate the role of peroxynitrite on mitochondrial function and cell death, we measured mitochondrial cytochrome c release (Fig. 3(b)) and quantified the epithelial cell death (Fig. 3(c)). As expected, we found that increased iNOS levels highly correlated with peroxynitrite levels leading to mitochondrial dysfunction and cell death, consistent with previous studies. Various studies have documented saffron for its significant antioxidant and antiinflammatory activities with a prominent role in lowering DNA damage and cell death (Soeda *et al.*, 2001; Vaali *et al.*, 1996; Verma *et al.*, 2012). Here, we tested the hypothesis that saffron may interfere with the peroxynitrite production and hence prevent the epithelial cell damage, which in turn will hamper the inflammatory cell infiltration into the lungs, during asthma. We tested the efficacy of saffron and its constituents under cell culture conditions. We found that safranal, one of the important components of saffron, lowered the NO production in bronchial epithelial cells more significantly than crocin (Fig. 3(d)). Safranal treatment was also associated with restoration of mitochondrial function, DNA damage, and reduced epithelial cell apoptosis (Fig. 3(c), (d) and Fig. 4(b)).

To investigate the *in vivo* efficacy of safranal, we developed a mouse model of allergic airway inflammation, mimicking allergic asthma in humans. Ovalbumin was used as an allergen to induce allergic airway inflammation in mice, which was characterized by increased airway cellular infiltration and AHR. Oral

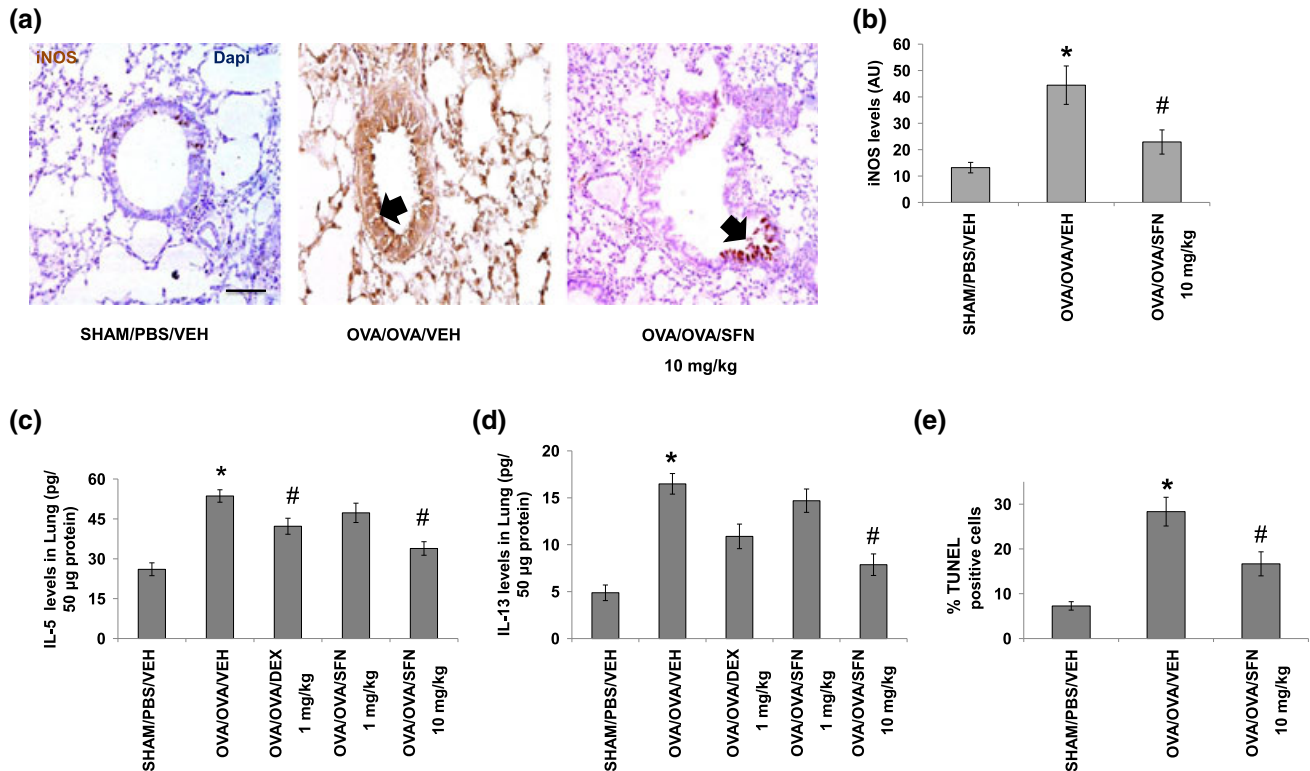


**Figure 6.** Safranal treatment reduced induced nitric oxide synthase (iNOS) levels and inflammatory cytokines in mice lungs. (a) Immunohistochemistry of iNOS in mice lungs showed an increased expression of iNOS in bronchial epithelial cells, which was significantly reduced by safranal treatment. (b) Semi-quantitative measurement showed reduced iNOS levels. Safranal treatment significantly reduced both IL-5 (c) and IL-13 (d) levels in the lung homogenates of ovalbumin sensitized and challenged mice lungs, DEX (Dexamethasone) also reduced the IL-5 and IL-13 levels. (e) Ovalbumin induced bronchial epithelial cell apoptosis, which was significantly reduced by safranal treatment. \* denotes  $P < 0.05$  versus SHAM and # denotes  $P < 0.05$  versus OVA/OVA/VEH.

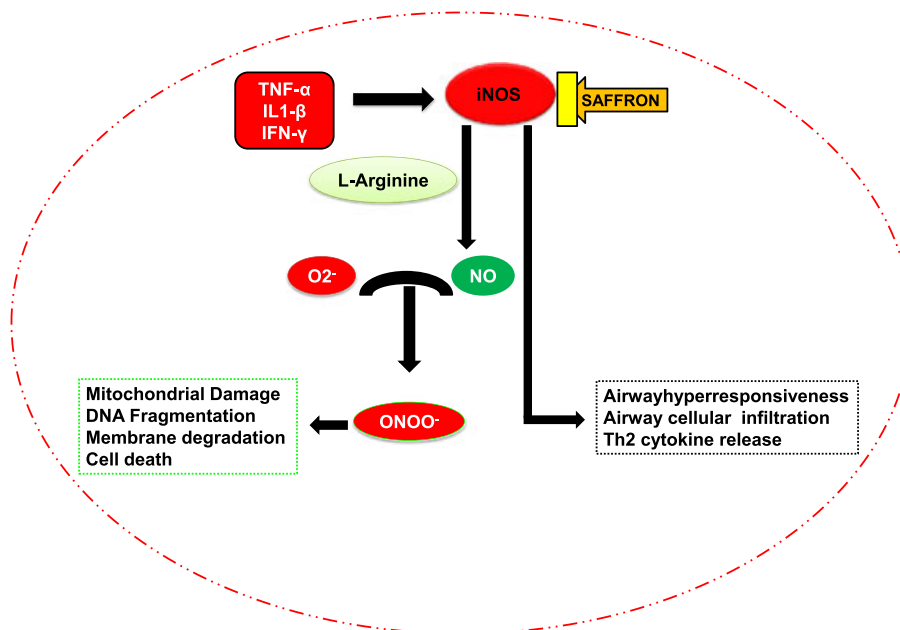


**Figure 7.** Schematic diagram shows the safron mediated attenuation of asthmatic features. During stress (here, asthma) induced nitric oxide synthase (iNOS) expression is induced in cells, which leads to the production of NO. Nitric oxide produced then reacts with superoxide ions ( $O_2^-$ ) and leads to the production of peroxynitrite ions ( $ONOO^-$ ). Peroxynitrite ion leads to mitochondrial damage, DNA fragmentation, and cell death. Damaged cells then release different cytokines, chemokines and recruit other inflammatory cells and hence contribute to asthma pathogenesis. Safron treatment inhibits iNOS levels and thereby attenuates asthmatic features by inhibiting the peroxynitrite ion formation. TNF, tumor necrosis factor.

administration of safranal during allergic airway inflammation was associated with reduced iNOS expression in bronchial epithelial cells and macrophages (Fig. 6). Safranal treatment also led to a significant decrease in airway cellular infiltration and AHR (Fig. 5). Furthermore, a substantial decrease in epithelial cell damage was found with safranal treatment.

Our aim in this study was to investigate the antiinflammatory and antioxidant role of saffron (Fig. 7), and further studies are warranted to investigate the specific signaling pathways and the targeting molecules involved. We do not exclude other possible mechanisms of saffron action, besides its role in reducing iNOS and lowering peroxynitrite levels.

## CONCLUSION

We concluded from this study that saffron and its constituents may have a broad spectrum of action but one of the prominent roles of its constituent, safranal, is to reduce oxidative stress and prevent epithelial cell damage during allergic airway inflammation.

## Acknowledgements

The authors thank the Department of Biotechnology, University of Jammu, for the financial assistance.

## AUTHOR'S CONTRIBUTION

Conceived and designed the experiments: Syed Imran Bukhari, Bijay Ranjan Pattnaik, and Manoj Kumar Dhar.

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Writing and editing of the manuscript: Syed Imran Bukhari, Sheikh Rayees, and Sanjana Kaul.

## Conflict of Interest

The authors declare no conflicts of interest related to this work.

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