

RESEARCH ARTICLE

Anti-asthma potential of crocin and its effect on MAPK signaling pathway in a murine model of allergic airway disease

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Abstract

Context: Crocin, a diterpenoid glucoside, has multitudinous activities such as anti-inflammation, anti-allergy, anti-oxidation and relaxing smooth muscles.

Objective: In this study, the potential of crocin as an anti-asthma agent was investigated in a murine model.

Materials and methods: BALB/c mice were sensitized and challenged by ovalbumin (OVA) to induce allergic airway inflammation, with crocin administered one hour before every OVA challenge. Airway hyper-reactivity was evaluated by lung function analysis systems. Leukocyte counts in bronchoalveolar lavage fluid (BALF) were measured by a hemocytometer and Diff-Quick-stained smears. Lung tissues were stained with hematoxylin–eosin, Congo red and methylene blue for histopathological inspection. Inflammatory mediators in serum, BALF and lung were measured by ELISA or RT-PCR. Effects of crocin on MAPK signaling pathways were investigated by western blot analysis.

Results: Crocin significantly suppressed airway inflammation and hyper-reactivity, reduced levels of BALF interleukin (IL-4), IL-5, IL-13 and tryptase, lung eosinophil peroxidase and serum OVA-specific IgE, and inhibited the expression of lung eotaxin, p-ERK, p-JNK and p-p38 in the OVA-challenged mice.

Conclusions: These results demonstrated that the suppression of crocin on airway inflammation and hyper-reactivity in a murine model, thus crocin might have a great potential to be a candidate for the treatment of asthma.

Keywords

Airway inflammation, airway hyper-reactivity, asthma, crocin, *Crocus sativus*

History

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Introduction

Allergic asthma is a prevalent and severe disease with ever-increasing worldwide incidence. Overall asthma mortality rates have been significantly decreased in developed countries, but increasing in developing countries^{1,2}. Allergic asthma is characterized by airway inflammation, hyper-reactivity, hypersecretion and reversible airway obstruction. A number of effector cells (mast cells, Th2 lymphocytes and eosinophils) and inflammatory mediators (cytokines, chemokines and adhesion molecules) contribute to the development, maintenance and aggravation of asthma^{3–5}.

Glucocorticoids are still the most effective agents in attenuating airway inflammation and airway remodeling. However, their long-term use with high doses often produce side effects such as slow growth in children, low bone mineral density in adults, high blood pressure in the eye and high blood sugar^{6–8}. Therefore, finding highly active anti-asthma drug candidates with no or lower toxicity is of great interest.

Crocus sativus, a commonly used herb for the treatment of asthma in European folk, has been proved to be effective against asthma^{9,10}. Its flower contains one of the primary (active) compound, crocin (Figure 1), a diterpenoid glucoside with activities such as anti-inflammation¹¹, anti-allergy¹², anti-oxidation¹³, anti-hyperplasia¹⁴, decreasing blood glucose and lipids¹⁵, relaxing smooth muscles¹⁶ and regulating the immune system¹⁷. Based on the fact that most of asthma is an allergic and inflammatory disease accompanied by airway obstruction, we examined the anti-asthma potential of crocin in a murine model of allergen-induced acute airway inflammation.

Materials and methods

Materials

Crocin (purity ≥ 98%) was obtained from Energy Chemical. Dexamethasone (purity ≥ 98%) was obtained from Sigma-Aldrich (St. Louis, MO). OVA-specific IgE ELISA kit was obtained from Shibayagi Co., Ltd (Shibukawa, Gunma, Japan). IL-4, IL-5, IL-13 and IL-10 ELISA kits were obtained from Bender MedSystems (Vienna, Austria). Tryptase ELISA kit was obtained from R&D Systems Inc. (Minneapolis, MN). RT-PCR reagents were obtained from Invitrogen

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(Waltham, MA). ERK, JNK, p38, ERK, p-ERK, p-JNK and p-p38 antibodies were obtained from Beyotime Biotechnology (Shanghai, China).

Animal

Thirty-two female BALB/c mice (5–7 weeks) were purchased from Yangzhou Laboratory Animal Center (Yangzhou, China) and reared in animal experiment center of Nanjing University of Traditional Chinese Medicine. Mice were treated according to laboratory guidelines for animal care, and the experimental protocol was approved by the Association for Assessment and Accreditation of Laboratory Animal Care. To accommodate their new environments, mice were provided ad lib with water and standard laboratory diet (Purina Lab Chow) for 2 week prior to experiments.

Antigen sensitization, challenge and treatment

Mice were randomly divided equally into four groups of eight animals each: control, model, crocin and Dex-treatment groups. All mice, except for the control group, all mice were sensitized on days 0, 7 and 14 by intra-peritoneal injection of 0.2 ml of normal saline containing 10 µg ovalbumin (OVA, grade V; Sigma, St. Louis, MO) adsorbed on 2 mg aluminum hydroxide (Sigma-Aldrich, Brøndby, Denmark). Mice in control group only received aluminium hydroxide gel. Two weeks after sensitization, all OVA-sensitized mice were aerosol challenged with 1% OVA in PBS by a nebulizer in an exposure chamber (50 cm × 35 cm × 35 cm), 30 min per day for 5 consecutive days. Mice in control group only received

PBS aerosol. One hour before each OVA challenge, mice in crocin group were intragastrically administered with 100 mg/kg crocin dissolved in 0.5% sodium carboxymethyl cellulose solution (CMC-Na) daily; mice in Dex group were treated with 1 mg/kg Dex by intraperitoneal injection, and control and model groups received intragastric feeding of 10 ml/kg 0.5% CMC-Na (Figure 2).

Evaluation of airway hyper-reactivity

Lung resistance (R_L) induced by acetylcholine chloride (Ach; Sigma-Aldrich, St. Louis, MO), reflecting the development of airway hyperreactivity, was evaluated using AniRes 2005 mouse lung function analysis system (SYNOL High-Tech, Beijing, China) 48 h after the last OVA challenge as described previously¹⁸. Mice were anesthetized with pentobarbital sodium (60 mg/kg) by intra-peritoneal injection, intratracheally intubated, placed in a rodent plethysmograph capable and mechanically ventilated at a tidal volume of 6 ml/kg and velocity of 90 breaths/min. By lung function analysis system, R_L was measured at every increasing doses (10, 30, 90 and 270 µg/kg) of Ach injected via caudalis vein through a micro-infusion pump every 5 min.

Measurement of OVA-specific IgE

Forty eight hours after the last OVA challenge, mice were bled from the retro-orbital venous plexus. Blood sample was centrifuged at 1000 × *g* for 10 min at 4 °C to collect serum. The serum was stored at –70 °C before use for estimation of the production of OVA-specific IgE according to the manufacturer's protocol.

Leukocyte counts of bronchoalveolar lavage fluid

Forty eight hours after the last OVA challenge, mouse bronchial was lavaged thrice by intratracheal instillation of 0.4 ml phosphate buffered saline (PBS). The BALF was centrifuged at 1000 × *g* for 10 min at 4 °C. The sediment of BALF was resuspended in PBS to a final volume of 0.5 ml. Total leukocytes or differential in BALF sediment were counted using a hemocytometer and Diff-Quick-stained smears under light microscopy.

Measurement of inflammatory mediator levels

The supernatants of centrifuged BALF were stored at –70 °C until being used to analyze the production of cytokines. Levels of BALF IL-4, IL-5, IL-13, IL-10 and tryptase

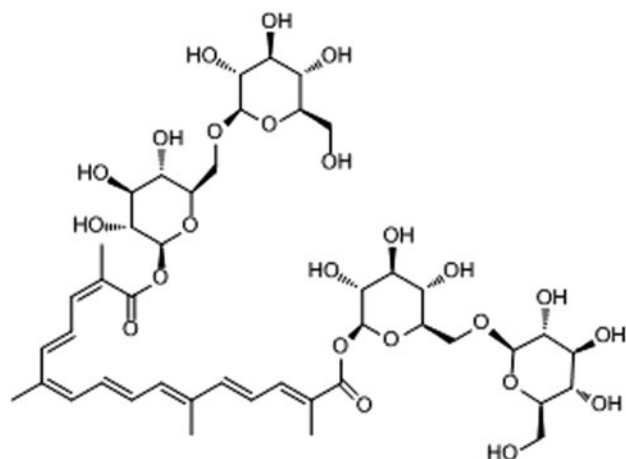


Figure 1. Molecular structure of crocin.

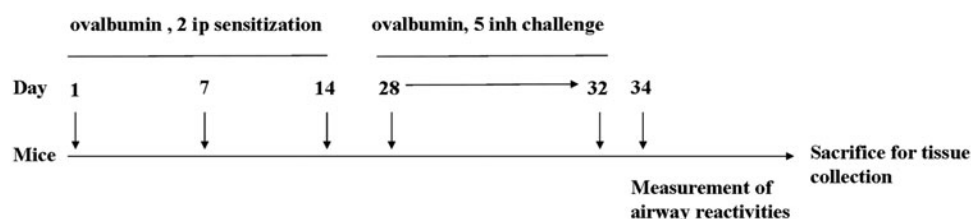


Figure 2. Empirical model-building: Female BALB/c mice were sensitized by intra-peritoneal injection of ovalbumin on days 0, 7 and 14 and challenged their airways by aerosol inhalation for 5 consecutive days from days 28 to induce allergic airway inflammation, with crocin administered 1 h before every ovalbumin challenge. To evaluate the protective effect, mice were given Congo orally from days 28 to 32. On day 34, airway reactivities was evaluated using a mouse lung function analysis systems, and then mice were sacrificed for tissue collection. ip: intra-peritoneal; inh: inhalation.

were measured by ELISA according to the manufacturer's protocol.

EPO activity

Lung EPO activity was measured as described previously¹⁹. Briefly, lung tissues were grinded in liquid nitrogen, centrifuged at $1000 \times g$ under 4°C for 10 min, and then the supernatants were transferred and stored at -70°C before use. The supernatants were thawed and added into 96-well plates at $75\mu\text{l}$ per well, and then a solution of 1.5 mmol/L *o*-phenylenediamine and 6.6 mmol/L H_2O_2 in 0.05 mol/L Tris-HCl (pH 8.0) was added into each well. The substrate reaction was conducted for 30 min and was stopped with $75\mu\text{l}$ of 0.2 mol/L citric acid under room temperature. Absorbance of the reactants was read at 492 nm in a microplate reader (Bio-Rad, Hercules, CA).

Histopathological analysis

After collection of BALF, the low right lobe of the lungs were obtained and fixed in 10% phosphate-buffered formalin, and embedded in paraffin for histological analysis. Tissues were cut into $2\text{-}\mu\text{m}$ sections and stained with H&E, Congo red and methylene blue for analysis of histological changes and detection of some inflammatory cells in lung tissue. For each section, eight visual field of high power lens ($400\times$) were used to calculate the mean number of cells per mm^2 . All images were captured using an Olympus DP50 digital camera (Olympus Optical Co, Sea Essex, UK) through a BX-51 microscope (Olympus American, Melville, NY) equipped with a reference measurement slide. Peribronchial inflammatory cells were quantified by counting the number of inflammatory surrounding airways, and normalizing for airway size, the square root of each component of wall area was divided by the perimeter of the basement membrane.

Reverse transcriptase PCR for eotaxin mRNA

By TRIzol reagent, RNA was extracted from lung tissues following the manufacturer's protocol. Eotaxin first-strand cDNA was generated by the technology of M-MLV reverse transcriptase. The cDNA was amplified using the following primers (downstream and upstream primer, respectively): eotaxin ($5\text{'-CTCTT TGCCC AACCT GGTCTT-3'}$, $5\text{'-GCTCA CGGTC ACTTC CTTCAC-3'}$), β -actin ($5\text{'-GCCGT CAGGC AGCTC GTAGC-3'}$, $5\text{'-GTCAC CAACT GGGAC GACATG-3'}$). PCR was performed using conditions as follows: initial denaturation at 94°C (5 min), 33 cycles composed of denaturation at 94°C (40 s), primer annealing at 56°C (30 s), extension at 72°C (35 s) and a final extension at 72°C (10 min). Products were quantified by 1% agarose gel electrophoresis.

Western blot for MAP kinases

Frozen lung tissue was homogenized with a homogenizer in ice-cold buffer (10 mM Tris-HCl (pH 7.4), 2 mM EDTA, 1 mM EGTA, 10 mM β -mercaptoethanol, 5 mM NaN_3 , $20\text{ }\mu\text{M}$ leupeptin, 0.2 mM PMSF, $0.15\text{ }\mu\text{M}$ pepstatin A, 50 mM NaF, 1 mM sodium orthovanadate, 0.4 nM microcystin and 0.32 M sucrose). The homogenate was centrifuged ($1000 \times g$, 10 min), and the supernatant was transferred to a new tube

and stored at -70°C until use. The total protein contents were determined by the Bio-Rad protein assay. Equal amount of proteins ($45\mu\text{g}$) were dissolved in 10% SDS-PAGE and transferred to polyvinylidene fluoride membrane by electroblotting. Membranes were blocked for 1 h in TBST buffer [0.05 M Tris-Cl (pH 7.5), 0.1% Tween 20, 0.85% NaCl] containing 5% skim milk, incubated with antibodies against ERK, JNK, p38, p-ERK, p-JNK and p-p38 overnight at 4°C , respectively, and then exposed to corresponding HRP-conjugated goat anti-rabbit IgG for 1 h at room temperature. The labeled bands were visualized using electrogenerated chemiluminescent solution.

Statistical analysis

Data are represented as means $\pm$ SEM. The SPSS software for Windows 16 (SPSS Inc., Chicago, IL) was used for statistical analysis. The Kruskal-Wallis and *post-hoc* Dunn's multiple comparison tests were used for non-parametric cases. The one-way ANOVA and *post-hoc* Bonferroni's multiple comparison tests were used for parametric cases. Values of $p < 0.05$ were considered statistically significant.

Results

Effect of crocin on airway hyper-reactivity

To investigate the effect of crocin on airway hyper-reactivity, the R_L of anesthetized mice in response to increasing doses of Ach was determined using a mouse lung function analysis systems. Compared with control group, R_L was dose-dependently increased in model group administered with Ach at dosages ranging from 30 to $270\text{ }\mu\text{g}\cdot\text{kg}^{-1}$ ($p < 0.05$, $p < 0.01$; Figure 3). Ach induced increase of R_L could be

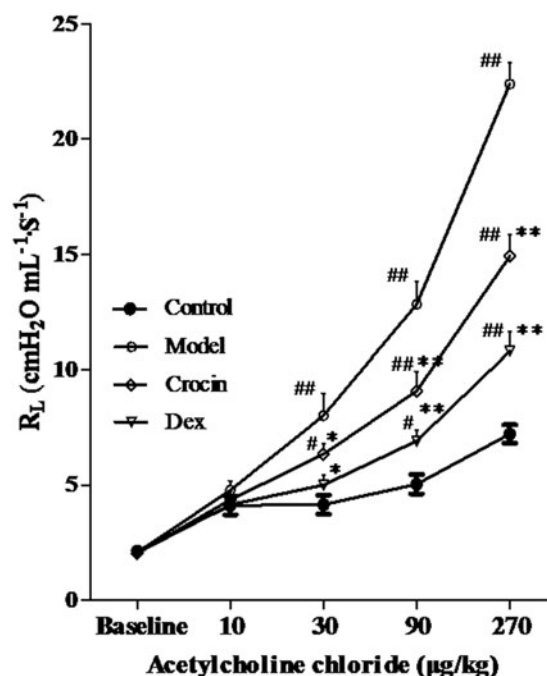


Figure 3. Effects of crocin on lung resistance (R_L) induced by acetylcholine (Ach) in a murine model of allergic airway inflammation. Values are mean $\pm$ SEM of eight mice per group. Significant at: # $p < 0.05$, ## $p < 0.01$ versus the normal group; * $p < 0.05$, ** $p < 0.01$ versus the OVA group. The differences between groups were evaluated by one-way ANOVA followed by Bonferroni's *post-hoc* test.

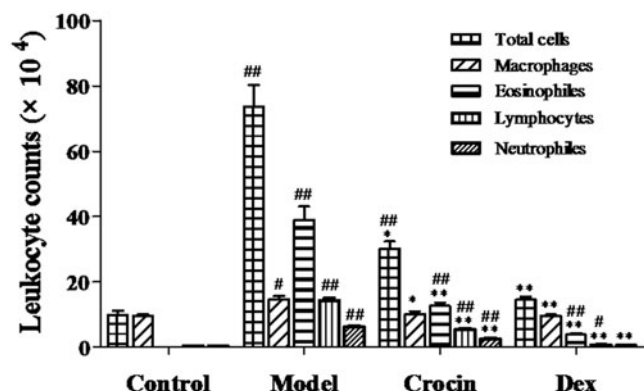


Figure 4. Effects of crocin on the numbers of BALF total leukocytes or differential in a murine model of allergic airway inflammation determined by a hemocytometer or Diff-Quick-stained smears. Values are mean $\pm$ SEM of eight mice per group. Significant at: # $p < 0.05$, ## $p < 0.01$ versus the normal group; * $p < 0.05$, ** $p < 0.01$ versus the OVA group. The differences between groups were evaluated by Kruskal–Wallis followed by *post-hoc* Dunn's multiple comparison tests.

greatly attenuated by treatment of crocin before OVA aerosol ($p < 0.05$, $p < 0.01$; Figure 3).

Effect of crocin on inflammatory cell recruitment into BALF

The acute animal model display a significant increase in the number of inflammatory cells including macrophages, eosinophils, lymphocytes and neutrophils in BALF after repeated OVA challenge. To investigate the curative effects of crocin on allergic asthma, the leukocyte counts, which reflect the severity of pulmonary inflammation, were evaluated in BALF. The numbers of total and differential leukocyte counts in BALF were markedly elevated in model mice as compared with control group ($p < 0.05$, $p < 0.01$; Figure 4). In contrast, the numbers of total and differential leukocyte counts in BALF of OVA-induced groups were significantly decreased by treatment of crocin ($p < 0.05$, $p < 0.01$; Figure 4).

Effects of crocin on OVA-induced lung histological changes

Forty eight hours after the last OVA challenge, lung tissues of mice were taken out and stained with H&E, Congo red and methylene blue for pathological inspection. In model group, mice exhibited a significant peribronchial and perivascular inflammatory infiltrate including eosinophils, and degranulation of mast cell in inflammatory sites ($p < 0.01$; Figure 5). As compared with model mice, the infiltration of inflammatory cell and degranulation of mast cell in inflammatory sites was markedly inhibited by crocin treatment, and bronchi wall and interstitial edema found in the model group was also attenuated ($p < 0.01$; Figure 5).

Effect of crocin on inflammatory mediators

Cytokines play a pivotal role in the development of allergic asthma. Therefore, we examined the effect of crocin on the levels of IL-4, IL-5, IL-13, IL-10 and tryptase in the BAL fluid, and on the levels of EPO in lung and the OVA-specific IgE in serum. Compared with control mice, the levels of IL-4,

IL-5, IL-13, tryptase, EPO and OVA-specific IgE were significantly increased in model mice ($p < 0.05$, $p < 0.01$; Figure 6). Crocin markedly inhibited the levels of IL-4, IL-5, IL-13, tryptase, EPO and OVA-specific IgE in the OVA-challenged group mice ($p < 0.01$; Figure 6), and the levels of IL-13, tryptase and EPO did not showed the statistical significance differences as compared with control mice ($p > 0.05$; Figure 6). The decreased levels of IL-10 in OVA-challenged groups could not be enhanced by crocin treatment ($p > 0.05$; Figure 6).

Effect of crocin on eotaxin

To assess the effect of crocin on chemokines, the expressions of eotaxin mRNA in lung tissues were analyzed by RT-PCR. As compared with control group, these expressions were greatly increased in model group ($p < 0.01$; Figure 7), which could be significantly decreased by treatment with crocin ($p < 0.05$; Figure 7).

Effect of crocin on activities of mitogen-activated protein (MAP) kinases

To investigate the effect of crocin on pathway of MAP kinases, the expressions of p-ERK, JNK, p-JNK, p38 and p-p38 protein were determined. Crocin significantly inhibited the increased level of phosphorylated MAP kinases in lung tissues of OVA-challenged mice ($p < 0.05$; Figure 8).

Discussion

In this study, the anti-asthma potential of crocin from *Crocus sativus* was investigated using a mouse model of acute allergic airway inflammation. Our results showed that crocin could significantly inhibited the recruitment of inflammatory cells to the airways challenged by OVA, which consistent with previous studies of crocin extract on prevention of allergic inflammation⁹. Moreover, the data also showed that crocin could significantly depressed airway hyper-reactivity, and decreased levels of OVA-specific IgE, IL-4, IL-5, IL-13, tryptase and EPO in BALF, serum or lung tissue and inhibited the expressions of eotaxin, p-ERK, p-JNK and p-p38 protein in the OVA challenged mice. These results demonstrated the potent anti-inflammatory effect of crocin in a murine model of allergic airway inflammation.

It is well known that increased production of Th2 cytokines (IL-4, IL-5 and IL-13) play vital roles in the inflammatory mechanism of allergic asthma²⁰. These cytokines are produced by different types of cells such as mast cells, lymphocytes, eosinophils, etc.^{21–24} IL-4 and IL-13 are essential in the switching of the B lymphocyte to IgE production, and in activating alternative macrophage and dendritic cell^{25–27}, and IL-5 is essential for the proliferation, survival and activation of eosinophil^{28,29}. In this study, levels of IL-4, IL-5 and IL-13 were significantly inhibited by the treatment of crocin. Interestingly, the decreased levels of IL-10 in OVA-challenged groups were not enhanced by crocin treatment. Even so, the results still indicated that crocin might effectively suppress the Th2 lymphocytes mediated response in allergic asthma.

Infiltration of mast cells/eosinophilic granule cells features the inflammatory events of asthma³⁰. Mast cells, which play a

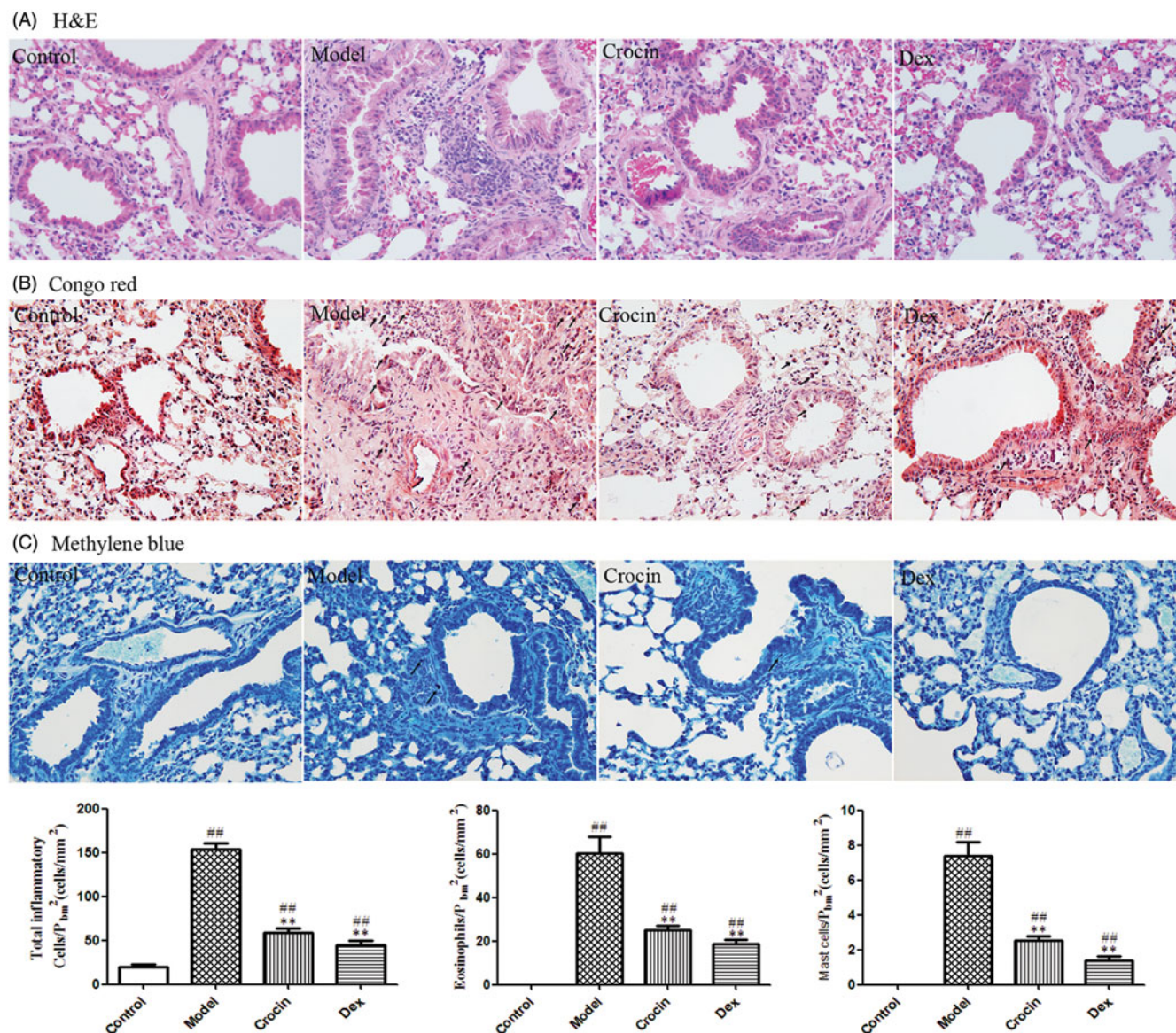


Figure 5. Effects of crocin on the OVA induced airway inflammation evaluated by H&E, Congo red and methylene blue staining in a murine model of allergic airway inflammation (magnification 400 $\times$). Values are mean $\pm$ SEM of eight mice per group. Significant at: ^{##} $p < 0.01$ versus the normal group; ^{**} $p < 0.01$ versus the OVA group. The differences between groups were evaluated by Kruskal–Wallis followed by *post-hoc* Dunn's multiple comparison tests.

pathogenic role in asthma, are recognized as one of the most important factors related to asthma pathophysiology including immediate hypersensitivity, early and late phase inflammation and tissue remodeling³¹. Likewise, infiltrative eosinophils in airway tissues are also recognized as one of the indispensable inflammatory effector cells contribute to asthma pathophysiology³². As a mast cell-specific and predominant protease, tryptase has the potential to induce both early- and late-phase airway responses³³. EPO is exclusively released by eosinophils and can be used as biomarkers for the severity of asthmatic events³⁴. Increased EPO in sputum and lung tissue of OVA-challenged mice is a key player in the generation of diffusible radical species and reactive oxidants by the phagocytes³⁵. Compared with the model group, crocin treatment could lead to an obvious inhibition of both tryptase and EPO in OVA-challenged mice which indicated that crocin might effectively inhibit early- and late-phase airway response by inhibiting the production of these proteases in asthma.

Recruitment of inflammatory cells into the airways is associated with specific chemokines, which are important for selective leukocyte recruitment³⁶. Eotaxin produced by lung fibroblast, epithelial cells and endothelial cells is one of the most efficient chemoattractants, playing an essential role in allergic airway inflammation³⁷. Abolition of eotaxin could markedly inhibit allergic airway inflammation and AHR in murine models³⁸. In this study, OVA-challenged mice treated with crocin were observed with a significant inhibition of eotaxin, which suggested that crocin could effectively inhibit the recruitment of eosinophils and Th2 lymphocytes into the airway by interfering with the expression of eotaxin protein.

Asthma as an airway inflammatory disease is characterized by airway hyper-reactivity, hypersecretion and remodeling, involving in many molecular regulatory pathways³⁹. Previous studies have shown that productions of many pro-inflammatory mediators in human asthma were dependent on MAP kinases⁴⁰. MAPK inhibitors have been reported to inhibit the

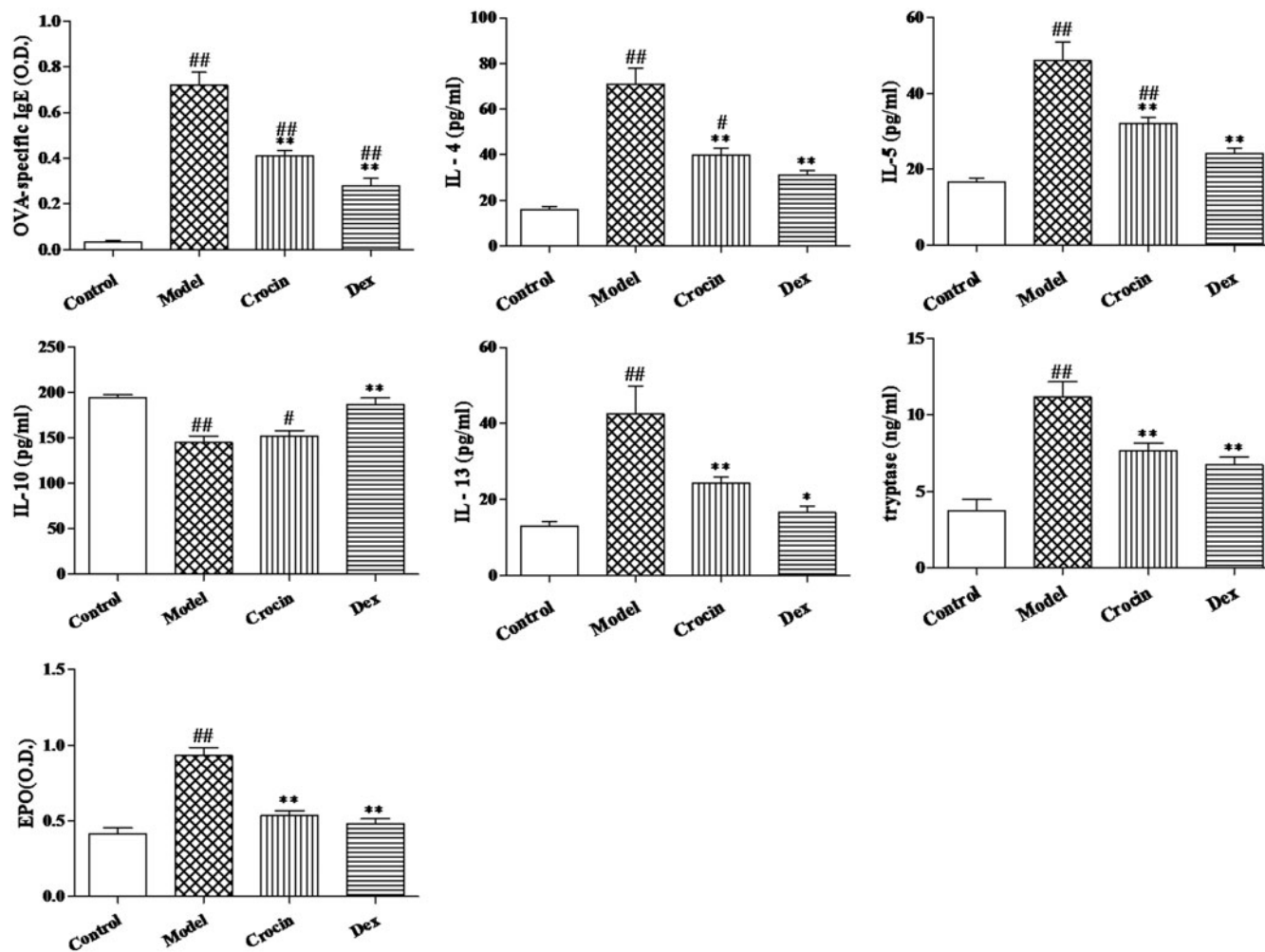


Figure 6. Effects of crocin on levels of OVA-specific IgE, IL-4, IL-5, IL-13, IL-10, tryptase and EPO determined by ELISA or chemical assay in a murine model of allergic airway inflammation. Values are mean $\pm$ SEM of eight mice per group. Significant at: # p < 0.05, ## p < 0.01 versus the normal group; * p < 0.05, ** p < 0.01 versus the OVA group. The differences between groups were evaluated by one-way ANOVA followed by Bonferroni's *post-hoc* test.

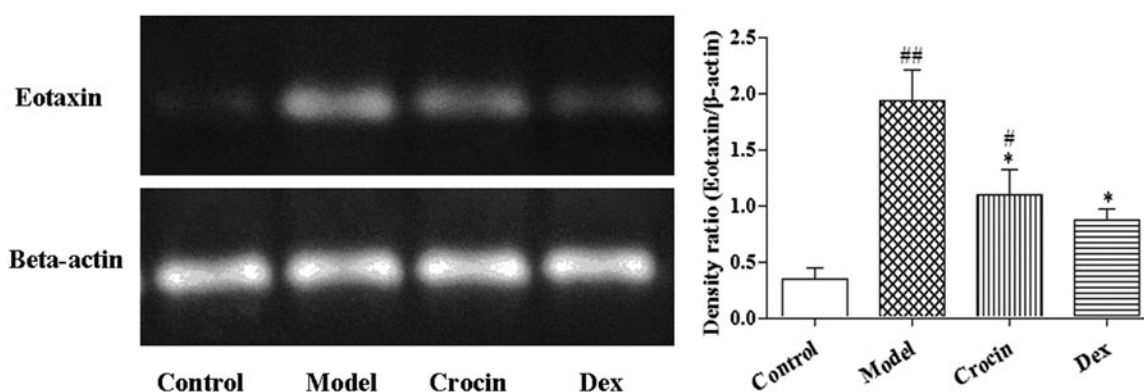


Figure 7. Effects of crocin on lung eotaxin mRNA expression analyzed by RT-PCR in a murine model of allergic airway inflammation. Each data presents three independent experiments. Data represent the mean $\pm$ SEM of three independent experiments (n = 3 per group). Significant at: # p < 0.05, ## p < 0.01 versus control group; * p < 0.05 versus OVA group. The differences between groups were evaluated by one-way ANOVA followed by Bonferroni's *post-hoc* test.

production of IL-4, IL-5 and IL-13 in OVA-challenged mice^{41,42}. The MAPK signaling pathway has been reported to be implicated in eotaxin production from airway smooth muscle cells stimulated by IL-4 and IL-13⁴³. To explore the mechanism underlying the inhibitory effect of crocin on

allergic airway inflammation, three major groups of MAPK including p38 MAPK, JNK and ERK were studied. The activities of p38 MAPK, JNK and ERK were increased due to OVA-challenge, which could be significantly inhibited by crocin. These data suggested that crocin could inhibit MAP

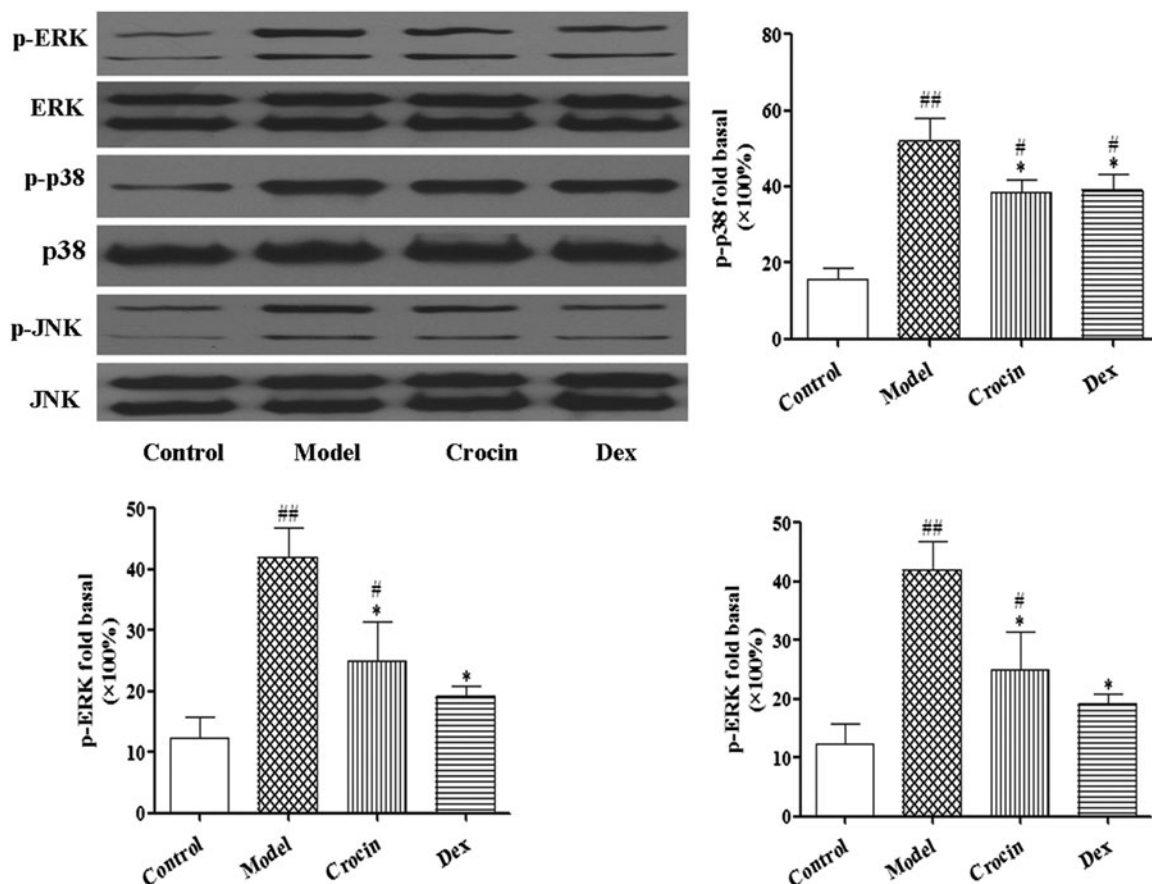


Figure 8. Effects of crocin on the expression of phosphorylated MAP kinase analyzed by western blot in a murine model of allergic airway inflammation. Data represent the mean $\pm$ SEM of three independent experiments ($n = 3$ per group). Significant at: ^{##} $p < 0.01$ versus control group; ^{*} $p < 0.05$ versus the OVA group. The differences between groups were evaluated by one-way ANOVA followed by Bonferroni's *post-hoc* test.

kinase pathways in the lung tissues of OVA challenged mice, thus suppressing the airway inflammation.

In our study, no signs of toxicity were observed in crocin treated mice. Previous studies also found that oral treatment of crocin did not alter behavior of treated mice even at high doses⁴⁴. The median lethal dose (LD₅₀) of crocin in mice is over 3.0 g/kg in our primary acute toxicity testing. These data indicated the safety of crocin, which together with its marked effect in the suppression of airway inflammation, hyper-reactivity and hypersecretion, supported the possible use of crocin as a therapeutic drug for patients incurred with allergic asthma.

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Declaration of interest

The authors declare no conflict of interest.

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