



Effects of anthraquinones from *Cassia occidentalis* L. on ovalbumin-induced airways inflammation in a mouse model of allergic asthma

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ABSTRACT

Ethnopharmacological relevance: *Cassia occidentalis* Linn. is a traditional ayurvedic edible shrub containing anthraquinones (AQs) as the principle active constituents. In folk medicine, it has a variety of uses including treatment of whooping cough ('pertussis') and inflammatory diseases. Despite these applications, limited data are available to validate the effects of *C. occidentalis* AQs on airways inflammation in asthma.

Aim of the study: To explore the anti-inflammatory potential of AQs extracted from *C. occidentalis* using an in vivo model of ovalbumin (OVA)-induced asthma.

Materials and methods: Extraction and optimization of AQs from *C. occidentalis* was performed by mechanicochemistry. Allergic asthma in BALB/c mice was sensitized and challenged by OVA, and the effects of AQs investigated in a mouse model. OVA-specific IgE concentrations in serum, and Th1/Th2 cytokine (IL-4, IL-5, IL-13 and IFN- γ) concentrations, inflammatory cell counts and classification in bronchoalveolar lavage fluid (BALF) were determined. Histopathological evaluation of lung tissue was performed using hematoxylin and eosin (H&E), and periodic acid-schiff (PAS) staining. Th1/Th2 cytokine mRNA expression was analyzed using the 2^{- $\Delta\Delta C_t$} method.

Results: Treatment with AQs decreased inflammatory cell counts and production of Th2 cytokines (IL-4, IL-5 and IL-13) in BALF, and OVA-specific IgE in serum. In contrast, Th1 cytokine IFN- γ production in BALF was promoted. AQs also decreased mRNA expression of Th1/Th2 cytokine in lung tissue. Histological studies demonstrated that AQs substantially inhibited OVA-induced cellular infiltration, mucus hypersecretion and goblet cell hyperplasia in the lung.

Conclusions: These findings demonstrated the inhibitory effects of AQs, derived from *C. occidentalis*, on OVA-induced allergic asthma in mice. The results suggest a promising ethnopharmacological use for AQs in patients with asthma.

1. Introduction

The prevalence of asthma has increased significantly over the past three decades, especially in developed countries (Wu et al., 2016). It has high associated morbidity and mortality, with at least 250,000 deaths worldwide annually (Fahy, 2015). Therefore, it presents a major public health and socioeconomic burden. Asthma is an allergic disease characterized by chronic, reversible airways inflammation, obstruction and hyperresponsiveness. It is also often associated with airways remodeling (Poon et al., 2012). The precipitant for these changes is classically considered to be an imbalance between T-helper cell types 1

and 2 (Th1 and Th2, respectively).

While there is considerable understanding about the pathophysiological processes underlying asthma, there remains much that needs to be elucidated. Current core pharmacological treatments are inhaled glucocorticoids and β_2 -agonists. However, 5–10% of patients do not respond well to these agents or to high doses of oral glucocorticoids such as prednisolone (Adcock and Ito, 2004). Consequently, efforts should be placed upon identifying new anti-asthmatic remedies that are well-tolerated and effectively mitigate the symptoms of asthma. Traditional herbal remedies with basic information on efficacy and safety could serve as the starting point for identifying natural products to treat

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this disease.

Cassia occidentalis Linn. (Caesalpiniaceae) is an edible rainforest shrub with important medicinal values that belongs to the ayurvedic pharmacopoeia (Yadav et al., 2010). It has antioxidant effects (Yadav et al., 2011), which relate to its phenolic content (Arya et al., 2011). It is employed in the treatment of respiratory tract infections in Dominica (Adjanohoun et al., 1985), and used to manage cough (Jain, 1991) and various allergic and inflammatory diseases in Indian traditional medicine (Sadique et al., 1987). Despite these therapeutic applications, limited data are available concerning its effects in allergic asthma and its anti-inflammatory activity in vivo. Phytochemical research has identified anthraquinones (AQs) as the main active constituent in *C. occidentalis*. It has potential properties that include antibacterial, antioxidant, anticancer, anti-allergy and anti-inflammatory effects (Duval et al., 2016). An ethanolic extract of *C. occidentalis* showed antagonistic effects against allergy, inflammation and lipid oxidation thereby reducing edema formation and mast cell degranulation (Sreejith et al., 2010), whereas a methanolic extract induced potent tracheal relaxation (Irié-N'guessan et al., 2011). Collectively, these findings indicate that the AQs found within *C. occidentalis* might have the potential to play a key therapeutic role in managing inflammation and asthma.

Mechanochemistry is an emerging technique that is used to increase the efficiency of extraction by changing the concentration of defects and enhancing the reactivity of solid material and its components (Lomovsky and Lomovsky, 2011). In the present study, the effects of AQs from *C. occidentalis* on allergic asthma in an OVA-induced chronic asthma murine model were investigated after mechanochemical treatment.

2. Materials and methods

2.1. Materials and reagents

C. occidentalis plants were cultivated and harvested by one of the researchers (WK Su) in January 2016 in Hangzhou, China (GPS coordinates: 30°17'44"N and 120°09'41"E), and identified by Professor Guiyuan Lv of Zhejiang Chinese Medical University (Hangzhou, China). A voucher specimen (COL-20160112-205) was deposited at the Key Laboratory of the College of Pharmaceutical Sciences, Zhejiang University of Technology (Hangzhou, China). Dexamethasone was purchased from Aladdin Co. Ltd. (Shanghai, China), Roswell Park Memorial Institute (RPMI) 1640 and Dulbecco's modified eagle medium from Gibco (Grand Island, NY, USA), and lipopolysaccharide (LPS), 3-(4,5-dimethyl tiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) and ovalbumin from Sigma (St. Louis, MO, USA). Griess reagent and phosphate buffered solution (PBS) were obtained from Jiancheng Bioengineering Institute of Nanjing (Jiangsu, China), and ELISA kits for IL-4, IL-5, IL-13, TNF- γ and IgE from R & D Systems (Minneapolis, MO, USA). Female BALB/c micewere purchased from JOINN Laboratories, Inc. (Jiangsu, China). All other reagents were of analytical grade and purity.

2.2. Mechanochemically assisted extraction and optimization

The dried aerial part of *C. occidentalis* was crushed using a Q400B pulverizer (Yuju, Shanghai, China), followed by mechanical treatment through a PM-200planetary mill (Retsch, Haan, Germany). After grinding, the powder was immersed in deionized water, extracted with mechanical stirring at room temperature for 10 min and then centrifuged. As a control, a mixture of unmilled *C. occidentalis* powder and solid reagent was extracted following the same process. Hydrochloric acid was used to adjust the supernatant pH to 3. The supernatant was then centrifuged to obtain the precipitant, which was dried as total AQs and stored at -20°C before use.

AQs were prescreened by UV spectroscopy and the concentration expressed as 1,8-dihydroxyAQ equivalents. The latter was based on

Table 1

Experimental parameters of Box-Behnken design and their total AQ extraction yield by mechanochemically assisted extraction.

Run	Coded Variables				Actual Variables				Yield (mg/g)
	x_1	x_2	x_3	x_4	X_1	X_2	X_3	X_4	
1	1	0	-1	0	450	17.50	10	17.50	7.79
2	0	0	0	0	325	17.50	25	17.50	13.93
3	0	1	-1	0	325	30.00	10	17.50	10.59
4	0	-1	0	1	325	5.00	25	30.00	6.01
5	0	0	1	-1	325	17.50	40	5.00	10.48
6	1	0	0	1	450	17.50	25	30.00	8.05
7	1	1	0	0	450	30.00	25	17.50	9.25
8	0	0	0	0	325	17.50	25	17.50	13.61
9	-1	-1	0	0	200	5.00	25	17.50	4.22
10	0	1	0	-1	325	30.00	25	5.00	11.09
11	0	-1	0	-1	325	5.00	25	5.00	6.16
12	0	-1	1	0	325	5.00	40	17.50	5.99
13	0	0	1	1	325	17.50	40	30.00	9.89
14	0	-1	-1	0	325	5.00	10	17.50	5.88
15	1	0	0	-1	450	17.50	25	5.00	9.25
16	-1	0	0	1	200	17.50	25	30.00	8.29
17	0	1	0	1	325	30.00	25	30.00	10.96
18	0	1	1	0	325	30.00	40	17.50	10.30
19	0	0	0	0	325	17.50	25	17.50	13.61
20	-1	0	1	0	200	17.50	40	17.50	7.04
21	1	-1	0	0	450	5.00	25	17.50	4.81
22	0	0	-1	1	325	17.50	10	30.00	9.52
23	0	0	-1	-1	325	17.50	10	5.00	8.79
24	0	0	0	0	325	17.50	25	17.50	13.61
25	-1	0	-1	0	200	17.50	10	17.50	6.94
26	-1	1	0	0	200	30.00	25	17.50	8.34
27	1	0	1	0	450	17.50	40	17.50	8.15
28	0	0	0	0	325	17.50	25	17.50	13.61
29	-1	0	0	-1	200	17.50	25	5.00	8.33

X_1 for rotational speed (rpm), X_2 for grinding time (min), X_3 for solvent to material ratio and X_4 for amount of solid reagents (%).

extrapolation from a calibration curve of standard 1,8-dihydroxyAQs concentrations (1.8, 3.6, 7.2, 10.8 and 14.4 $\mu\text{g/mL}$) in an equal volume mixture of sodium hydroxide 5% solution and aqueous ammonia 2% at 516 nm. The mechanochemically assisted extraction was further optimized with response surface methodology (RSM).

To explore the effect of independent variables on response within the preset range, a central composite rotate design with four independent variables (X_1 , rotational speed; X_2 , milling time; X_3 , solvent to material ratio and X_4 , loading of solid reagent) at three levels (Dong et al., 2011) was performed, with some modifications (Table 1). The experimental results were fitted to a second-order polynomial model, and the regression coefficients determined.

2.3. Macrophage cell viability assay

The viability of RAW264.7 cells was measured in vitro by MTT assay (Yang et al., 2015). Briefly, macrophages were seeded at 1×10^5 cells per well in 96-well plates with RPMI-1640 media, and 100 μL of different concentrations of AQ (1, 5, 10, 50 and 100 $\mu\text{g/mL}$) was added. The cells were incubated at 37°C in a humidified atmosphere with 5% CO_2 for 24 h. The supernatant was then discarded and 10 μL of combined MTT (5 mg/mL) was added into each well. After incubation for 4 h, the supernatant was removed and 100 μL DMSO was added to dissolve the produced formazan crystals. After shaking gently for 10 min, the absorbance was determined at 570 nm by a microplate reader.

2.4. In vitro anti-inflammatory activity

The murine macrophage cell line RAW 264.7 (2×10^5 cells/well, 1.8 mL/well) was seeded into a 24-well plate, and incubated at 37°C in a humidified atmosphere with 5% CO_2 for 24 h. The supernatant was

then discarded and 1.8 mL of RPMI-1640 media added to each well. After incubation for 4 h, 100 μ L of LPS 1 μ g/mL was incubated alone or with 100 μ L of AQ 1, 5, 10, 50 or 100 μ g/mL for 24 h. The isolated supernatants were then collected for nitric oxide (NO) determination by Griess reaction (Yao et al., 2015). Finally, 100 μ L of each supernatant was mixed with an equal volume of Griess reagent and left at room temperature for 10 min. The absorbance was measured at 540 nm by a microplate reader. NO secretion was calculated according to a least squares linear regression analysis of a sodium nitrite standard curve.

2.5. Animals and ethical statement

Female BALB/c mice, weighing approximately 19–20 g, were housed at room temperature (20–25 °C) and constant humidity (40–70%) under a 12 h light-dark cycle in a SPF grade laboratory. The study was conducted in accordance with the Health Guide for the Care and Use of Laboratory Animals (Ministry of Science and Technology of China, 2006) and was approved by the animal ethics committee of Zhejiang University of Technology.

2.6. Establishment of the allergic asthma model and treatment

The experiment was carried out as described by others (Yang et al., 1998) with some modification (Fig. 4A). After one week for acclimatization, BALB/c mice were randomly assigned into six groups (of at least six mice each) as follows: (1) Normal; (2) OVA; (3) Dexamethasone (5 mg/kg); (4) AQ-1 (250 mg/kg); (5) AQ-2 (500 mg/kg); (6) AQ-3 (2000 mg/kg). The mice were sensitized on days 0 and 14 by intraperitoneal injection with 20 μ g of OVA in PBS mixed with equal volumes of alum as an adjuvant in a total volume of 200 μ L. During the third week (days 15–21), the groups were administered their respective treatments (400 μ L) by mouth every 12 h for 14 doses; for the control (normal) group, carboxymethylcellulose 0.5% was given. On days 22, 23 and 24, the mice were exposed to aerosolized OVA (1% OVA in PBS) or PBS alone for 30 min per day.

2.7. Serum, bronchoalveolar lavage fluid collection and cell enumeration

On day 25, blood was harvested by orbit intubation for determination of IgE concentrations. Serum was obtained by centrifuging (4500 $\times$ g) blood for 10 min at 4 °C, and then stored at –80 °C until analysis. Mice were subsequently sacrificed and the left lungs lavaged with 0.5 mL of autoclaved sodium chloride 0.9% (normal saline) three times. Recovery of the bronchoalveolar lavage fluid (BALF) was about 40–60% (0.6–0.9 mL). BALF was centrifuged (2000 $\times$ g) immediately for 10 min at 4 °C, and the supernatants stored at –80 °C for subsequent testing. The cell pellets were resuspended in PBS, and cytospin preparations of the BALF cells were stained with Giemsa solution before enumerating based on morphology and staining profile. The total number of each cell type was determined by multiplying the percentage by the total number of cells.

2.8. Enzyme-linked immunosorbent assay

Concentrations of OVA-specific IgE in serum, and IL-4, IL-5, IL-13 and IFN- γ in BALF were measured by commercial enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's specifications. The lowest limits of detection were: IL-4 (4 ng/L), IL-5 (7 ng/L), IL-13 (3 ng/L), IFN- γ (30 ng/L) and IgE (0.8 μ g/mL).

2.9. Quantitative real-time PCR

Lung tissue samples (up to 30 mg each) were treated with TRIzol (Sigma, St. Louis, MO, USA), and RNA was isolated according to the manufacturer's instructions. cDNA was synthesized using a reverse transcriptase kit (Sigma, St. Louis, MO, USA) and followed by

quantitative qPCR analysis (SYBR Green; Applied Biosystems, Waltham, MA, USA). The expression of mRNA was normalized to β -actin expression, and the results analyzed using the $2^{-\Delta\Delta C_t}$ method. The primers used were: IL-4 forward, 5'-ACAGGAGAAGGGACGCCAT-3', IL-4 reverse, 5'-GAAGCCCTACAGACGAGCTCA-3'; IL-5 forward, 5'-AGCACA GTGGTGAAGAGACCTT-3', IL-5 reverse, 5'-TCCAATGCATAGCTGGT GATTT-3'; IL-13 forward, 5'-GGAGCTGAGCAACATCACACA-3'; IL-13 reverse, 5'-GGTCCTGTAGATGGCATTGCA-3'; β -actin forward, 5'-GTA TGGAATCCTGTGGCATC-3', β -actin reverse, 5'-CGTACTCCTGCTTGCT GATC-3'.

2.10. Lung histology

Histological examination was used to further assess disease progress. The left lung was removed, washed in phosphate buffer and fixed in formaldehyde 4% buffered with a phosphate solution (0.1 M, pH 7.4) at room temperature. Organ fragments were dehydrated in graded concentrations of ethanol, and then embedded in paraffin. Thick sections of 3 μ m were made from each tissue and stained with hematoxylin and eosin (H&E) and periodic acid-schiff (PAS) to evaluate the general morphology (Di Valentin et al., 2009; Imaoka et al., 2007).

2.11. Statistical analysis

Results are presented as mean $\pm$ S.E.M. One-way ANOVA was used for multiple comparisons, followed by Dunnett's test where further nonparametric analysis was appropriate. A *p*-value less than 0.05 was considered significant.

3. Results

3.1. Extraction and optimization

AQ yields, with and without mechanical treatment, were 6.160 and 0.493 mg/g, respectively. The findings of the preliminary screening experiments, the design matrix using 29 runs of Box-Behnken design and their corresponding responses (yield of AQs) are shown in Table 1, and the significance of each coefficient in Table 2. The determination coefficient ($R^2 = 0.9914$) of the quadratic regression model and the associated *p*-value (Prob > F, < 0.00001) indicated that the model was a good fit and that the model terms were significant. The 3D plots and their respective contour plots visually showed the interactions between two variables and their optimum levels (Fig. 1). With the decrease in the four variables ($X_1 \times X_2$, X_3 and X_4) until a threshold level, the extraction rate was positively affected, while the interactions between any two variables were not significant. Furthermore, initial experimentation showed factors X_1 and X_2 were statistically significant and played a vital role in determining yield. On the basis of the response surfaces, the optimal conditions were predicted as 331.50 rpm rotational speed, 21.82 min grinding time, 25.56:1 ratio of solvent to material, and 16.97% solid reagent. Under optimal conditions, the model gave a maximum predicted yield of 14.1 mg/g. For operational convenience, the extraction parameters were set as 332 rpm, 22 min, 26:1 and 17%, respectively. Triplicate experiments were performed, with a resultant yield of 13.7 mg/g i.e. near to the predicted value.

3.2. In vitro anti-inflammatory activity

An ethanolic extract of *C. occidentalis* leaves has been reported to significantly reduce carrageenan-induced inflammation (Sadique et al., 1987). To assess whether there is a potential positive correlation between AQs and anti-inflammatory activity, we first focused on its effects on stimulated macrophages. We also investigated the influence of test extracts on the viability of RAW 264.7 cells using the MTT test. As shown in Fig. 2, AQ significantly reduced cell viability (80% with respect to untreated controls) at the higher concentrations of 50 and

Table 2
Analysis of variance (ANOVA) of the fitted second-order polynomial model.

Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F	
Model	211.70	14	15.12	114.98	< 0.0001	significant
X_1	1.43	1	1.43	10.89	0.0053	
X_2	62.77	1	62.77	477.32	< 0.0001	
X_3	0.46	1	0.46	3.48	0.0831	
X_4	0.16	1	0.16	1.23	0.2852	
$X_1 \times X_2$	0.025	1	0.025	0.19	0.6717	
$X_1 \times X_3$	0.017	1	0.017	0.13	0.7213	
$X_1 \times X_4$	0.34	1	0.34	2.56	0.1321	
$X_2 \times X_3$	0.041	1	0.041	0.31	0.5836	
$X_2 \times X_4$	2.403E-004	1	2.403E-004	1.827E-003	0.9665	
$X_3 \times X_4$	0.43	1	0.43	3.29	0.0913	
X_1^2	88.90	1	88.90	676.01	< 0.0001	
X_2^2	71.05	1	71.05	540.27	< 0.0001	
X_3^2	35.49	1	35.49	269.88	< 0.0001	
X_4^2	17.82	1	17.82	135.49	< 0.0001	
Residual	1.84	14	0.13			
Lack of Fit	1.76	10	0.18	9.00	0.0242	significant
Pure Error	0.078	4	0.020			
C.V.%	3.98			R-Squared		0.9914

100 µg/mL, and this was an effect similar to that seen in the LPS and dexamethasone groups. As cell viability at high concentrations was close to that of the positive control groups, AQ 50 and 100 µg/mL were tested for NO production. The results showed that the test extracts reduced stimulated NO production compared with the LPS-stimulated positive control group (Fig. 3). The AQ group did not have any significant influence on macrophage cell viability at the low test concentrations (1, 5 and 10 µg/mL), excluding the possibility that the observed effects were mainly due to unspecific cytotoxicity. Although high concentrations of AQ showed some toxicity on macrophages, these data demonstrated the NO inhibitory effect of *C. occidentalis* extracts. This supports the potential anti-inflammatory effects of *C. occidentalis*, perhaps being due to AQ constituents, with potential application in asthma treatment.

3.3. Observation on viability and clinical signs

During the modeling period, mice sometimes wheezed, coughed, bristled and fed less. With the exception of the control group, the mice generally lost weight. During the administration period, there was no significant improvement in the OVA group, but symptoms were

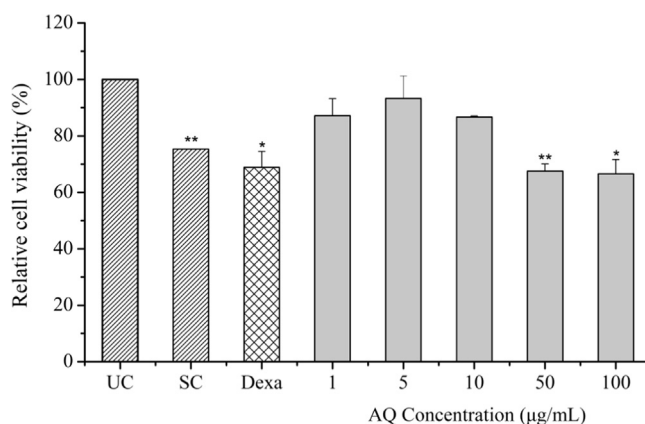


Fig. 2. Influence of AQs on RAW 264.7 cell viability. Values (n = 3) were expressed as mean ± S.E.M. A significant difference compared with the untreated control group was designated as * $p < 0.05$ or ** $p < 0.01$. UC = untreated control, defined as 100%; SC = stimulated control: LPS 1 µg/mL; Dexa = dexamethasone: 10 µg/mL.

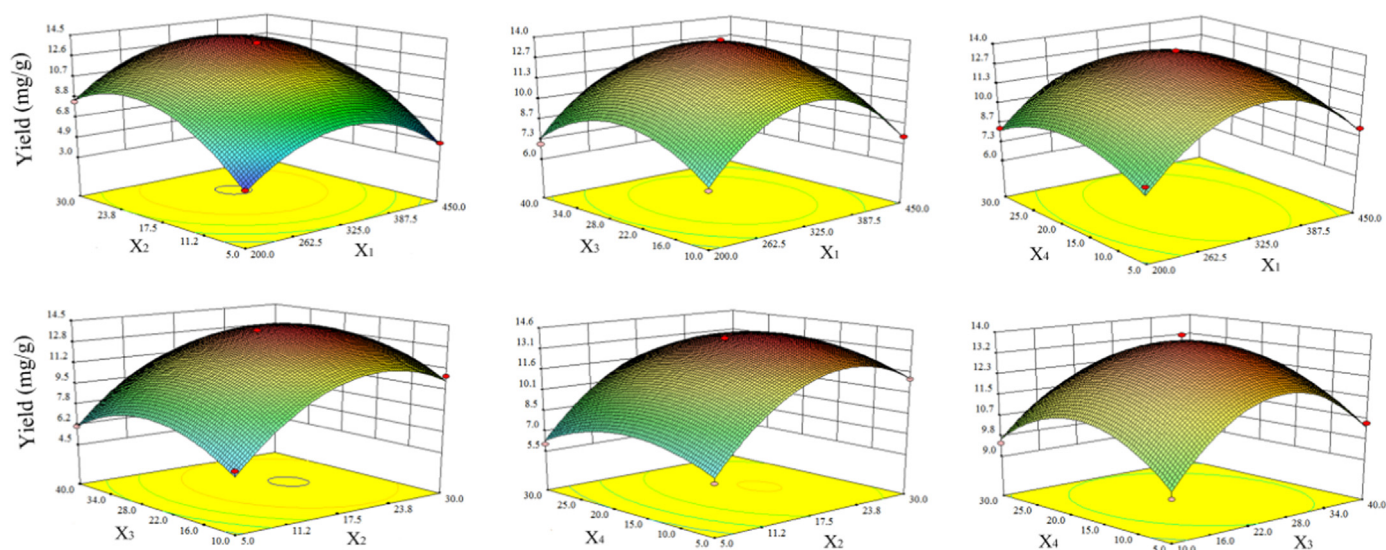


Fig. 1. Response surface for the independent variable interactions on extraction yield [X_1 for rotational speed (rpm); X_2 for grinding time (min); X_3 for solvent to material ratio; X_4 for amount of solid reagents (%)].

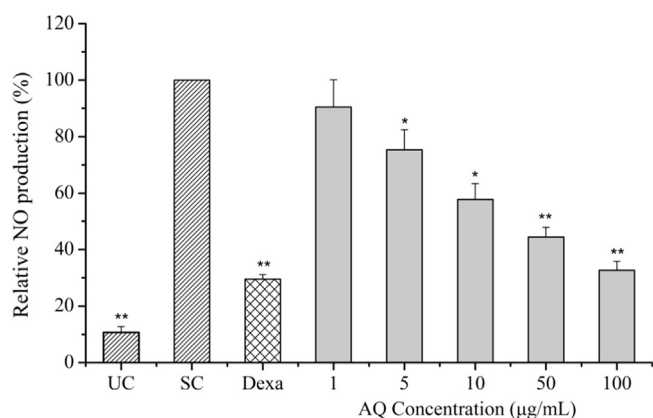


Fig. 3. Influence of AQs on RAW 264.7 NO production. Values ($n = 3$) were expressed as mean $\pm$ S.E.M. A significant difference compared with the stimulated control group was designated as * $p < 0.05$ or ** $p < 0.01$. UC = untreated control; SC = stimulated control: LPS 1 µg/mL, defined as 100%; Dexa = dexamethasone as positive control: 10 µg/mL.

alleviated in the treatment groups without observation of a dose-response relationship.

3.4. Effects on OVA-specific IgE in serum

An increase in blood IgE production is a central feature of allergic asthma (Scirica et al., 2007), and OVA-specific IgE is a central component of the allergic asthma model. A marked increase in OVA-specific IgE concentrations was observed in the OVA group (Fig. 4B), but treatment groups showed lower IgE concentrations. AQ-2 (500 mg/kg) showed a similar inhibitory effect to dexamethasone, and this was comparable to normal concentrations. While there was no dose-dependent relationship for AQ-mediated inhibitory effects, it may be that even the lowest dose administered was already at the top of the dose-

response curve.

3.5. Effects on inflammatory cell counting in BALF

Lungs were lavaged at the end of experimentation, and the inflammatory cells in BALF were counted to evaluate the inhibitory effect of AQs on the influx of inflammatory cells. As shown in Fig. 4C, total inflammatory cell numbers in the OVA group were markedly increased compared with the normal group. Eosinophilic inflammation is a hallmark of asthma and, as expected, treatment with AQs (250, 500, and 2000 mg/kg) reduced the number of total cells including eosinophils in BALF. Moreover, the percentage of eosinophils amongst total BALF cells was lower in the treatment groups than in the control group. This indicated that AQ treatment inhibited cellular infiltration in the airways.

3.6. Cytokines production and mRNA expression levels in lung tissue

The effects of AQ on IL-4, IL-5, IL-13 and INF- γ production in BALF were analyzed by ELISA (Fig. 5). Compared with the normal group, OVA challenge led to increased concentrations of Th2 cytokines (IL-4, IL-5 and IL-13) in BALF, whereas AQ and dexamethasone both suppressed Th2 cytokine elevation compared to the OVA group. However, the result for the Th1 cytokine differed, with INF- γ slightly higher after treatment with AQ compared with the OVA group, to a level that was similar to the dexamethasone group.

We measured mRNA levels of several cytokines and transcription factors in lung tissue (Fig. 6). Relative mRNA expression of IL-4, IL-5 and IL-13 were significantly inhibited by AQ compared with the OVA group, to a level that was comparable to the dexamethasone group. Expression of IL-4 and IL-13 mRNA in the AQ-2 group was comparable to the Dexa and normal group.

3.7. Lung histopathology

H&E staining was performed to enable pulmonary morphologic

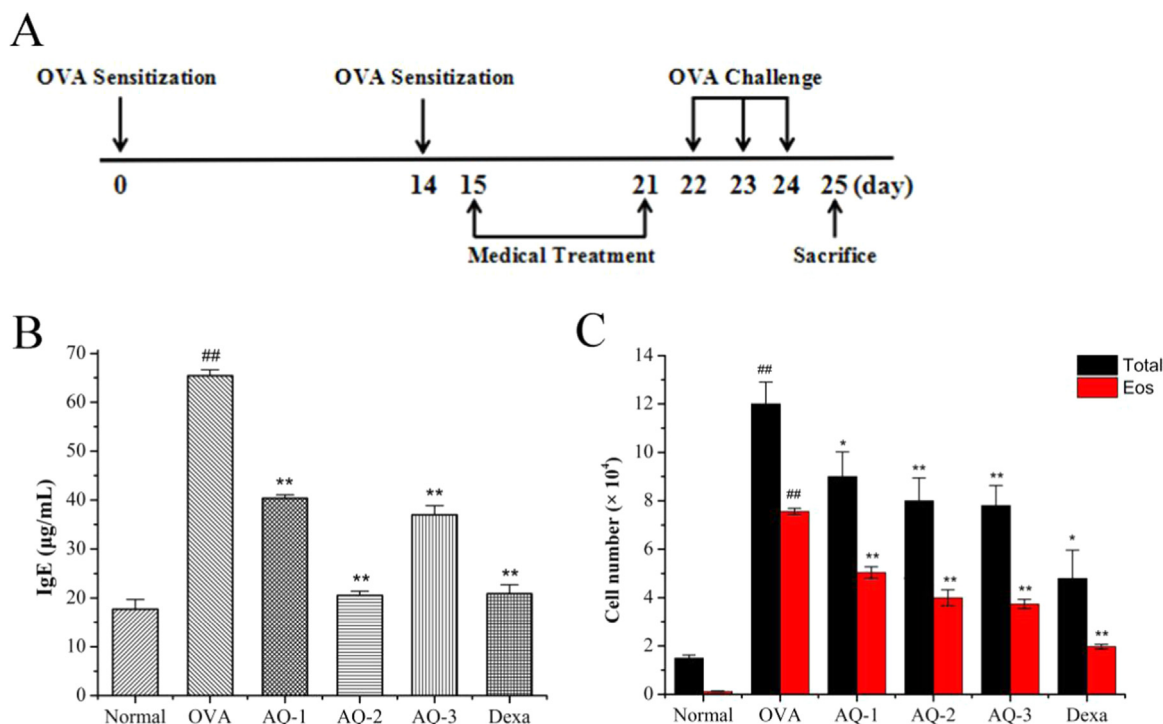


Fig. 4. Effect of AQs on an OVA-induced asthma model. (A) Experimental scheme for measuring the effects of AQs in mice. (B) Concentration of OVA-specific IgE in serum. (C) Number of total leukocytes and eosinophils in BALF. All data are means $\pm$ S.E.M. ## $p < 0.01$ compared to non-treated mice, * $p < 0.05$ compared to OVA-sensitized mice. The experiments used a minimum of six mice in each group.

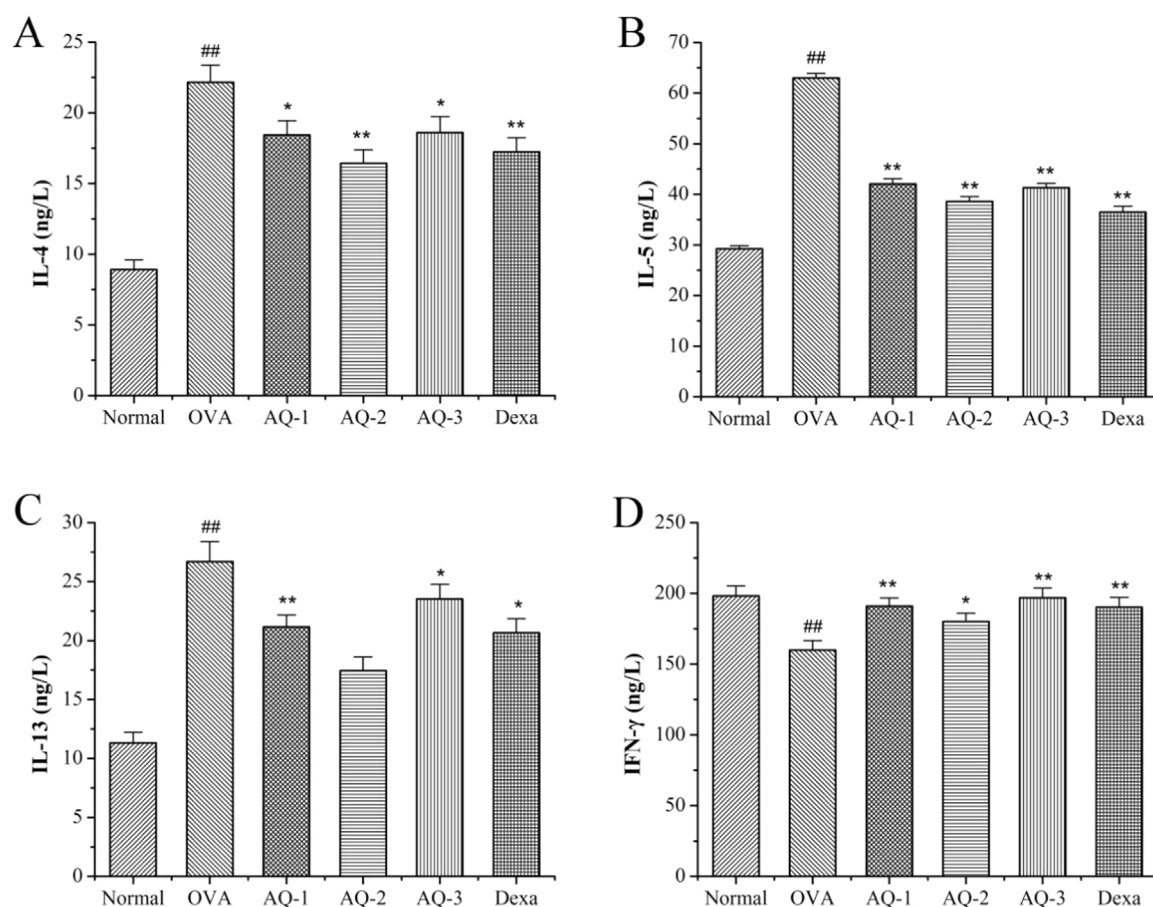


Fig. 5. Concentrations of cytokines (IL-4, IL-5, IL-13 and IFN- γ) in lung homogenates. ## $p < 0.01$ compared to non-treated mice, and * $p < 0.05$ and ** $p < 0.01$ compared to OVA-sensitized mice. The experiments were performed twice with similar results and used a minimum of six mice per group.

changes resulting from the various treatments to be observed via light microscopy. Goblet cell hyperplasia and mucus hyperproduction were also evaluated via PAS staining and quantification of PAS-stained cells. As shown in Fig. 7, lung sections from the normal group showed a normal bronchoalveolar structure with few inflammatory cells. In contrast, lung tissues from the OVA group were significantly damaged. They showed dense perivascular and peribronchial infiltration of leukocytes and mucus secretion within the bronchi, thickening of the alveolar wall, and narrowing of the alveolar cavity. However, histologic damage was less pronounced in the treatment groups compared with the OVA group, with a great reduction in the mean number of PAS-stained goblet cells observed. Dexamethasone also markedly attenuated the pulmonary histologic changes.

4. Discussion and conclusions

In this work, mechanochemically assisted extraction produced a high yield of AQs from *C. occidentalis* compared to the traditional method (Lomovsky, 2011). The protective effect of these AQs was then investigated in mice with OVA-induced asthma. Asthma is regarded as a Th2 cell-associated inflammatory disease of the lung characterized by eosinophil recruitment, inflammatory mediator release, IgE production, mucus hypersecretion and bronchoconstriction (Lloyd and Hessel, 2010). Upon exposure to an allergen, Th2 cell activation is thought to trigger the asthmatic inflammatory cascade, which leads to clonal proliferation as well as IgE class switching of B lymphocytes (Saenz et al., 2008). IgE molecules bind to their surface receptors on mast cells causing release of mediators that recruit inflammatory cells and stimulate secretion of cytokines (including IL-4, IL-5 and IL-13), which are thought to drive disease pathology in asthmatic patients (Lloyd and

Hessel, 2010). Antagonism of IL-4 and IL-13 resulted in decreased serum IgE concentrations, mucus secretion and airway eosinophilia in allergen-sensitized mice (Renz et al., 1995). IL-4 has also been shown to inhibit Th1 cell development, promote Th2 cell development and up-regulate B cell costimulatory molecules and the expression of the low affinity IgE receptor (Alvaro et al., 2013). IL-5 is a key cytokine for inducing eosinophil differentiation, survival and growth (Barnes, 2008). In the OVA-induced asthma model, BALB/c mouse strains are most commonly used for antigen challenge models because they develop a good Th2-biased immunological response (Nials and Uddin, 2008). In our study, AQs from *C. occidentalis* suppressed type 2 cytokine (IL-4, IL-5, and IL-13) concentrations in lung tissues from asthmatic model mice. This suggests that these AQs could be used as a cytokine-blocking drug to protect against OVA-induced airways inflammation by reducing production of pro-inflammatory cytokines. INF- γ is a type 1 cytokine that is critical for innate and adaptive immunity against viral, bacterial (some) and protozoal infections. It is an important activator of macrophages and inducer of class II major histocompatibility complex molecule expression. Thus, the slightly increased INF- γ concentrations in the AQ groups compared with the OVA group (Fig. 5), might be because the increased INF- γ production helps generate antiviral, immunoregulatory and anti-inflammatory activities (Schroder et al., 2004). As these specific cytokines have core functions in the pathogenesis of asthma, they have become major receptor targets for new asthma therapies.

Th2 cells are established as having an important role in the immune system, particularly in the adaptive immune system. As mRNA expression of IL-4, IL-5 and IL-13 were reported to be significantly up-regulated in asthmatic patients (Humbert et al., 1996; Ocman et al., 2005), we determined mRNA expression of these cytokines in the lung

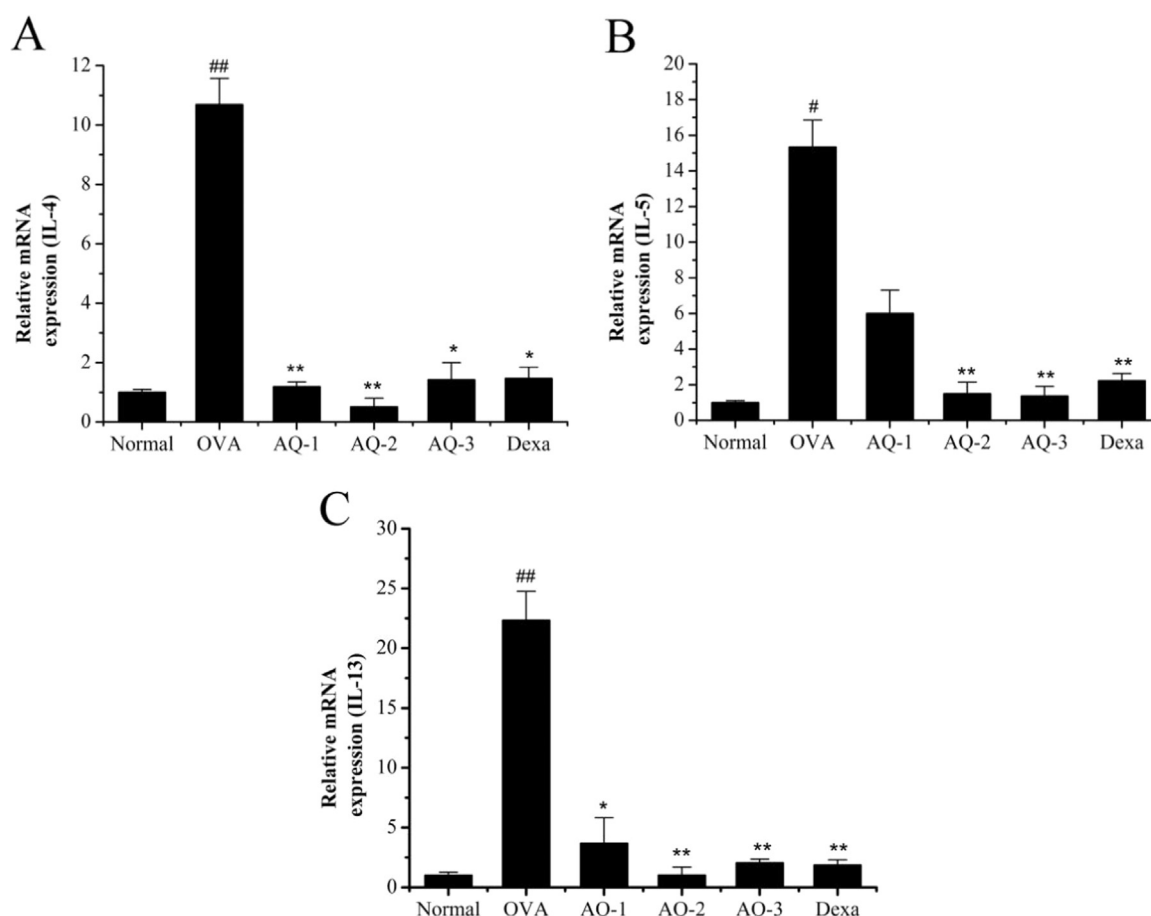


Fig. 6. Inhibitory effect of AQs on the mRNA expression of IL-4, IL-5 and IL-13 in the asthma model lung tissue. ## $p < 0.01$ compared to non-treated mice, * $p < 0.05$ and ** $p < 0.01$ compared to OVA-sensitized mice. The experiments used a minimum of six mice in each group.

tissue of asthmatic mice using real-time PCR. Our results (Fig. 6) indicated that AQ significantly reduced mRNA expression of these cytokines, supporting this as a mechanism for the anti-inflammatory effects. mRNA expression of IL-4, IL-5 and especially IL-13 (Fig. 6) in lung tissue increased more than 10-fold compared to the normal group, with AQ decreasing IL-4, IL-5 and IL-13 levels towards normal. Thus, we believe that AQs may also be able to play a therapeutic role in other Th2 cell-mediated immune diseases.

IL-5 has been suggested to be a key cytokine for inducing eosinophil maturation (Barnes, 2008), such that generation of eosinophils is suppressed if IL-5 production is inhibited. In our study, AQs significantly reduced eosinophil count and the level of IL-5 ($p < 0.01$, Figs. 4C and 5), supporting the conclusions of the previous study. Furthermore, concentrations of OVA-specific IgE, together with IL-4 and IL-13, were remarkably decreased in the AQ-treated groups compared with the OVA group. This is in accordance with previous reports that B lymphocytes secrete IgE upon induction of IL-4 and IL-13 (Hajoui et al., 2004). In brief, AQs from *C. occidentalis* may reduce production of IL-4, IL-5 and IL-13 by inhibiting mRNA expression of these interleukins, and may also suppress IgE secretion via decreased IL-4 and IL-13 production. Ultimately, the AQs could suppress eosinophil maturation and decrease OVA-specific IgE in serum, thereby alleviating inflammation and asthma. A dose-response relationship was not demonstrated for AQs, but this could be due to dosing above the maximal response level. The toxicity profile of AQs has not been well defined, although toxicity associated with *C. occidentalis* is usually attributed to various AQs and their derivatives, as well as other alkaloids (Vashishtha et al., 2009). Work needs to be undertaken to clarify the adverse effects expected with this class, if they were to be considered for treatment of

inflammatory diseases.

Further study is also needed to clarify the best dose for human use. In our study, we elected to use AQ doses of 250, 500 and 2000 mg/kg following consideration of the ethnopharmacological relevance and several convincing works about *C. occidentalis* L. (Sadique et al., 1987; Sreejith et al., 2010). These previously published works demonstrated that the anti-inflammatory activity of *C. occidentalis* in carrageenan-induced rat paw edema was maximal at a dose of 2000 mg/kg. Therefore, we decided to use AQ in doses up to this maximal dose to explore effects and to determine whether such high doses would cause toxicity. In addition, the 'dose' of the whole plant extract for human internal use has generally been 40 g (Watt, 1889). Based on the formula for the dose conversion between humans and animals (Nair and Jacob, 2016), if an average human weighs around 60 kg, the equivalent dose for a mouse is about 8 g/kg. According to the literature (Zeng et al., 2016) and our test experiments, the ethanol extract of *C. occidentalis* L. contains about 20% AQ. This means that the AQ dose from 8 g/kg of the whole plant extract is around 1600 mg/kg for the mouse. While the upper dose of AQ used in our study is quite high, it is rational especially when investigating toxicity alongside the beneficial effects. It remains unclear what exact dose of AQ should be used in humans, and further detailed research should be carried out to determine the ideal therapeutic dose.

In conclusion, natural products that specifically target cytokines or their receptors may have potential benefit for the treatment of asthma. AQ extract from *C. occidentalis* effectively decreased the mRNA expression and concentrations of IL-4, IL-5 and IL-13, as well as reduced OVA-specific IgE concentrations and the counts of eosinophils and other inflammatory cells in the BALF. Exposure to AQs also substantially attenuated lymphocyte infiltration around the bronchia, goblet cell

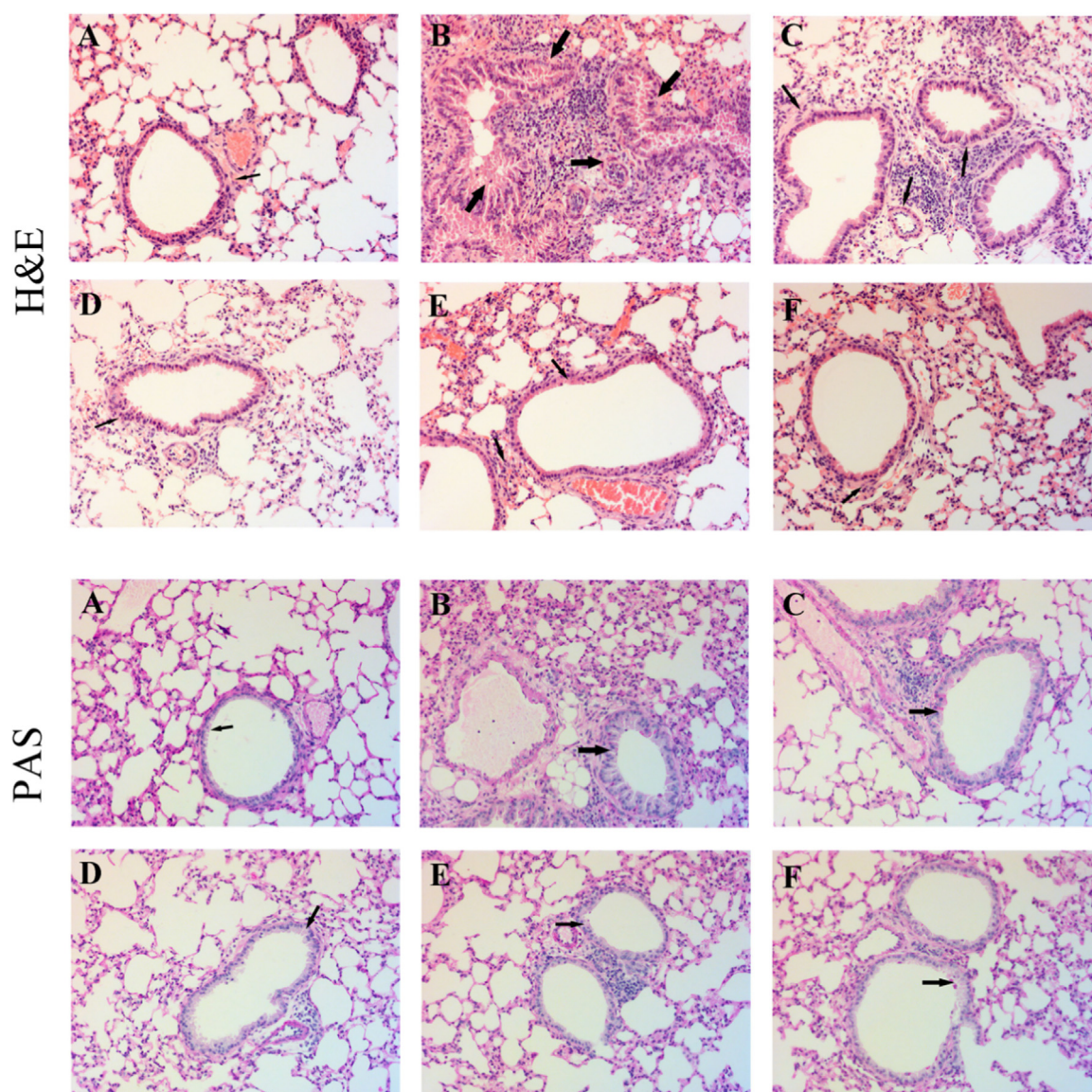


Fig. 7. Effects of AQs on lung tissue histology in OVA-induced asthmatic mice (images, Original magnification $\times 10$). (A) Normal; (B) OVA; (C) Dexamethasone (5 mg/kg); (D) AQ-1 (250 mg/kg); (E) AQ-2 (500 mg/kg); (F) AQ-3 (2000 mg/kg). Black arrows indicate cellular infiltration in H&E staining, and goblet cell metaplasia in PAS staining. The size of the arrows indicates the degree of infiltration and metaplasia with H&E and PAS staining, respectively.

hyperplasia and excessive mucus secretion by the airway epithelial cells. This is the first report indicating that total AQs from *C. occidentalis* have anti-inflammatory and anti-asthmatic effects. However, some questions remain to be answered including clarification of the specific key bioactive compound(s), the exact molecular target of AQ and the interactive mechanism. Our present results identify AQs from *C. occidentalis* as a potential novel therapeutic agent for application in allergy and asthma.

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Conflict of interest

None.

Authors contribution

- Conceived and designed the experiments: Wenhao Xu, Meiqun Hu, Qihong Zhang.
- Performed the experiments and analyzed the data: Meiqun Hu.
- Drafted and edited the manuscript: Meiqun Hu.
- Reviewed: Jingbo Yu, Qihong Zhang.

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