

# Protective effects and active ingredients of *Salvia miltiorrhiza* Bunge extracts on airway responsiveness, inflammation and remodeling in mice with ovalbumin-induced allergic asthma



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## ABSTRACT

**Background:** *Salvia miltiorrhiza* Bunge (*S. miltiorrhiza*), a traditional Chinese medicine, has demonstrated anti-oxidant, anti-inflammatory, and antibacterial activities. However, its effects against asthma that shows chronic inflammation and oxidative damage remain unknown.

**Purpose:** To assess the effects of *S. miltiorrhiza* extracts on airway responsiveness, inflammation, and remodeling in ovalbumin (OVA)-induced asthmatic mice.

**Methods:** Mice with ovalbumin (OVA)-induced allergic asthma were treated with *S. miltiorrhiza* extracts, and airway resistance ( $R_L$ ) to methacholine, inflammatory cell infiltration, Th1/Th2 cytokine levels, and airway remodeling were assessed. TGF- $\beta$ 1-induced BEAS-2B and MRC-5 cells were used to evaluate the effects of five *S. miltiorrhiza* compounds on epithelial-mesenchymal transition and fibrosis.

**Results:** OVA-challenge resulted in remarkably increased  $R_L$ , inflammatory cell infiltration, Th1/Th2 cytokine levels in BALF, goblet cell hyperplasia, collagen deposition, and airway wall thickening. Daily treatment with *S. miltiorrhiza* ethanolic (EE, 246 mg/kg) or water (WE, 156 mg/kg) extract significantly reduced OVA-induced airway inflammatory cell infiltration, Th1/Th2 cytokine amounts, and goblet cells hyperplasia. However, only WE remarkably decreased  $R_L$ , collagen deposition, and airway wall thickening. Moreover, Chromatography showed that salvianic acid A and caffeic acid levels were much higher in WE than EE, while rosmarinic acid was slightly lower; salvianolic acid B and tanshinone IIA levels were much higher in EE than WE. Interestingly, caffeic acid and rosmarinic acid were more potent in reducing E-cadherin and vimentin levels in TGF- $\beta$ 1-induced BEAS-2B cells, and  $\alpha$ -SMA and COL1A1 amounts in TGF- $\beta$ 1-induced MRC-5 cells.

**Conclusions:** Both *S. miltiorrhiza* WE and EE alleviate airway inflammation in mice with OVA-sensitized allergic asthma. *S. miltiorrhiza* WE is more potent in reducing responsiveness and airway remodeling.

## Introduction

Asthma is a chronic airway inflammatory disease associated with a wide range of symptoms, including prolonged inflammation, airway hyper-responsiveness (AHR), mucus hypersecretion, and airway remodeling (Reddy and Gupta, 2014; Xiong et al., 2014). Airway inflammation is one of the distinct characteristics of asthma that is directly linked to a Th2-associated disorder due to TH1/Th2 imbalance

(Wang et al., 2014). Th2 cytokines, such as IL-4, IL-5, and IL-13, are important clinical indicators and have been demonstrated to play a critical role in the pathogenesis of asthma (Lee et al., 2001). Airway remodeling is reversible and constitutes an important cause of airway hyper-responsiveness in clinical asthma. Airway remodeling in asthmatic patients is characterized by loss of epithelial integrity, goblet cell hyperplasia, mucus hypersecretion, basement membrane thickening, smooth muscle hypertrophy and hyperplasia, and sub-epithelial fibrosis

**Abbreviations:** AHR, airway hyper-responsiveness; BALF, bronchoalveolar lavage fluid; DEX, dexamethasone; Mch, methacholine; OVA, ovalbumin; PAS, periodic acid-Schiff; RL, airway resistance; ROS, reactive oxygen species; EE, ethanol extracts; WE, water extracts; WEH, High concentration of WE; WEL, Low concentration of WE; EEH, High concentration of EE; EEL, Low concentration of EE

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(Berair and Brightling, 2014; Zhang and Li, 2011). Current treatment strategies for asthma mainly include bronchodilators and anti-inflammatory therapy (Reddy and Gupta, 2014; Ohta et al., 2014). Glucocorticoids are among the most effective agents that attenuate airway inflammation and hyper-responsiveness. However, such products could lead to serious side effects and drug resistance. Meanwhile, current asthma therapy does not reverse all disease associated symptoms, and new effective drugs are urgently needed.

*Salvia miltiorrhiza* Bunge (*S. miltiorrhiza*, Danshen) is a well-known traditional Chinese medicine that is widely used for the treatment of patients with cardiovascular and cerebrovascular diseases (Gao et al., 2014; Zhou et al., 2012). Previous studies have revealed that active components of *S. miltiorrhiza* mainly include two groups (Zhou et al., 2012; Fang et al., 2008): hydrophilic phenolic acids such as salvianic acid A, caffeic acid, and rosmarinic acid; lipophilic tanshinones such as tanshinone IIA. The hydrophilic components of *S. miltiorrhiza* have shown direct radical scavenging and antioxidant activities, while lipophilic components exhibit anti-inflammatory, antibacterial, and anti-tumor activities. Moreover, previous studies have shown that *S. miltiorrhiza* and its preparations have promising therapeutic applications in hypertension and inflammatory diseases (Woo et al., 2013). However, there is currently no direct evidence suggesting the anti-asthmatic effects of *S. miltiorrhiza* extracts. In addition, the effects of *S. miltiorrhiza* components on asthmatic airway responsiveness, inflammation, and remodeling have not been fully confirmed.

Therefore, the present study established a murine model of allergic asthma by ovalbumin (OVA) sensitization and challenge, and assessed the effects of *S. miltiorrhiza* extracts on airway responsiveness, inflammation, and remodeling. Test parameters included airway resistance ( $R_L$ ) to methacholine (Mch), inflammatory cell infiltration, Th1/Th2 cytokine levels in the BALF, goblet cell hyperplasia, collagen deposition, and airway wall thickening. Differences in efficacy and chemical composition of water (WE) and ethanolic (EE) extracts were assessed, and five active components of *S. miltiorrhiza* were evaluated for their effects on E-cadherin and vimentin levels in TGF- $\beta$ 1-induced BEAS-2B cells, as well as  $\alpha$ -SMA and COL1A1 amounts in TGF- $\beta$ 1-induced MRC-5 cells.

## Methods

### Reagents

OVA, aluminum hydroxide, and methacholine (Mch) were purchased from Santa Cruz (Dallas, TX, USA). Pentobarbital sodium and dexamethasone (DEX) were purchased from Shanghai Chemical Reagent Company (Shanghai, China). Salvianic acid A (SML0679,  $\geq 98\%$ ), salvianolic acid B (49724,  $\geq 95\%$ ), tanshinone IIA (T4952,  $\geq 97\%$ ), caffeic acid (C0625,  $\geq 98\%$ ), and rosmarinic acid (536954,  $\geq 96\%$ ) were purchased from Sigma-Aldrich (St Louis, MO, USA). IFN- $\gamma$ , IL-4, IL-5, and IL-13 ELISA kits were purchased from R&D system (Minneapolis, MN, USA). Mouse OVA-specific immunoglobulin E (IgE) ELISA kit was purchased from R&D Systems (Minneapolis, MN, USA). Antibodies against  $\beta$ -actin (No. 3700),  $\alpha$ -SMA (No. 14968), E-cadherin (No. 14472), and vimentin (No. 3390) were purchased from Cell Signaling Technology (Danvers, MA, USA). COL1A1 (sc-8784) and suitable HRP-conjugated secondary antibodies were purchased from Santa Cruz (Dallas, TX, USA).

### Preparation of *S. miltiorrhiza* extracts

The dried root of *S. miltiorrhiza* was purchased from a local herb shop in Nanchang City, and identified by Prof. Zhuxin Wang (Hunan university of Chinese medicine, Changsha, China). Subsequently, the *S. miltiorrhiza* material was certified according to Chinese Pharmacopoeia (Version 2015), with respect to qualification and quantification tests, and crushed into powder with an electric grinder. The dried powder of

*S. miltiorrhiza* (40 g) was extracted for 2 h at room temperature with 1000 ml of water or 75% ethanol. *S. miltiorrhiza* extracts were then concentrated and freeze-dried to yield crude water (WE) and ethanolic (EE) extracts, respectively.

### HPLC-PDA analysis

A Shimadzu HPLC system, equipped with an LC-20A pump, a CTO-10AS column oven, and an SPD-20A detector (Shimadzu, Kyoto, Japan) was used for the analysis of WE and EE, with an Agilent TC-C18 column (4.6  $\times$  250 mm, 5  $\mu$ m) (Agilent Technol., Santa Clara, CA, USA) at temperature of 25 °C. The mobile phase consisted of 0.2% phosphoric acid solution (mobile phase A) and acetonitrile (mobile phase B) at a flow rate of 1 ml/min. The gradient was started at  $t = 0$  min with 92% (v/v) A, held for 5 min, and changed linearly to 50% (v/v) A in the following 45 min, changed linearly to 0% (v/v) A in the subsequent 10 min, and held for 10 min. Injection volume was 10  $\mu$ l, and detection was performed at 280 nm. The above method has been validated in a previous report (Saran et al., 2013). The limit of detection (LOD) and limit of quantification (LOQ) were determined with signal to noise ratios of 3 and 10, respectively. Five distinct concentrations of mixed standards were used to generate the analytical curves. EE was tested in six replicates to evaluate the system suitability. Mixed standard solutions were tested in triplicate runs at three levels per day for three consecutive days to evaluate intraday and inter-day precisions. Accuracy was evaluated by the recovery of three levels of mixed standards added to EE in triplicate sets, and presented as percentage deviation between the amounts of substances detected and those added. The stability of the mixed standard solution within 48 h was evaluated by comparing chromatographic areas of various substances at 48 h to those obtained at 0 h.

### In vivo study

#### Animal antigen sensitization, challenge and treatment

Animal experiments were carried out in accordance with the Guidelines for Animal Experimentation of Nanchang University (Nanchang, China), and approved by the Animal Ethics Committee of the above institution (No.20150712003). A total of seventy female BALB/c mice were obtained from the Experimental Animal Center of Nanchang University and housed in a SPF facility. The mice were acclimatized for a week before random assignment to seven groups, including: (a) normal control (Con), (b) OVA-induced allergic asthma (OVA), (c) DEX treatment (DEX, 2 mg/kg body weight, *i.p.*), (d) Low dose of WE oral treatment (WEL, 31.2 mg/kg, equivalent to 1.2 g raw herb/kg body weight), (e) High dose of WE oral treatment (WEH, 156 mg/kg, equivalent to 6.0 g raw herb/kg body weight), (f) Low dose of EE oral treatment (EEL, 49.2 mg/kg, equivalent to 1.2 g raw herb/kg body weight), and (g) High dose of EE oral treatment (EEH, 246 mg/kg, equivalent to 6.0 g raw herb/kg body weight). On days 0, 7, and 14, groups (b)–(g) were immunized by intraperitoneal injection of 20  $\mu$ g OVA (0.2 ml, emulsified in 2.25 mg aluminum hydroxide). From day 21, all mice except the Con group were challenged by exposure to 1% aerosolized OVA solution (w/v) using an ultrasonic nebulizer for 30 min per day, 3 days per week for 8 weeks. The DEX group was administered daily with DEX for 8 consecutive weeks, while groups (b)–(f) received different concentrations of WE or EE, respectively. The Con group was treated with the same schedule for sensitization and challenge by PBS. After sensitization and challenge, 5 animals in each group were used for airway resistance ( $R_L$ ) measurement, and the remaining 5 animals in each group were used for bronchoalveolar lavage fluid (BALF) and lung tissue collection.

#### Assessment of airway responsiveness and serum OVA-specific IgE

Airway responsiveness to Mch was analyzed 24 h after the last OVA challenge by invasive airway measurement as described previously

(Wei et al., 2015). Briefly, mice were anesthetized by pentobarbital sodium (50 mg/kg body weight, *i.p.*) and fixed on a board. The trachea was surgically exposed, and an incision was made beneath the center line of the laryngeal cartilage. A small caliber “Y-shaped” tube was inserted and connected to detect airway pressure and respiratory flow. Then, total  $R_L$  representing airway responsiveness was recorded in response to increasing concentrations of Mch (3.125, 6.25, 12.5, 25, and 50 mg/ml). After  $R_L$  measurement, the animals were anesthetized by pentobarbital sodium (50 mg/kg body weight, *i.p.*) and bled from the retro-orbital venous plexus. The serum levels of OVA-specific IgE were measured by ELISA.

#### BALF collection and cell counting

BALF samples were obtained from the left lung by tracheal intubation, lavaged with PBS (1 ml PBS; washed for three times) and centrifuged at 800 g for 5 min. The supernatant was collected and assessed for BALF IFN- $\gamma$ , IL-4, IL-5, and IL-13 levels by ELISA, according to the manufacturer's instructions. The pellets were re-suspended in 50  $\mu$ l PBS, and inflammatory cell count and classification in the BALF were performed on an automated cell counter.

#### Histological examination

Right lung samples were obtained and fixed in 4% paraformaldehyde overnight, paraffin embedded and sectioned. The tissue sections (3.5  $\mu$ m) were deparaffinized in xylene, rehydrated in a graded series of ethanol solutions, and stained with periodic acid-Schiff (PAS), or submitted to Masson's trichrome staining. The stained sections were observed under a light microscope by pathologists blinded to grouping. PAS-positive epithelial cells (goblet cells), airway basement membrane thickness, and collagen deposition were evaluated by a semi-quantitative scoring method. Three animals in each group (five high power fields in each section *per* animal) were randomly selected and assessed.

#### In vitro study

##### Cell culture and MTT assay

BEAS-2B cells and MRC-5 cells (human lung fibroblasts) were purchased from ATCC, and cultured in DMEM containing 10% FBS, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin. The cells were maintained at 37 °C in an atmosphere with 5% CO<sub>2</sub> in 25 cm<sup>2</sup> cell culture flasks. For the MTT assay, cells were seeded into 96-well plates (6 × 10<sup>3</sup> cells/well) and incubated for 24 h. Then the culture medium was replaced by serum-free DMEM containing several concentrations of salvianic acid A, salvianolic acid B, tanshinone IIA, caffeic acid, and rosmarinic acid (3, 10, 30, 100, 300, 1000  $\mu$ M), respectively, for 24 h. the concentration of compounds were calculated by treating the purity of standards as 100%. Next, 10  $\mu$ l of MTT solution (5 mg/ml) was added into each well, and the cells were incubated for another 4 h. After careful aspiration of the medium, 150  $\mu$ l of dimethyl sulfoxide was used to solubilize the formazan crystals. Absorbance was read on a microplate reader at 570 nm, and cell viability was calculated as the ratio of optical densities.

##### Real-time qPCR

Cells were seeded into 6-well plates (1 × 10<sup>5</sup> cells/well) and incubated for 24 h with standard culture medium, and pretreated with salvianic acid A, salvianolic acid B, tanshinone IIA, caffeic acid, and rosmarinic acid (10 or 100  $\mu$ M, 2 ml, dissolved in serum-free DMEM medium), respectively, for 1 h. Then, TGF- $\beta$ 1 was added into plates at a final concentration of 40 ng/ml, followed by incubation for 1 h or 6 h. Total RNA was extracted with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Reverse transcription was performed with Oligo (dT) 15 Primer and SuperScript™ II

**Table 1**  
Primers for quantitative RT-PCR analysis.

| Gene           | Primer sequence                                           |
|----------------|-----------------------------------------------------------|
| E-cadherin     | F: CTGAAGTGACTCGTAACGAC<br>R: ACGAGCAGAGAATCATAAGG        |
| Vimentin       | F: ACATTG AGA TTG CCA CCT AC<br>R: AACCGTCTTAATCAGAAGTGTG |
| COL1A1         | F: AACCAAGGCTGCAACCTGGA<br>R: GGCTGAGTAGGGTACACGCAGG      |
| $\alpha$ -SMA  | F: AGCGTGGCTATTCTTCG<br>R: CTCATTITCAAAGTCCAGAGCTACA      |
| $\beta$ -actin | F: CCTGTACGCCAACACAGTGC<br>R: ATACTCTGCTTGCTGATCC         |

reverse transcriptase reagents. Real time PCR was performed in triplicate with primers listed in Table 1, on an ABI 7300 Real-Time PCR System in 20  $\mu$ l reactions. Master Mix without cDNA template was used as a negative control. Melting curves were evaluated to ensure the identity and purity of PCR products. Relative mRNA levels of target genes were normalized to  $\beta$ -actin mRNA amounts.

#### Western blot

Total protein from BEAS-2B and MRC-5 cells was extracted with the Radio-Immunoprecipitation Assay (RIPA) buffer and assessed for the concentrations with a BCA Protein Assay Kit. For Western blot, 30  $\mu$ g of total protein in each cell lysate was loaded for separation by SDS-PAGE, followed by transfer onto nitrocellulose membranes. After blocking with 5% BSA in PBST, the membranes were incubated with primary antibodies (diluted 1:1000) against  $\alpha$ -SMA, E-cadherin, vimentin, and COL1A1, respectively, at 4 °C overnight. Next, the membranes were extensively washed, and incubated with suitable secondary antibodies (diluted 1:5000) for 1 h at room temperature. After extensive washing, the membranes were treated with the ECL reagent for HRP detection and exposed to autoradiography films for band visualization. The relative amounts of target proteins were quantified with the Image J software, with  $\beta$ -actin as a loading control.

#### Statistical analysis

Data were expressed as Mean  $\pm$  SEM. Differences were assessed by one-way analysis of variance (ANOVA) for multiple groups and Student's *t*-test for two groups, with the SPSS software (SPSS 19, Chicago, IL, USA). A *p*-value of less than 0.05 was considered statistically significant.

## Results

#### *S. miltiorrhiza* components in extracts as analyzed by HPLC-PDA

A quantitative method for *S. miltiorrhiza* extracts was established and validated. The typical chromatograms of WE, EE and standards are shown in Fig. 1 with salvianic acid A, caffeic acid, rosmarinic acid, salvianolic acid B, and tanshinone IIA peaks marked in order. As shown in Table 2, validation data were used to confirm the accuracy of this analytical method. WE chromatogram was slightly different from that of EE. The concentrations of the above five components in WE and EE are shown in Table 3. WE and EE both contained a few weak hydrophilic phenolic acids such as rosmarinic acid and salvianolic acid B. However, strong hydrophilic phenolic acids such as salvianic acid A and caffeic acid, were detected at much higher concentrations in WE compared with EE. Meanwhile, lipophilic tanshinones such as tanshinone IIA were detected at much higher concentrations in EE compared with WE.

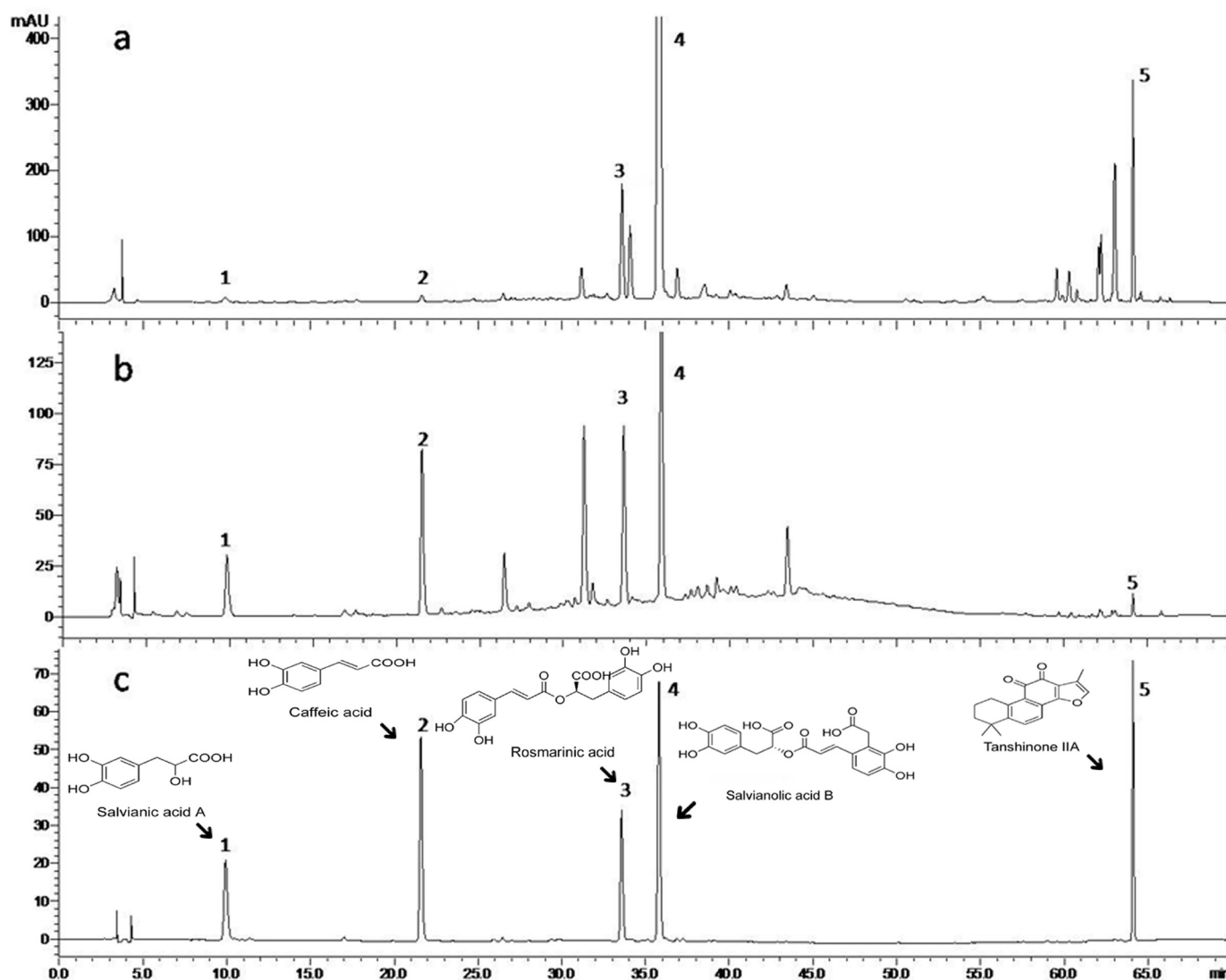


Fig. 1. The chromatograms of *S. miltiorrhiza* EE (a) and WE (b), and mixed standards (c). Peaks 1, 2, 3, 4, and 5 indicate salvianic acid A, caffeic acid, rosmarinic acid, salvianolic acid B, and tanshinone IIA, respectively. The detection was performed at 280 nm.

#### Effects of *S. miltiorrhiza* extracts on AHR to Mch and serum OVA-specific IgE levels in OVA-induced allergic asthma mice

$R_L$  is a useful index in estimating the effect of *S. miltiorrhiza* extracts (EE and WE) on airway responsiveness. As shown in Fig. 2A, there was no significant difference in  $R_L$  among groups at baseline. The Con group showed a slight and concentration-dependent increase of  $R_L$  in response to aerosolized Mch. In contrast,  $R_L$  in response to Mch was significantly enhanced in mice sensitized and challenged with OVA, compared with

the Con group. Compared with the OVA group,  $R_L$  in response to Mch in groups (c)–(g) was decreased after 8 weeks of treatment, especially in the DEX (decreased to 37.2% in response to Mch 50 mg/ml) and WE (WEL and WEH, decreased to 48.9% and 66.6%, respectively, in response to Mch 50 mg/ml) treatment groups.

To confirm that OVA antigen treatment results in sensitization, serum OVA-specific IgE levels were measured by ELISA (Fig. 2B). Serum IgE levels were significantly increased in the OVA group ( $107.7 \pm 11.6$  ng/ml) compared with Con group ( $11.9 \pm 5.5$  ng/ml).

Table 2

Method validation parameters for quantification of five substances in *S. miltiorrhiza*.

| Parameters                          | Salvianic acid A    | Caffeic acid        | Rosmarinic acid     | Salvianolic acid B | Tanshinone IIA      |
|-------------------------------------|---------------------|---------------------|---------------------|--------------------|---------------------|
| LOD (ng/ml)                         | 71                  | 92                  | 63                  | 25                 | 37                  |
| LOQ (ng/ml)                         | 237                 | 307                 | 210                 | 83                 | 123                 |
| Regression equation                 | $y = 10878x + 1391$ | $y = 55886x + 2232$ | $y = 26699x - 1431$ | $y = 7419x - 2981$ | $y = 11406x - 3271$ |
| Linear range ( $\mu\text{g/ml}$ )   | 1.41–140.50         | 1.03–103.53         | 1.34–134.02         | 40.41–4040.56      | 5.02–502.24         |
| Correlation coefficient (R)         | 0.997               | 0.998               | 0.996               | 0.998              | 0.999               |
| System suitability(% CV, $n = 6$ )  | 0.86                | 0.62                | 1.06                | 0.71               | 0.53                |
| Intraday precision (% CV, $n = 3$ ) | 1.02–1.55           | 0.97–1.60           | 0.83–1.78           | 0.98–1.59          | 0.51–1.64           |
| Interday precision (% CV, $n = 3$ ) | 1.08–1.51           | 0.58–1.46           | 1.06–1.82           | 0.74–1.77          | 0.87–1.57           |
| Accuracy(% , $n = 3$ )              | 97.27–102.24        | 101.49–103.35       | 96.87–103.27        | 97.79–99.85        | 97.31–100.88        |
| Stability (%)                       | 98.57               | 97.82               | 97.49               | 99.12              | 98.43               |

**Table 3**

Concentrations of salvianic acid A, caffeic acid, rosmarinic acid, salvianolic acid B, and tanshinone IIA in WE and EE of *S. miltiorrhiza*.

| Peak No. | Substances         | Concentrations (mg/kg) |        |
|----------|--------------------|------------------------|--------|
|          |                    | WE                     | EE     |
| 1        | Salvianic acid A   | 38.8                   | 6.8    |
| 2        | Caffeic acid       | 16.0                   | 2.4    |
| 3        | Rosmarinic acid    | 36.8                   | 68.4   |
| 4        | Salvianolic acid B | 1282.8                 | 3476.0 |
| 5        | Tanshinone IIA     | 10.4                   | 187.2  |

Compared with the OVA group, serum IgE levels were decreased in all drug treatment groups, especially in the DEX, EEH, and WEH groups, with IgE levels notably reduced to 31.3%, 30.4%, and 32.8%, respectively.

#### Effects of *S. miltiorrhiza* extracts on airway inflammation in mice with OVA-induced allergic asthma

To evaluate airway inflammation, all inflammatory cells in the BALF were counted and classified. As shown in Table 4, total inflammatory cells were significantly elevated in the OVA group compared with Con group, especially eosinophils (EOS) and neutrophils (NEU), which were increased to 23.5- and 6.1- fold, respectively. Although the ratio of mononuclear macrophages (MONO) was remarkably declined, with no significant change in lymphocytes (LYM) proportion in the OVA group, the absolute numbers of these two cell types were increased remarkably. DEX treatment resulted in significantly reduced numbers of total inflammatory cells in the BALF from mice with OVA-induced allergic asthma (decreased to 19.7%, compared with the OVA group), especially EOS and NEU, which decreased to 9.7% and 20.9%, respectively. Moreover, both EE and WE treatment reduced inflammatory cell infiltration in the BALF from mice with OVA-induced allergic asthma. Total inflammatory cells, EOS, and NEU were significantly reduced in BALF samples from the EEH and WEH groups (decreased to 30.6%, 8.9%, and 50.4% in the EEH group, respectively, and to 26.5%, 12.6%, and 38.6% in the WEH group, respectively, compared with the OVA group).

#### Effects of *S. miltiorrhiza* extracts on Th1/Th2 cytokines levels in the BALF

To evaluate the Th1/Th2 immune imbalance, Th2 cytokines such as IL-4, IL-5, and IL-13, as well as the Th1 cytokine IFN- $\gamma$  in BALF were

assessed by ELISA. As shown in Fig. 3, OVA sensitization and challenge markedly increased IL-4 (13.8 fold), IL-5 (6.8 fold), and IL-13 (8.2 fold) levels in the BALF from mice (vs. Con group, all  $p < 0.01$ ). Treatment with DEX or high concentration of *S. miltiorrhiza* extracts (both EEH and WEH) resulted in significantly decreased IL-4, IL-5, and IL-13 levels (vs. OVA group, all  $p < 0.05$ ). In contrast, IFN- $\gamma$  concentration in the BALF was significantly decreased to 9.5% in the OVA group compared with the Con group ( $p < 0.05$ , Fig. 3D). Meanwhile, DEX treatment significantly increased IFN- $\gamma$  concentration in the BALF from mice with OVA-induced allergic asthma (increased to 7.3 fold, compared with the OVA group). Moreover, IFN- $\gamma$  concentration was significantly increased in all mice treated with any concentration of EE or WE, especially the EEH and WEH groups (increased to 9.1- and 9.8- fold, respectively; vs. OVA group,  $p < 0.01$ ).

#### Effects of *S. miltiorrhiza* extracts on airway remodeling in mice with OVA-induced allergic asthma

Goblet cell hyperplasia, collagen deposition and airway wall thickening represent the key factors contributing to the pathologic changes of asthma, and are commonly used to evaluate the severity of airway remodeling. In the present study, goblet cell hyperplasia was assessed by PAS staining. As shown in Fig. 4a and c, positive staining of goblet cells and mucus production were significantly increased in bronchial airways of the OVA group (compared with the Con group,  $p < 0.05$ ). Compared with the OVA group, DEX, EEH, and WEH treatments, respectively, significantly alleviated mucus secretion and goblet cell hyperplasia (vs. OVA group, all  $p < 0.05$ ). The inhibition rates in the WEH treatment group were slightly higher compared with those of the EEH group, with no statistically significant difference as analyzed by *t*-test.

Collagen deposition and airway wall thickness were detected by Masson's trichrome staining. As shown in Fig. 4b, d, and e, collagen deposition and airway wall thickness were remarkably increased in the OVA group (vs. Con group,  $p < 0.05$ ). Compared with the OVA group, EEH treatment slightly alleviated collagen deposition and airway wall thickening, while both DEX and WEH showed a significant inhibition (vs. OVA group,  $p < 0.05$ ).

#### Effects of five *S. miltiorrhiza* compounds on the viability of BEAS-2B and MRC-5 cells

To determine optimal concentrations for the five compounds of *S. miltiorrhiza* extracts, their cytotoxicity effects on BEAS-2B and MRC-4

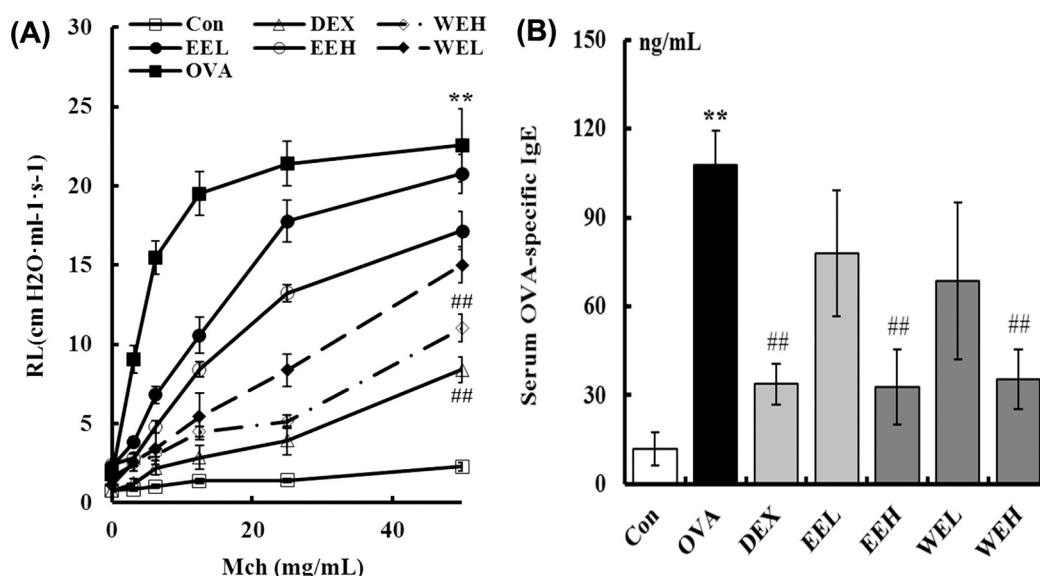


**Fig. 2.** Effects of *S. miltiorrhiza* extracts on airway resistance ( $R_L$ ) and serum OVA-specific IgE levels in mice with OVA-induced allergic asthma. (A)  $R_L$  was recorded in response to increasing concentrations of Mch (3.125, 6.25, 12.5, 25, and 50 mg/ml). (B) The serum OVA-specific IgE levels was assessed by ELISA. Data were given as Mean  $\pm$  SEM ( $n = 5$ ). WEH, High dose of WE (156 mg/kg); WEL, Low dose of WE (31.2 mg/kg); EEH, High dose of EE (246 mg/kg); EEL, Low dose of EE (49.2 mg/kg). \*represents significant difference compared with the Con group, \*\* $p < 0.01$ ; #represents significant difference compared with the OVA group, ## $p < 0.01$ .

**Table 4**  
Leukocytes counting and classification in the BALF of mice.

| Group | Total leukocytes ( $\times 10^8/l$ ) | Leukocytes classification (%) |                             |                             |               |
|-------|--------------------------------------|-------------------------------|-----------------------------|-----------------------------|---------------|
|       |                                      | EOS                           | NEU                         | MONO                        | LYM           |
| Con   | 4.38 $\pm$ 0.28                      | 16.3 $\pm$ 2.1                | 20.8 $\pm$ 1.9              | 55.3 $\pm$ 3.5              | 1.2 $\pm$ 0.3 |
| OVA   | 29.16 $\pm$ 1.01**                   | 57.6 $\pm$ 28.7**             | 19.0 $\pm$ 6.2*             | 15.3 $\pm$ 6.7*             | 1.7 $\pm$ 0.5 |
| DEX   | 5.74 $\pm$ 0.84 <sup>#</sup>         | 28.3 $\pm$ 0.9 <sup>#</sup>   | 20.2 $\pm$ 1.5              | 44.1 $\pm$ 3.7 <sup>#</sup> | 1.3 $\pm$ 0.2 |
| EEL   | 20.25 $\pm$ 1.39                     | 58.2 $\pm$ 15.6               | 19.5 $\pm$ 5.2              | 20.2 $\pm$ 5.3              | 1.5 $\pm$ 0.2 |
| EEH   | 8.93 $\pm$ 1.42 <sup>#</sup>         | 16.7 $\pm$ 4.8 <sup>#</sup>   | 31.3 $\pm$ 5.2 <sup>#</sup> | 49.3 $\pm$ 3.6 <sup>#</sup> | 1.3 $\pm$ 0.5 |
| WEL   | 17.85 $\pm$ 1.69                     | 55.2 $\pm$ 8.9                | 12.6 $\pm$ 2.9              | 18.6 $\pm$ 4.6 <sup>#</sup> | 1.6 $\pm$ 0.2 |
| WEH   | 7.74 $\pm$ 0.95 <sup>#</sup>         | 27.3 $\pm$ 5.9 <sup>#</sup>   | 27.6 $\pm$ 4.9              | 37.9 $\pm$ 5.6 <sup>#</sup> | 1.3 $\pm$ 0.3 |

Abbreviations: Con, control group; OVA, ovalbumin; DEX, dexamethasone; EEL, low dose of EE; EEH, high dose of EE; WEL, low dose of WE; WEH, high dose of WE; EOS: eosinophils; NEU: neutrophil; MONO: monocyte; LYM: lymphocyte. \* represents compared with the Con group, \*\*  $p < 0.01$ ; <sup>#</sup> represents compared with the OVA group, <sup>#</sup> $p < 0.05$ , <sup>##</sup>  $p < 0.01$ .

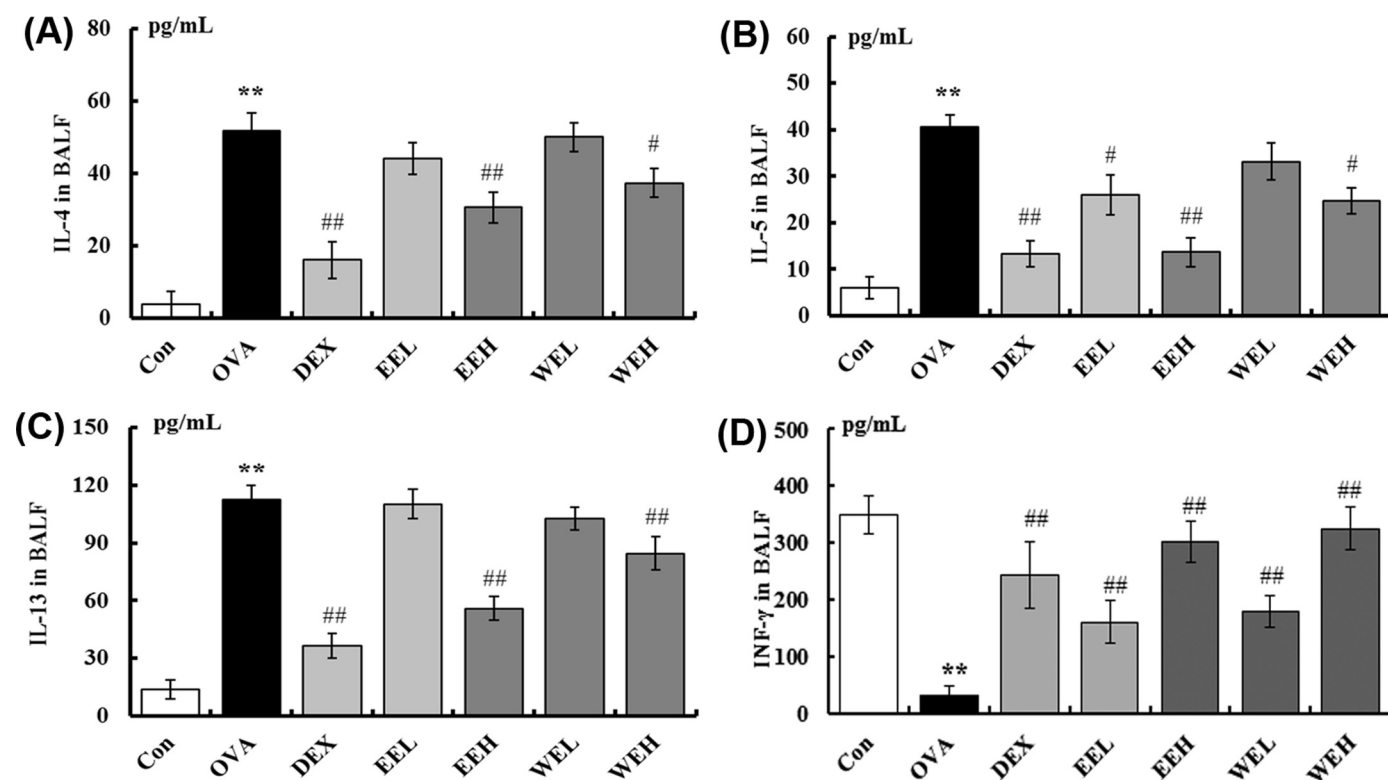


**Fig. 3.** Effects of *S. miltiorrhiza* extracts on IL-4, IL-5, IL-13, and IFN- $\gamma$  levels in the BALF. Data were given as Mean  $\pm$  SEM (n = 5). WEH, High dose of WE (156 mg/kg); WEL, Low dose of WE (31.2 mg/kg); EEH, High dose of EE (246 mg/kg); EEL, Low dose of EE (49.2 mg/kg). \*represents significant difference compared with the Con group, \*\* $p < 0.01$ ; <sup>#</sup>represents significant difference compared with the OVA group, <sup>##</sup> $p < 0.01$ , <sup>#</sup> $p < 0.05$ .

cells were measured by the MTT assay. Results in Fig. 5 showed that all five compounds concentration-dependently suppressed the proliferation of both BEAS-2B and MRC-5 cells, with concentrations above 330  $\mu$ M administered for 24 h showing significant cytotoxic effects, especially for caffeic and rosmarinic acids. Considering there were no remarkable cytotoxic effects after 24 h of treatment for salvianic acid A, salvianolic acid B, tanshinone IIA, caffeic acid, and rosmarinic acid at concentrations below 100  $\mu$ M, 10 and 100  $\mu$ M were selected, respectively, to assess whether these compounds affect the expression of  $\alpha$ -SMA, E-cadherin, vimentin, and COL1A1.

#### Effects of the five compounds on $\alpha$ -SMA, E-cadherin, vimentin, and COL1A1 levels in cells induced by TGF- $\beta$ 1

As shown in Fig. 6A–D, both mRNA and protein levels of E-cadherin and vimentin were significantly increased in TGF- $\beta$ 1-induced BEAS-2B cells (increased to 2.35- and 3.54- fold, respectively), while  $\alpha$ -SMA and COL1A1 amounts were remarkably increased in TGF- $\beta$ 1-induced MRC-

5 cells (increased to 5.24- and 3.29- fold, respectively). These results were consistent with previous findings demonstrating that TGF- $\beta$ 1 induces epithelial-mesenchymal transition and fibrosis. Pretreatment of cells with salvianic acid A, salvianolic acid B, tanshinone IIA, caffeic acid, and rosmarinic acid, respectively, at 10 or 100  $\mu$ M, caused a certain degree of reduction in TGF- $\beta$ 1-induced mRNA expression of  $\alpha$ -SMA, E-cadherin, vimentin, and COL1A1, especially in the caffeic acid and rosmarinic acid groups (vs. TGF- $\beta$ 1 group, all  $p < 0.05$ ).

As shown in Fig. 6E, E-cadherin and vimentin protein levels in BEAS-2B cells were significantly increased after incubation with TGF- $\beta$ 1 for 6 h (increased to 2.01- and 2.51- fold, respectively). Compared with the Con group, both  $p < 0.05$ ). Moreover, TGF- $\beta$ 1-induced E-cadherin and vimentin upregulation was not remarkably attenuated by pretreatment of BEAS-2B cells with salvianic acid A, salvianolic acid B, and tanshinone IIA, respectively, at concentrations of 10 or 100  $\mu$ M. However, both caffeic acid and rosmarinic acid (10 and 100  $\mu$ M) significantly suppressed TGF- $\beta$ 1-induced E-cadherin and vimentin upregulation in BEAS-2B cells.

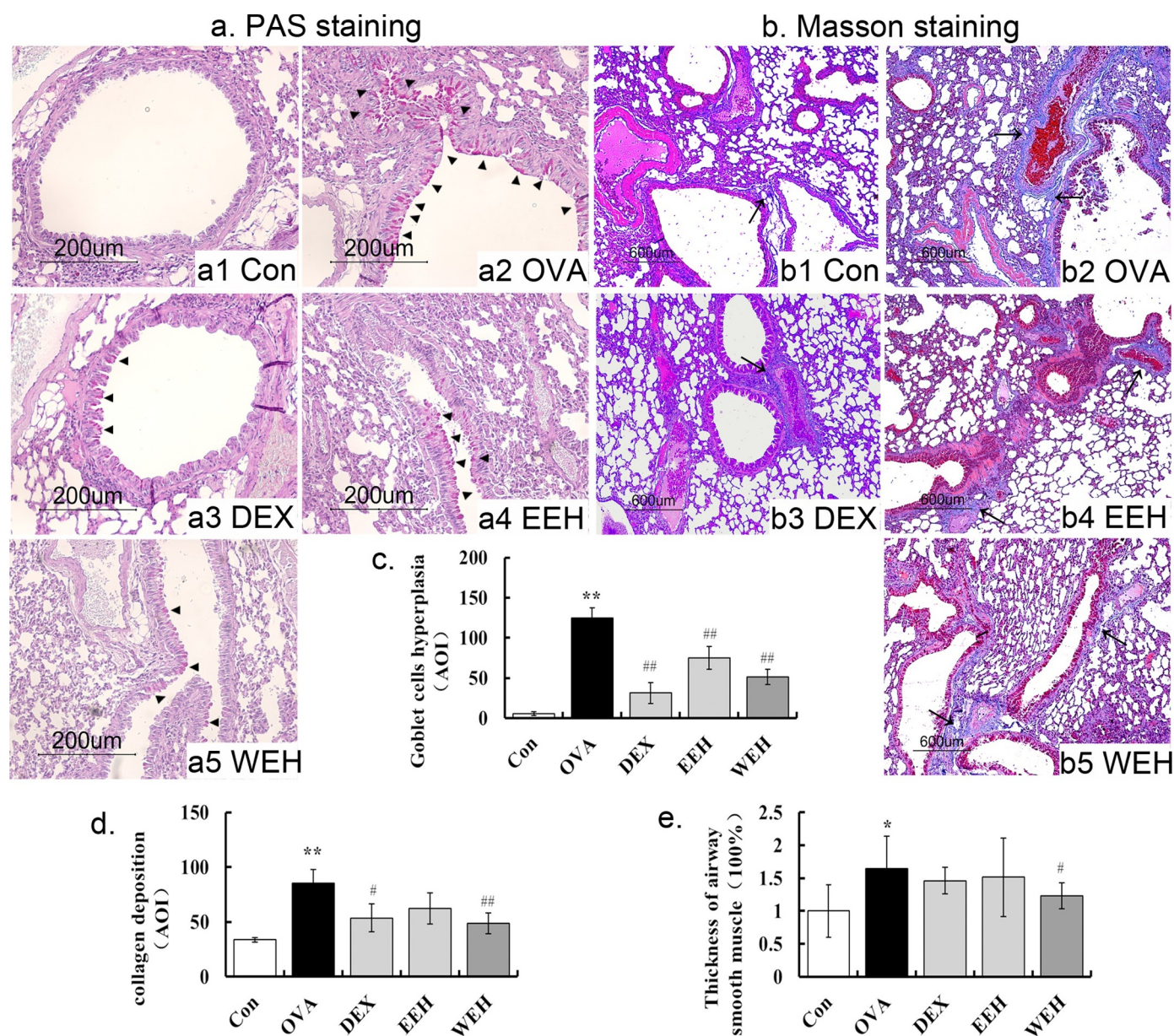


**Fig. 4.** Morphopathological changes in trachea and lung tissue. The non-lavaged left lung was removed, fixed, paraffin embedded, cut into 3.5  $\mu\text{m}$  sections, and then these sections were stained with PAS (a1–a5) and Masson's trichrome staining (b1–b5) for histological evaluation. Goblet cell hyperplasia ( $\blacktriangle$ ), collagen deposition ( $\dagger$ ) and airway wall thickness of these animals were semi-scored under light microscopy ( $n = 3$ ).

As shown in Fig. 6F,  $\alpha$ -SMA and COL1A1 protein amounts in MRC-5 cells were remarkably increased after 6 h of TGF- $\beta$ 1 induction. Pretreatment of cells with salvianic acid A, salvianolic acid B, and tanshinone IIA (10 or 100  $\mu\text{M}$ ), respectively, did not attenuate TGF- $\beta$ 1-induced increase of cytoplasmic  $\alpha$ -SMA and COL1A1 protein levels. Meanwhile, both caffeic acid and rosmarinic acid (10 and 100  $\mu\text{M}$ ) significantly suppressed TGF- $\beta$ 1-induced increase of  $\alpha$ -SMA and COL1A1 protein amounts in MRC-5 cells.

## Discussion

OVA is the most common stimulus used to induce allergic asthma. Two OVA-induced asthma models have been reported in previous studies. One is modeled by OVA sensitization on days 0 and 14, and challenge on days 21–23. The other is a chronic asthma model established by OVA sensitization on days 0, 7 and 14, with challenge 3 days per week for 6–8 weeks. In the present study, female BALB/c mice were

sensitized (on days 0, 7, and 14) and challenged (3 days per week for 8 weeks) with OVA, and the chronic allergic asthma model was successfully developed, with symptoms such as remarkably increased airway AHR, Th1/Th2 cytokine imbalance, inflammatory cell infiltration, goblet cell hyperplasia, collagen deposition, and airway wall thickening around the bronchia. In addition, the ratio of NEU in the OVA group (about 19.0%) was much higher in this study, while that of EOS (nearly 57.6%) was a somewhat lower compared with BALF data reported in previous 24 days asthmatic mice (Shin et al., 2014; Zhou et al., 2014). The current results were consistent with previous 6–8 week chronic asthma models (Hou et al., 2015; Lin et al., 2015), and differences in inflammation between these two models may be explained by that chronic OVA challenge possibly induces airway remodeling as well as a complex immune response other than EOS associated inflammation.

The positive control DEX showed strongest inhibitory effects on AHR and airway inflammation (Su et al., 2016), but was less powerful

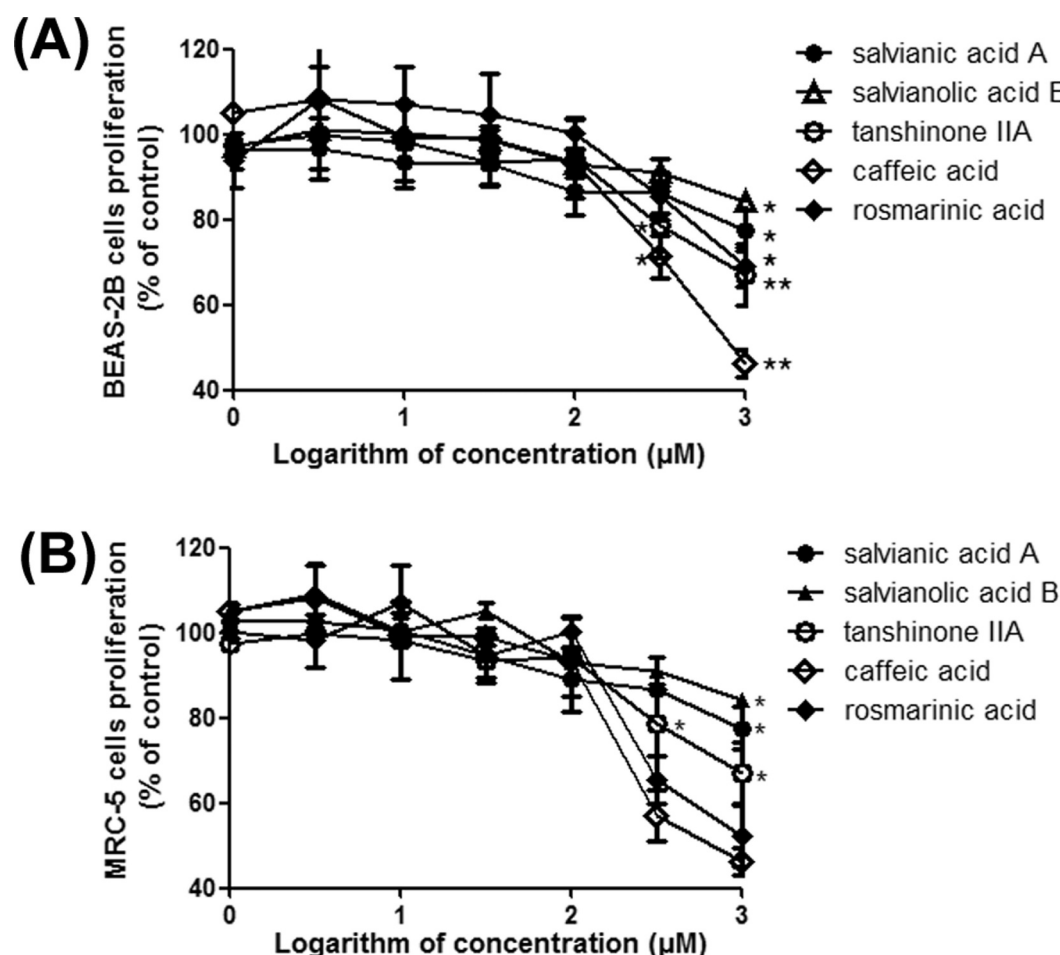


**Fig. 5.** Effects of five *S. miltiorrhiza* compounds on the viability of BEAS-2B and MRC-5 cells. Concentration-dependent curves of BEAS-2B cells (A) and MRC-5 cells (B) incubated with concentrations (3, 10, 30, 100, 300, 1000 μM) of salvianic acid A, salvianolic acid B, tanshinone IIA, caffeic acid, and rosmarinic acid, respectively. After 24 h of incubation, the living cells were detected by the MTT assay. The cell viability of the DMSO vehicle control group was set at 100%, the mean value was calculated from three repeated experiments. \*represents significant difference compared with the control group, \* $p < 0.05$ ; \*\* $p < 0.01$ .

in suppressing airway remodeling compared with high concentration of WE. Considering high WE was more potent than EE in inhibiting airway remodeling and AHR, but weaker in suppressing airway inflammation, we hypothesized that the various components of WE and EE responsible for such differences.

In order to assess the active components of WE and EE, these extracts were analyzed by HPLC-PDA, and five main compounds were quantitate using reference standards. As shown above, WE had much higher concentrations of highly hydrophilic phenolic acids such as salvianic acid A and caffeic acid, which have been widely reported to exhibit potent antioxidant effects. Moreover, previous studies have demonstrated that caffeic acid phenethyl ester not only exerts potent anti-allergic effects by decreasing reactive oxygen species (ROS) production and nuclear factor-kappaB DNA binding activity (Jung et al., 2008), but also alleviates asthma by regulating the airway micro-environment via the ROS-responsive MAPK/Akt pathway (Ma et al., 2016). Therefore, salvianic acid A and caffeic acid likely play a decisive role in inhibiting airway wall remodeling in mice with OVA-induced allergic asthma.

Previous reports have confirmed that rosmarinic acid attenuates airway inflammation and hyperresponsiveness in mice with allergic asthma (Sanbongi et al., 2004; Liang et al., 2016), and exhibits anti-fibrotic effects in experimental liver fibrosis (Li et al., 2010). However, existing data are still limited in verifying that the above active components inhibit collagen deposition and airway wall thickening. Therefore, we further compared the effects of five compounds in *S.*

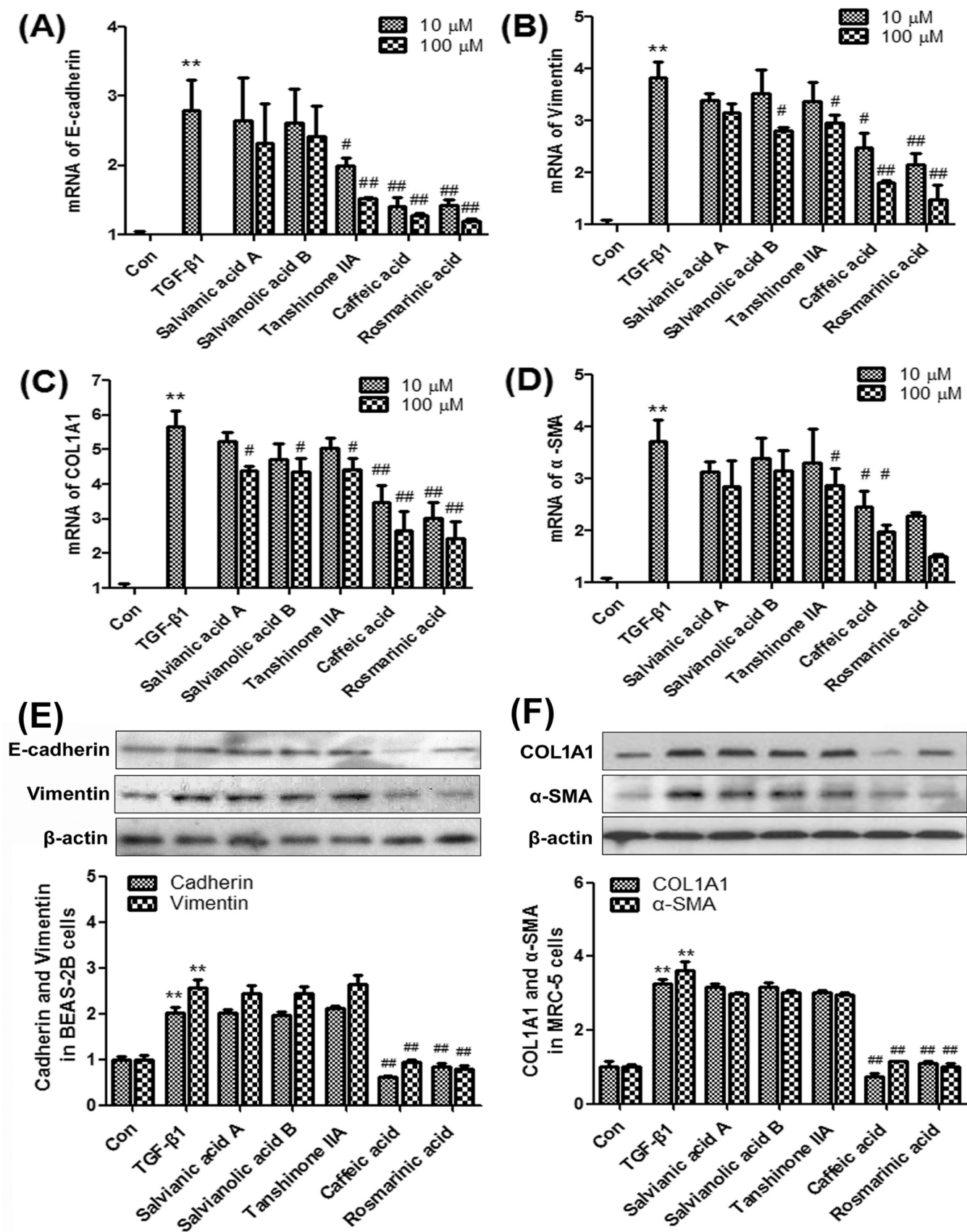
*miltiorrhiza* extracts on TGF-β1-induced E-cadherin and vimentin up-regulation in TGF-β1-induced BEAS-2B cells, as well as α-SMA and COL1A1 upregulation in TGF-β1-induced MRC-5 cells. These in vitro models faithfully reflect the pathological processes involved in the epithelial-mesenchymal transition and fibrosis (Kamitani et al., 2011; Honda et al., 2010). As shown above, both caffeic acid and rosmarinic acid potently reduced E-cadherin, vimentin, α-SMA, and COL1A1 amounts. However, HPLC-PDA data showed that total rosmarinic acid and caffeic acid amounts in EE were 70.8 mg/kg, somewhat higher than WE (52.8 mg/kg). Since WE was more potent in inhibiting airway remodeling compared with EE, there are likely other components in WE suppressing airway remodeling besides rosmarinic acid and caffeic acid.

## Conclusions

Both *S. miltiorrhiza* WE and EE alleviate airway inflammation in mice with OVA-sensitized allergic asthma. *S. miltiorrhiza* WE is more potent in reducing responsiveness and airway remodeling.

## Author's contributions

Fang Zou, Wei Zou and Chunsong Yan conceived and designed the experiments; Junming Luo, Li Zhang, Xinyi Zhang, Yingying Long, and Wei Zou performed the experiments; Junming Luo, Li Zhang, and Wei Zou analyzed the data; Wei Zou contributed reagents, materials, and analysis tools; Junming Luo and Li Zhang wrote the paper. All authors



(caption on next page)

**Fig. 6.** Effects of five *S. miltiorrhiza* compounds on  $\alpha$ -SMA, E-cadherin, vimentin, and COL1A1 levels induced by TGF- $\beta$ 1. (A) and (B): mRNA expression of E-cadherin and vimentin in TGF- $\beta$ 1-induced BEAS-2B cells were detected by qPCR; (C) and (D): mRNA expression of  $\alpha$ -SMA and COL1A1 in TGF- $\beta$ 1-induced MRC-5 cells were detected by qPCR; (E): E-cadherin and vimentin protein amounts in BEAS-2B cells were detected by western blot; (F)  $\alpha$ -SMA and COL1A1 protein amounts in MRC-5 cells were detected by western blot. The relative amount of target proteins was analyzed and compared with  $\beta$ -actin, the mean value was calculated from three repeated experiments. \*represents significant difference compared with the Con group, \*\* $p < 0.01$ , #represents significant difference compared with the TGF- $\beta$ 1 group, ## $p < 0.01$ , # $p < 0.05$ .

have read and approved the final manuscript.

## Conflicts of interest

The authors declare no conflict of interest.

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