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## Effects of indole alkaloids from leaf of *Alstonia scholaris* on post-infectious cough in mice



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### ABSTRACT

**Ethnopharmacological relevance:** Leaf of *Alstonia scholaris* (L.) R. Br. (Apocynaceae), a wide used ethnic-medicine in many Asia and Africa counties, has also been recorded as the common traditional Chinese medicine for treatment of illnesses in respiratory system by Dai people.

**Aim of the study:** To provide experimental data of clinical adaption of total indole alkaloids (TA) from leaf of *A. scholaris* for treating post-infectious cough in phase II clinical trial.

**Materials and methods:** To model post-infectious cough, all animals except control group were instilled intratracheal with lipopolysaccharide (LPS) (80 µg/50 µL/mouse), followed by subsequent exposure to cigarette smoke (CS) for 30 min per day for a total of 30 days. Mice were orally given TA at dose of 10, 25, 50 mg/kg, and four main alkaloids (Sch: scholaricine, Epi: 19-episolaricine, Val: vallesamine, Pic: picrinine) once daily. Cellular infiltration was assessed in the broncho-alveolar lavage fluid (BALF). Expression of interleukin-6 (IL-6) and C-reactive protein (CRP) in the serum was determined, the superoxide dismutase (SOD) activity as well as malondialdehyde (MDA) content in the serum and homogenate were examined. Finally, histopathological examination in the lungs was assessed by H. E. staining.

**Results:** After administration of TA and four major alkaloids respectively, the symptoms of cough in mice were obviously attenuated. Total white blood cells (WBC) and neutrophils (NEU) amounts in BALF were reduced obviously and the pathological damage of lung was also attenuated. There was also significant reduction in IL-6, CRP, MDA and a marked improvement in SOD.

**Conclusions:** The efficacy of indole alkaloids against post-infectious cough (PIC) was shown in the down-regulation of inflammatory cells, cytokines, and the balance of antioxidants. What's more, the pharmacological effects of TA were better than single indole alkaloid, which might be related to the synergic effect of four major alkaloids.

### 1. Introduction

Coughing is the classic symptoms of a cold, which has been divided into three types on the basis of its duration: acute cough (< 3 weeks), sub-acute cough (3–8 weeks), and chronic cough (> 8 weeks) (Irwin et al., 2006). Post-infectious cough (PIC) belongs to a type of sub-acute cough, and the percentage of it is 40% – 50%. Among the causes that

can induce cough, the most common is represented by the infection of upper respiratory track, generally of viral origin. The most frequently implicated are picornavirus (rhinovirus and enterovirus) and coronavirus, followed by adenovirus, metapneumovirus, parainfluenza and influenza viruses, respiratory syncytial virus, and bocavirus (Dicpinigaitis et al., 2009; Footitt and Johnston, 2009; Jones and Stewart, 2002; Pappas et al., 2008; Regamey et al., 2008). Cough is the

**Abbreviations:** TA, total alkaloids; PIC, post-infectious cough; Sch, scholaricine; Epi, 19-episolaricine; Val, vallesamine; Pic, picrinine; DXM, dexamethasone; BALF, broncho-alveolar lavage fluid; IL-6, Interleukin-6; CRP, C-reactive protein; MDA, malondialdehyde; SOD, superoxide dismutase; ELISA, enzyme-linked immunosorbent assay

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most common and troublesome symptom during airway infections, which is a major cause of significant morbidity and indeed mortality, and may last for several weeks or even months in some patients (Jones and Stewart, 2002), that bring the patient to the doctor (Footitt and Johnston, 2009).

The process of virus's replication in the airways includes the following parts: virus attachment to epithelial cells, cell membrane fusion and release of viral nucleocapsid and proteins into the cell cytoplasm (Jackson and Johnston, 2010). Some mediators, come from airway epithelial cells, activate the immune response by promoting the recruitment and activation of leukocytes (neutrophils, mast cells, monocytes, lymphocytes and cells NK) which provide a more effective response to different infections. Furthermore these cells release cytokines, chemokines, proteases, and oxygen free radicals that aggravate the injury of the airway structures caused by the virus (Biagioli et al., 1998; Jackson and Johnston, 2010; Rossi and Colin, 2015).

The pathogenesis of the PIC is as follows: extensive disruption of epithelial integrity, widespread airway inflammation, bronchial hyperresponsiveness, and cough hypersensitivity (Betz et al., 2001; Cho et al., 2003; Morice, 2010; Zimmerman et al., 2000). Studies showed that the incidence of coughing after infection has an increased trend in recent years, which raised to the range from 25% to 50% during outbreaks of atypical pathogens' infections (Ryan et al., 2012).

Although, a little of the complex pathogenesis of cough has been revealed by humans until now, but it at best increased the treatment options available (Morice et al., 2015). Many patients use over-the-counter drugs, for instance, cough suppressants and expectorants/mucolytics, which often lack a clinically proven efficacy or have some activity, but at the expense of unpleasant or intolerable side effects. Two classes of cough suppressants are available: those with central action (codeine, dextromethorphan, and cloperastine) and those acting peripherally (levodropropizine). The only expectorant capable of inhibiting the cough reflex is guaifenesin. Meanwhile common side effects from these drugs are nausea, dry mouth, insomnia, dizziness, constipation, anorexia, fatigue, sleepiness and so on. A large part of income was spent on the treatment of cough globally (Morice, 2008). However, satisfactory results of all the medicine were not achieved due to the symptomatic treatment, side effects, and relapse after treatment. Additionally, montelukast, medicaments of a gleam for treating respiratory tract infection, was unresponsive to treatment for post-infectious cough (Wang et al., 2014). Therefore, an unmet need exists for the development of an effective therapeutic approach to prevent PIC.

*Alstonia scholaris* is distributed in deciduous, evergreen forests and even in plains widely over the tropical regions of Africa and Asia (Li et al., 1995). Its leaves have long been used in "dai" ethno-pharmacy for the treatment of whooping cough (post infectious cough), chronic bronchitis, asthma, and other respiratory tract infections in Yunnan Province, PR China (Compiling Group of Yunnan Traditional Chinese Medicine, 1977). Phytochemical constituents of different parts of the plant were investigated intensively by our group (Cai et al., 2010, 2008a, 2008b, 2007; Chen et al., 2016; Du et al., 2007a, 2007b; Feng et al., 2009, 2008; Liu et al., 2015; Pan et al., 2016; Qin et al., 2015a, 2015b; Xu et al., 2009; Yang et al., 2015a, 2015b, 2014a, 2014b; Zhang et al., 2014; Zhou et al., 2005). Meanwhile, the chemical profiling and metabolites of alkaloidal extract of *A. scholaris* were reported (Cao et al., 2015), in which scholaricine, 19-episolaricine, vallesamine, and picrinine were the major indole alkaloids of its leaf (Fig. 1.), and

their pharmacokinetic behavior in rats was also investigated (Zhao et al., 2017). Moreover, *A. scholaris* extracts and alkaloids have shown antitussive, anti-asthmatic, expectorant (Shang et al., 2010a), analgesic, anti-inflammatory (Shang et al., 2010b), anti-airway inflammation (Zhao et al., 2016), and airways anti-allergic effect (Zhao et al., 2017) *in vivo*. The alkaloids also triggered  $\beta_2$  adrenergic receptor ( $\beta_2$ AR) activation (Hou et al., 2012a) and inhibited the nuclear factor- $\kappa$ B (NF- $\kappa$ B) (Hou et al., 2012b) bioactivities *in vitro*. Based on the pre-clinical studies, the total indole alkaloids from *A. scholaris* (TA) has been registered as investigational new botanical drug (No. 2011L01436) and was approved for phase I/II clinical trials by China Food and Drug Administration (CFDA). A phase-I Single-center, randomized, double-blind, and placebo-controlled clinical trial has been completed, and the results support further phase-II clinical trials. The traditional use and our pharmacological evaluation assumed that the alkaloids from the leaf of *A. scholaris* might be used in treatment of respiratory diseases related to airway sub-acute inflammation. In this paper, we present the effect of alkaloids on the model of post-infectious cough.

## 2. Materials and methods

### 2.1. Plant material

Leaves of *A. scholaris* were collected in June 2013 in Pu'er city of Yunnan Province, People's Republic of China, and identified by Dr. Xiao-Dong Luo, Kunming Institute of Botany, Chinese Academy of Sciences. A voucher specimen (Luo20130601) has been deposited in State Key Laboratory of Phytochemistry and Plant Resources in West China, Chinese Academy of Sciences.

### 2.2. Alkaloids preparation

The dried and powdered leaves of *A. scholaris* (1 kg) were extracted with 90% EtOH under reflux conditions (3 h  $\times$  4) and the solvent was evaporated *in vacuo* to get the ethanolic extract. The ethanolic extract was dissolved in 0.3% aqueous HCl solution and filtered; the residue was recognized as non-alkaloid fraction. Then the acidic solution, adjusted to pH 9–10 with 10% aqueous ammonia, was extracted with EtOAc to give TA fraction (10 g). Picrinine (10%), vallesamine (6%), scholaricine (6%), and 19-episolaricine (2%) were isolated (Fig. 1) and kept in refrigerator in a previous phytochemical investigation from TA.

### 2.3. Chemicals

Lipopolysaccharide (LPS) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Hong mei brand cigarettes were purchased from Hongta Tobacco Group Co., Ltd. ELISA reagent sets for IL-6 and CRP were purchased from R&D Systems (Minneapolis, MN, USA). Malondialdehyde (MDA) and superoxide dismutase (SOD) determination kits were purchased from Jiancheng Bioengineering Institute of Nanjing (Jiangsu, China). All the other chemicals and solvents were of highest purity grade available.

### 2.4. Animals

Special pathogen-free male ICR mice, weighing approximately

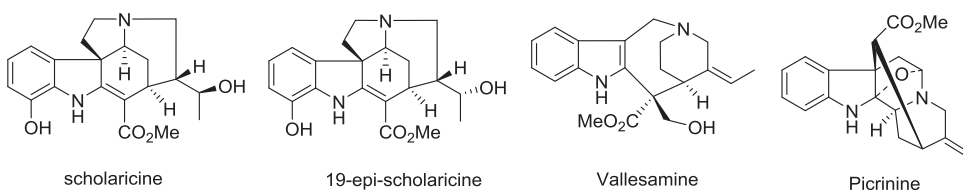


Fig. 1. Four major alkaloids from leaf of *A. scholaris*.

22–25 g, were purchased from Kunming Medical University (licence number SYXK 2011–0004). All the animals were housed at room temperature (20–25 °C) and constant humidity (40–70%) under a 12 h light-dark cycle in SPF grade laboratory. Food and water were supplied *ad libitum*. Animals were acclimated for one week before treatment. Animal study was performed according to the international rules considering animal experiments and the internationally accepted ethical principles for laboratory animal use and care.

## 2.5. Experimental design

The experiment was performed according to previously described method with some modifications (Zhu and Li, 2014). To model post-infectious cough, mice were anesthetized by intraperitoneal injection of sodium pentobarbital. The trachea was intubated with a cannula (30 mm length and outer diameter 1.5 mm). Mice were placed in a supine position with the head elevated. With use of a micropipette and an intravenous catheter inserted through the intubated cannula, 50  $\mu$ L of sterile saline containing 80  $\mu$ g lipopolysaccharide and corresponding vehicle with 50  $\mu$ L saline were instilled. On 8th day, all mice except control were placed in a smoke chamber and challenged with exposure to smoke of 5 cigarettes for 30 min/day for a total of 30 days. On 39th day after intra-tracheal instillation, the mice were randomly divided into 10 groups and treated as follows: (1) Control group (did not receive any intervention); (2) Model group: .5% carboxymethylcellulose intragastrically (i.g.), 20 mL/kg; (3) DXM: dexamethasone (i.g.), 1 mg/kg; (4) TA-H: total indole alkaloids (i.g.), 50 mg/kg; (5) TA-M: total indole alkaloids (i.g.), 25 mg/kg; (6) TA-L: total indole alkaloids (i.g.), 10 mg/kg; (7) Sch: scholaricine (i.g.), 3 mg/kg; (8) Epi: 19-episolaricine (i.g.), 1 mg/kg; (9) Val: vallesamine (i.g.), 3 mg/kg; (10) Pic: picricine (i.g.), 5 mg/kg. Each group contained ten mice. A schematic diagram of the treatment schedule is shown in Fig. 2.

## 2.6. Serum collection

On 46th day, animals were euthanized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg) after overnight food deprivation. Then blood samples were collected via the orbital vein of mice. The serum samples were obtained by centrifuging blood samples at 4 °C, 4000  $\times$ g for 15 min, and serum samples were prepared and stored at – 80 °C for the analysis of IL-6, CRP (Neumann et al., 2013), SOD, and MDA (Lemos et al., 2014). Operation was performed according to the manufacturer's specifications.

## 2.7. Bronchoalveolar lavage fluid (BALF) and cell enumeration

The right lungs were ligated and the left lungs were lavaged with 1.5 mL of autoclaved PBS for 3 times. The BALF was immediately centrifuged at 4 °C, 300 g for 10 min. The cell pellets were re-suspended in PBS, and the total cell number was counted using blood counting instrument (Wang et al., 2010; Zheng et al., 2009).

## 2.8. Lung homogenate

Lung tissue supernatants were prepared as described previously (Tsai et al., 2014). After BALF collection, 50 mg lungs were homogenized in 0.45 mL of ice-cold PBS, the assay mixture was cooled on ice, followed by centrifugation at 1000 g for 10 min at 4 °C to obtain the supernatant, and stored at – 80 °C until required for SOD and MDA analysis.

## 2.9. Lung histopathology

The right lung tissues were embedded in paraffin, cut into sections of 5  $\mu$ m thickness, and stained with hematoxylin and eosin (H. E.) to evaluate the general morphology, as reported previously (Ichiki et al., 2005). The degrees of peribronchial and perivascular inflammation were scored in a double-blind screen with two independent blind investigators using a subjective scale of 0–5 (0, normal lung; 1, structural lesions of bronchial mucosa and alveolar wall were mild; 2, focal lesions of the bronchial mucosa and alveolar wall structures; 3, lesions of bronchial mucosa and alveolar wall were patchy; 4, lesions of bronchial mucosa and alveolar wall were less than 1/2 lobectomy; 5, lesions of bronchial mucosa and alveolar wall were more than 1/2 lobectomy) as described elsewhere (El-Agamy, 2011; Li et al., 2011; Ma et al., 2012).

## 2.10. Statistical analyses

Results are presented as mean  $\pm$  SEM (n = 10). Comparisons between 2 groups were made by using a 2-tailed Mann-Whitney test. Multiple comparisons were made by using 1-way ANOVA with the Turkey post-test or Kruskal Wallis analysis with the Dunn post-test, in which nonparametric analyses were appropriate. A *P* value of less than 0.05 was considered as significant for all the analyses.

## 3. Results

### 3.1. Observation on general symptom

In contrast to control, the mice model developed cough, dyspnea, bristling, huddling, reducing diet, reduction, and weight loss. During administration period, the symptoms of the treatment groups alleviated without dose-response dependence compared to the model group.

### 3.2. Effects of indole alkaloids on inflammatory cell count in BALF

Lungs were lavaged at the end of experiment and differential cells in BALF were counted to investigate the effect of TA on inflammatory cells influx. As shown in Table 1, number of WBC (*p* < 0.05 vs. control group) and neutrophils (*p* < 0.05 vs. control group) in BALF increased in mice model, they were  $3.8 \pm 0.8$  and  $1.1 \pm 0.08$ , respectively, while the number of WBC in TA-L group and Epi group were  $1.1 \pm 0.3$  and  $1.6 \pm 0.5$  (both *p* < 0.05 vs. model group), respectively. The number of neutrophils in all the three TA groups as well as Pic group was  $0.43 \pm 0.08$ ,  $0.54 \pm 0.08$ ,  $0.43 \pm 0.08$ , and  $0.51 \pm 0.09$  (all

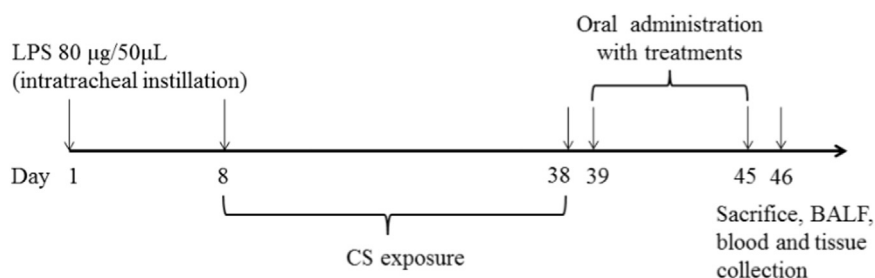


Fig. 2. Experimental protocol for the establishment of LPS and CS induced PIC model and the treatment schedule.

**Table 1**  
The production of total leukocytes in BALF after treatment.

Groups	WBC ( $\times 10^5$ /mL)	NEU ( $\times 10^5$ /mL)
Control	1.1 $\pm$ 0.5	0.54 $\pm$ 0.12
Model	3.8 $\pm$ 0.8 $\Delta$	1.10 $\pm$ 0.08 $\Delta$
DXM	1.1 $\pm$ 0.2*	0.53 $\pm$ 0.16*
TA-H	1.5 $\pm$ 0.4	0.43 $\pm$ 0.08**
TA-M	1.6 $\pm$ 0.4	0.54 $\pm$ 0.08**
TA-L	1.1 $\pm$ 0.3*	0.43 $\pm$ 0.08**
Sch	2.5 $\pm$ 1.1	0.66 $\pm$ 0.26
Epi	1.6 $\pm$ 0.5*	0.64 $\pm$ 0.17
Val	1.8 $\pm$ 0.5	0.75 $\pm$ 0.16
Pic	2.1 $\pm$ 0.6	0.51 $\pm$ 0.09**

Note: Data are expressed as mean  $\pm$  SEM. Statistical differences are represented as  $\Delta p < 0.05$  vs. control, \* $p < 0.05$ , \*\* $p < 0.01$  vs. model (n = 10 mice per group). WBC, white blood (cell) count. NEU, neutrophils. All groups were intra-gastrically administered.

Control: challenged with saline.

Model: established by intratracheal instillation of lipopolysaccharide (LPS) and passive smoking.

DXM: dexamethasone, 1 mg/kg.

TA-H: administrated total alkaloids, 50 mg/kg.

TA-M: administrated total alkaloids, 25 mg/kg.

TA-L: administrated total alkaloids, 10 mg/kg.

Sch, scholaricine, 3 mg/kg.

Epi, 19-episolaricine, 1 mg/kg.

Val, vallesamine, 3 mg/kg.

Pic, picrinine, 5 mg/kg.

$p < 0.01$  vs. model group), respectively.

### 3.3. Effect of indole alkaloids on IL-6 and CRP production in serum

IL-6 and CRP production in serum were analyzed by ELISA. As shown in Table 2, LPS instillation and CS significantly increased the levels of pro-inflammatory cytokines IL-6 and CRP in the serum as compared to model animals (both  $p < 0.05$  vs. control group), these were  $26.5 \pm 1.1$  and  $24.8 \pm 0.5$ , respectively. However, TA-L and Sch groups significantly reduced the level of IL-6- $22.7 \pm 0.6$  and  $22.2 \pm 0.9$ , respectively, (both  $p < 0.05$  vs. model group). The content of CRP was markedly decreased to  $22.7 \pm 0.5$  and  $22.9 \pm 0.4$  in TA-H and TA-L groups, respectively. DXM group showed similar effects on the above two parameters (all  $p < 0.05$  vs. model group). The remaining treatment group had the trend of decrease, but there was no significant difference ( $p > 0.05$  vs. model group).

### 3.4. Effect of indole alkaloids on SOD and MDA activities in serum and lung

Oxidative stress plays an important role in the development of PIC. SOD and MDA activities in the serum and lung homogenate were determined to evaluate the effect of indole alkaloids on oxidative stress. As

**Table 2**  
The production of IL-6 and CRP in serum after treatment.

Groups	IL-6 (pg/mL)	CRP (pg/mL)
Control	20.8 $\pm$ 0.7	20.8 $\pm$ 0.9
Model	26.5 $\pm$ 1.1 $\Delta$	24.8 $\pm$ 0.5 $\Delta$
DXM	22.0 $\pm$ 0.6*	22.3 $\pm$ 0.6*
TA-H	23.2 $\pm$ 1.3	22.7 $\pm$ 0.5*
TA-M	24.1 $\pm$ 0.8	23.5 $\pm$ 0.6
TA-L	22.7 $\pm$ 0.6*	22.9 $\pm$ 0.4*
Sch	22.2 $\pm$ 0.9*	24.4 $\pm$ 0.8
Epi	25.2 $\pm$ 1.2	24.8 $\pm$ 0.7
Val	25 $\pm$ 1.1	23.8 $\pm$ 0.6
Pic	23.9 $\pm$ 1.2	23.5 $\pm$ 0.7

Note: Data are expressed as mean  $\pm$  SEM. Statistical differences are represented as  $\Delta p < 0.05$  vs. control, \* $p < 0.05$ , \*\* $p < 0.01$  vs. model (n = 10 mice per group). IL-6, interleukin-6. CRP, C-reactive protein. All groups were intra-gastrically administered.

**Table 3**  
The production of SOD in serum and homogenate after treatment (mean  $\pm$  SEM, U/mL).

Groups	serum	homogenate
Control	93.5 $\pm$ 1.7	61.3 $\pm$ 1.1
Model	85.5 $\pm$ 2.4 $\Delta$	53.4 $\pm$ 2.1 $\Delta$
DXM	86.9 $\pm$ 3.0	63.9 $\pm$ 1.8*
TA-H	92.2 $\pm$ 2.7	54.0 $\pm$ 5.3
TA-M	97.7 $\pm$ 2.5*	64.2 $\pm$ 1.5**
TA-L	94.7 $\pm$ 2.2*	63.1 $\pm$ 2.2*
Sch	93.6 $\pm$ 3.9	58.4 $\pm$ 2.2
Epi	89.7 $\pm$ 6.9	54.2 $\pm$ 3.4
Val	91.7 $\pm$ 3.9	58.4 $\pm$ 1.8
Pic	91.2 $\pm$ 3.4	56.0 $\pm$ 4.0

Note: Data are expressed as mean  $\pm$  SEM. Statistical differences are represented as  $\Delta p < 0.05$  vs. control, \* $p < 0.05$ , \*\* $p < 0.01$  vs. model (n = 10 mice per group). SOD, superoxide dismutase. All groups were intra-gastrically administered.

shown, model group resulted in marked decline of SOD activities in serum and homogenate (Table 3) compared with the control group (both  $p < 0.05$ ) which decreased to 91.5% and 83.0%, respectively. Encouragingly, TA-M and TA-L groups significantly promoted the percentage of SOD activities, respectively, to 114.3% and 110% in serum (both  $p < 0.05$  vs. control group), and to 120.3% ( $p < 0.01$  vs. control group) and 118.3% ( $p < 0.05$  vs. control group) in lung homogenate.

Meanwhile, the percentage of MDA content in serum and homogenate (Table 4) was increased to 140.4% ( $p < 0.05$  vs. control group) and 116.3% ( $p < 0.01$  vs. control group) in model group, TA-L group also reduced MDA levels to 79.8% and 83.8% on the two samples (both  $p < 0.05$  vs. model group). Val and Sch decreased the content of MDA significantly ( $p < 0.05/0.01$  vs. model group), while Pic and Epi showed improving tendency ( $p > 0.05$  vs. model group) in the two indices compared to the model group.

### 3.5. Lung histopathology

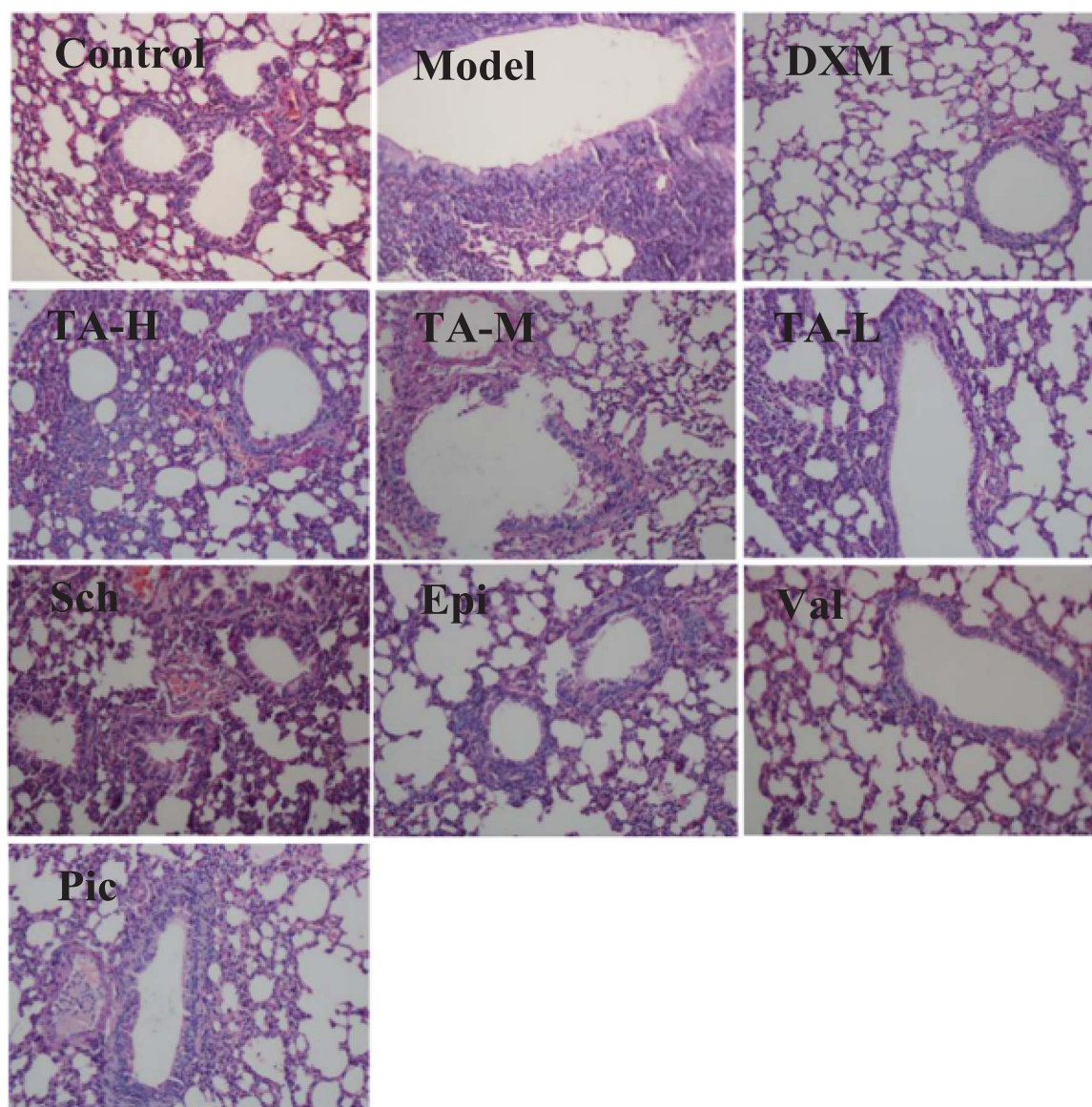
The production of inflammatory cells into the lungs of mice was also investigated by histopathological studies. LPS instillation and CS exposure provoked mice a marked infiltration of neutrophils around peribronchial and perivascular spaces compared to normal animals (Fig. 3). Accordingly, the scores for total lung inflammation increased significantly (Table 5). Besides, bronchia mucosal epithelium appeared swelling and shedding, and there was inflammatory cell infiltration exudate in the airway lumen. The infiltration of neutrophils in the bronchial mucosa was observed in control group. A few parts of infiltration of neutrophilic granulocytes were observed in pulmonary interstitial around the peribronchovascular. The results indicated that the infiltration of inflammatory cells reduced significantly in TA-treated mice compared to LPS and CS challenged mice, and four alkaloids (Sch, Epi, Val, and Pic) also prevented infiltration of inflammatory cells in

**Table 4**  
The production of MDA in serum and homogenate after treatment (nmol/mL).

Groups	Serum	Homogenate
Control	9.8 $\pm$ 0.9	6.4 $\pm$ 0.2
Model	13.7 $\pm$ 0.7 $\Delta$	7.4 $\pm$ 0.2 $\Delta\Delta$
DXM	14.0 $\pm$ 1.2	6.2 $\pm$ 0.2**
TA-H	11.9 $\pm$ 0.7	6.6 $\pm$ 0.4
TA-M	12.1 $\pm$ 2.0	6.2 $\pm$ 0.3
TA-L	11.0 $\pm$ 0.8*	6.1 $\pm$ 0.4*
Sch	12.2 $\pm$ 1.1	6.1 $\pm$ 0.4*
Epi	11.6 $\pm$ 0.9	6.4 $\pm$ 0.5
Val	11.1 $\pm$ 0.4**	7.1 $\pm$ 0.5
Pic	11.2 $\pm$ 1.4	6.2 $\pm$ 0.5

Note: Data are expressed as mean  $\pm$  SEM. Statistical differences are represented as  $\Delta p < 0.05$ ,  $\Delta\Delta p < 0.01$  vs. control, \* $p < 0.05$ , \*\* $p < 0.01$  vs. model (n = 10 mice per group). MDA, malondialdehyde. All groups were intra-gastrically administered.





**Fig. 3.** Representative microscopic sections of tissue demonstrating lung histology. The lung sections were stained with H&E and examined by light microscopy. Original magnification  $\times 200$ .

**Table 5**  
Pathological changes after intratracheal LPS instillation and CS exposure.

Groups	Dose (mg/kg)	Scores (means $\pm$ SEM)
Control	–	0.9 $\pm$ 0.2
Model	–	3.0 $\pm$ 0.4 $\Delta\Delta$
DXM	1.0	1.6 $\pm$ 0.3 <sup>**</sup>
TA-H	50.0	2.0 $\pm$ 0.2 <sup>*</sup>
TA-M	25.0	1.8 $\pm$ 0.3 <sup>*</sup>
TA-L	10.0	1.7 $\pm$ 0.3 <sup>*</sup>
Sch	3.0	2.0 $\pm$ 0.2 <sup>*</sup>
Epi	3.0	2.2 $\pm$ 0.2
Val	3.0	2.3 $\pm$ 0.4
Pic	5.0	2.0 $\pm$ 0.3 <sup>*</sup>

Note: Semi-quantitative analyses of inflammatory cell infiltrations in lung sections were performed as described in Materials and Methods. Lung tissues were fixed, sectioned at 5  $\mu$ m, and stained with H&E for tissue neutrophils infiltration. Data are expressed as mean  $\pm$  SEM. Statistical differences are represented as  $\Delta\Delta p < 0.01$  vs. control, <sup>\*</sup> $p < 0.05$ , <sup>\*\*</sup> $p < 0.01$  vs. model (n = 10 mice per group).

varying degrees. DXM at 1 mg/kg treatment, used as the reference drug, improved the pulmonary histologic changes remarkably.

#### 4. Discussion

The respiratory tract can be infected by a variety of bacteria, both gram positive and gram negative. The incidence of pneumonia is much higher for gram-negative infections than for gram-positive infections. Lipopolysaccharides (LPS), a large molecule consisting of a lipid and a polysaccharide, also known as lipoglycans and endotoxins, it was found in the outer membrane of Gram-negative bacteria and stimulate strong immune responses in animals, which may cause PIC with an inflammatory response (Kitamura et al., 2001; Saluk-Juszczak and Wachowicz, 2005). Several studies have shown that cigarette smoke has an impact on the immune function of the respiratory system, which induces the recruitment of inflammatory cells into the lungs and the release of pro-inflammatory cytokines, chemotactic factors, oxygen radicals and proteases (Joad et al., 2004; Karlsson et al., 1991).

In our present work, the content of pro-inflammatory cytokines (IL-6 and CRP) and lipid peroxidation products increased significantly.

Besides, pathological change in the lung showed that many inflammatory cells were accumulated and infiltrated. A successful animal model of PIC was established and used by intra-tracheal instillation of LPS and CS exposure in mice, since the etiologies and mechanisms of PIC have been extensively investigated in number of experimental models.

Neutrophils are key blood cells, which play an important role in the pathogenesis of lung inflammation. A high number of neutrophils activate the reactive oxygen system and aggravate the development of respiratory diseases (Barnes, 2013; Cepkova and Matthay, 2006). Bronchoalveolar lavage fluid (BALF) has been extensively utilized for evaluating the alterations of lung microenvironment, including pulmonary epithelial integrity, cellular damage, and surface release/accumulation of cellular secretory products. As expected, the animals exposed to LPS and CS exhibited a significant increase of neutrophils in BALF, and was consistent with histological examination of the lung tissue in our study. Then, after the treatment of indole alkaloids, the number of total cells and neutrophils in BALF were inhibited, meanwhile lung's inflammatory cell infiltration alleviated.

Inflammation is controlled by various types of inflammatory mediators, including cytokines and chemokines which are associated with the recruitment of inflammatory cell and tissue destruction (Liudet et al., 2002). Our research focused on the examination of interleukin (IL-6) and C-reactive protein (CRP). IL-6, an inflammatory and fibrogenic cytokine, is thought to play an important role in the development of lung disease (Jasiewicz et al., 2015). Recent studies demonstrated that IL-6 is marked increased in CS exposed animals (Li et al., 2009). CRP, an acute-phase protein, whose elevation accompanied by a remarkable pulmonary inflammation (Vos et al., 2009). Clinical trials reported that serum CRP concentrations of patients with COPD were higher than healthy controls (de Torres et al., 2006; Gan et al., 2004). In our investigation, mice intragastrically treated with indole alkaloids can effectively suppressed the elevated content of IL-6 and CRP produced by the exposure of LPS and CS in serum except the control group. The results suggest that the modulatory effect of pro-inflammatory cytokines may be one of the mechanisms of defending against infection.

It has been reported that the cigarette smoke contains many oxidants and free radicals, which causes the injury of alveolar epithelial cells and reduce anti-oxidizing abilities, and further leads to a large number of protein denaturation and apoptosis of surrounding tissues, and induces airway inflammation (William et al., 1983). SOD is an enzyme that exists in cells for removing oxyradicals, whose activity variation may represent the degree of tissue injury. Extracellular SOD might modulate neutrophil inflammation by reducing cytokine, released from macrophages, suggesting that extracellular SOD is an anti-inflammatory enzyme as well as a major anti-oxidant (Mates, 2000). Previous studies reported an increase of MDA concentration in the blood, BALF, and pulmonary tissues after the LPS administration (Shen et al., 2009), which was also observed in our experiment. In current study, we analyzed the anti-oxidant parameters (SOD and MDA) of serum and lung tissues, and found that the SOD activities were run-down significantly in all LPS and CS treated groups, but remarkably recovered in animals treated with alkaloids. Meanwhile, the promoted MDA in LPS and CS treated animals could be reduced.

Glucocorticoids with anti-inflammatory and anti-allergic activities are the most potent therapeutic agents, which are used to alleviate respiratory failure caused by neutrophilic granulocyte and alveolar macrophages in their metabolically activated states (Caramori and Adcock, 2003). Then, dexamethasone (DXM) was selected as a positive control of alkaloids to treat PIC. The results indicated that indole alkaloids from the leaf of *Alstonia scholaris* could well treat PIC in mice, which roughly equals to DXM in general ( $p > 0.05$  vs. DXM group).

## 5. Conclusions

In conclusion, our study displayed that indole alkaloids could fight

against PIC induced by LPS and CS exposure. However, the inhibition effect of TA was not appeared in a dosage dependent manner and superior to single alkaloids, simultaneously, four major alkaloids had different effects on different indicators, which assumed that the synergic effect between each component may compensate the drawback of single compound on PIC. And more importantly, this effect might be associated with the reduction of inflammatory infiltration and the improvement between oxidation and anti-oxidation.

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