

Airways antiallergic effect and pharmacokinetics of alkaloids from *Alstonia scholaris*



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ABSTRACT

Background: *Alstonia scholaris* (L.) R. Br. (Apocynaceae), an important herbal medicine, has been widely used to treat respiratory tract diseases, such as cough, asthma, phlegm, and chronic obstructive pulmonary disease.

Purpose: To evaluate pharmacological effect of alkaloids from *A. scholaris* on ovalbumin induced airways allergic inflammatory model, and explore whether the dosing frequency is related to pharmacokinetics.

Study design: After oral administration of total alkaloids, the pharmacokinetic study of it was investigated. In addition, anti-allergic studies were carried out on ovalbumin-sensitized airways allergic inflammatory model of mice.

Methods: The pharmacokinetics of total alkaloids (TA) was investigated in SD rat plasma by a fully-validated LC-MS/MS method. Then, an ovalbumin (OVA)-sensitized airways allergic inflammatory model was established, in which mice were intra-gastrically administrated by 3 times a day (8.3 and 16.7 mg/kg) based on the pharmacokinetic behavior of TA and single (25, 50 mg/kg) treatment regimen. Dexamethasone was used as a positive control for corticosteroid drugs. Cellular infiltration was assessed in the broncho-alveolar lavage fluid (BALF). Expressions of interleukin-4 (IL-4) and interleukin-10 (IL-10) in the BALF were determined, levels of immunoglobulin E (IgE) and eotaxin in serum were measured, and superoxide dismutase (SOD) activities as well as malondialdehyde (MDA) content in the serum and BALF were examined. Finally, histopathological examination in the lung was assessed by H. E. staining.

Results: The time course of plasma concentration of 4 bioactive indole alkaloids fitted an open two-compartment model after oral administration of total alkaloids at doses of 10, 25, and 50 mg/kg. The area under the curve and the maximum concentration values of four major alkaloids increased dose-dependently, and half-life suggested a short-lasting pharmacological effect. Then, an ovalbumin-provoked airways allergic inflammatory model indicated that the pharmacological effect of administration of total alkaloids 3 times a day was a little better than that of single dose daily. The percentage of eosinophils in BALF was reduced obviously and the pathological damage of lung was also attenuated. There was also a significant reduction in IL-4 and promotion in IL-10 in the BALF. Serum IgE and eotaxin expression also significantly decreased in treated animals. Furthermore, the activity of SOD elevated remarkably and lipid peroxidation product (MDA) decreased in the administrated mice.

Conclusion: The pharmacological effects administrated for 3 times a day had precedence over single dose daily, which was related to the prolonged retention time and the maintained plasma concentration. Moreover, scholaricine and vallesamine might be responsible for the treatment of allergic asthma, mainly in total alkaloids.

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Abbreviations: TA, total alkaloids; Sch, scholaricine; Epi, 19-epispholaricine; Val, vallesamine; Pic, picrinine; DXM, dexamethasone; OVA, ovalbumin; BALF, broncho-alveolar lavage fluid; IgE, immunoglobulin E; MDA, malondialdehyde; SOD, superoxide dismutase; ELISA, enzyme-linked immunosorbent assay; H.E., hematoxylin and

eosin; AUC, area under the curve; C_{max}, maximum concentration; T_{1/2}, half-life; β₂AR, β₂ adrenergic receptor; T_{max}, time of maximum concentration.

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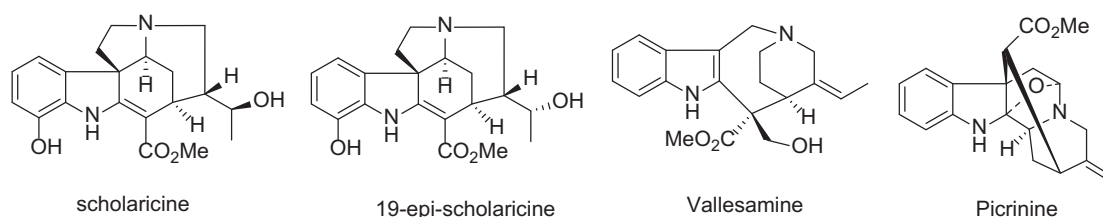


Fig. 1. Four major alkaloids from *A. scholaris*.

Introduction

Asthma is a global health problem, with more than 300 million people worldwide suffering from it (Yin et al., 2009), which results from a complex interplay between genetic and environmental factors. The disease is caused by nonspecific stimuli such as environmental pollutants, allergens, exhaust gases, infections, cold air, and strenuous exercise. Each of these stimuli irritates a variety of effector cells including inflammatory cells, epithelial cells, neurons, and narrowing the airway indirectly through the release of mediators (Factor, 2003). Investigation showed that pro-inflammatory mediators released from mast cells and eosinophils adjusted by T cells were particularly relevant to the progress of asthma (Holgate et al., 1999).

Alstonia scholaris (L.) R. Br. (Apocynaceae) 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 respiratory diseases in Yunnan Province, PR China (Compiling Group of Yunnan Traditional Chinese Medicine, 1977). *A. scholaris* extracts and alkaloids have shown antitussive, anti-asthmatic, expectorant (Shang et al., 2010a), analgesic, anti-inflammatory effects (Shang et al., 2010b), and airway anti-inflammation *in vivo* (Zhao et al., 2016). In our previous pharmacological studies, they also triggered β_2 adrenergic receptor (β_2 AR) activation (Hou et al., 2012a) and inhibited the nuclear factor- κ B (NF- κ B) (Hou et al., 2012b) activities *in vitro*. Moreover, the methanolic extract of *A. scholaris* exhibited anti-inflammatory and antioxidant activities against carrageenan induced rat paw oedema (Subraya and Gupta, 2012). These results indicated that alkaloids from the leaves of *A. scholaris* may also possess anti-inflammatory and analgesic effects. In the meantime, chemical profiling and metabolites of alkaloidal extract of *A. scholaris* have been reported (Cao et al., 2016). Based on pre-clinical studies on *A. scholaris*, “total alkaloids” 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 mono-center, randomized, double-blind, and placebo-controlled trial has been completed, and the results support further phase-II clinical trials.

In our previous phytochemical studies on *A. scholaris*, four indole alkaloids, scholaricine (Sch), 19-episolaricine (Epi), vallesamine (Val) (Feng et al., 2009), and picrinine (Pic) were isolated as major components in “total alkaloids (TA)” (Fig. 1). Previous pharmacological results indicated the efficiency of “total alkaloids” but adaptations and dosing frequency need to establish before further clinical applications. In the present work, a pharmacokinetic study was designed to develop and validate a highly sensitive liquid chromatography tandem mass spectrometry (LC-MS/MS) method for the quantitative determination of major alkaloids in biological samples collected from rat experiments *in vivo*. This method was applied to elucidate the pharmacokinetic behavior of Sch, Epi, Val, and Pic after oral administration of TA, which is highly important to understand their biological effects. Then, based on the pharmacokinetic profiles of 4 major bioactive alkaloids, evaluation of protective effect on airways allergic inflammation induced by ovalbu-

min (OVA) in mice was carried out to further provide remedy principle for phase II clinical trials.

Materials and methods

Plant material

The 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.

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 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). Sch (6%), Epi (2%), Pic (10%) (Shang et al., 2010a), and Val (6%) were isolated and kept in refrigerator in a previous phytochemical investigation from TA. The established UHPLC/UV method was used to determine four major alkaloids in the TA (See supporting information, S2.4).

Chemicals

Acetonitrile, methanol, and formic acid (Mallinckrodt Baker, Phillipsburg, NJ, USA) were of HPLC grade. Deionized water was obtained from a Milli-Q system (Millipore, MA, USA). Heparin sodium was purchased from Solarbio (Beijing, China). High-purity nitrogen (99.9%) and helium (99.99%) were purchased from Gas Supplies Center of Peking University Health Science Center (Beijing, China). Ovalbumin (OVA) was purchased from Source Biological Technology Co. Ltd. (Shanghai, China). Aluminum hydroxide was purchased from Sigma-Aldrich (St. Louis, MO, USA). ELISA reagents sets for IgE, eotaxin, IL-4, and IL-10 (Lot 10/2014) were purchased from R&D Systems (Minneapolis, MN, USA). Malondialdehyde (MDA), superoxide dismutase (SOD) and nitric oxide (NO) determination kits were purchased from Jiancheng Bioengineering Institute of Nanjing (Jiangsu, China). All the other chemicals and solvents were of highest purity grade.

Preparation of calibration standard, quality control, and internal standard stock solutions

Reference standards (0.5 mg/ml each for Sch, Val, and Pic) were dissolved in methanol to prepare individual stock solutions, and Epi (0.5 mg/ml) was dissolved in 50% aqueous methanol. These stock solutions were mixed and then serially diluted to obtain calibration standard (CS) stock solutions (125 μ g/ml for Sch, 62.5 μ g/ml for Epi and Val, and 250 μ g/ml for Pic). Quality control (QC) stock

solutions were prepared at three concentration levels as high QC (QC-H), middle QC (QC-M), and low QC (QC-L) (given in Table S1) and lower limit of quantification (LLOQ) was based on linear ranges of analytes (given in Table S2). Vincamine was used as an internal standard (IS) and was dissolved in acetonitrile to produce a 0.01 µg/ml IS stock solution. All the solutions were sealed and stored at −20 °C until use.

Sample preparation

For CS and QC samples, 150 µl of CS or QC stock solution were added to 150 µl blank rat plasma, followed by the addition of 150 µl IS solution and 150 µl acetonitrile. For unknown samples, 150 µl plasma was mixed with 150 µl IS solution and 150 µl organic phase (acetonitrile: methanol, 1:1, v/v). The above mixtures were vortexed (2200 rpm) for 1 min, ultrasonicated in a water bath for 5 min, vortexed (2200 rpm) for another 1 min, and then centrifuged (9000 rpm, 4 °C) for 10 min. The supernatant was separated and evaporated to dryness at 35 °C using a speedvac concentrator. The residue was stored at −20 °C and reconstituted in 150 µl 67% methanol before analysis to obtain the sample solution. After being filtered through 0.22 µm nylon membrane, a 5 µl aliquot was subjected to LC-MS/MS analysis.

Animals

Male Sprague-Dawley rats (8–10 weeks, 200–230 g) were provided by the Experimental Animal Center of Peking University Health Science Center (Beijing, China). Pathogen-free female ICR mice, weighing approximately 24–28 g, were purchased from Kunming Medical University (licence number SYXK 2011-0004). All 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.

Pharmacokinetics study

For total alkaloids (TA) administration, the rats were randomly divided into 3 groups ($n=10$ for each group) at doses of 10 mg/kg (L), 25 mg/kg (M), and 50 mg/kg (H) TA by oral gavage. Retro-orbital blood samples were collected into heparinized tubes before and 0.03, 0.08, 0.17, 0.25, 0.42, 1.0, 2.0, 4.0, 8.0, 12, and 24 h after administration. Blood samples were immediately centrifuged at 6000 rpm (4 °C) for 10 min to obtain plasma. The plasma samples were stored at −80 °C until LC-MS/MS analysis.

LC-MS/MS conditions

The LC/MS/MS system consisted of a Finnigan Surveyor LC instrument connected to a Finnigan TSQ Quantum triple quadrupole mass spectrometer via an ESI interface (ThermoFisher, CA, USA). The samples were separated on a Waters XTerra column (5 µm, ID 4.6 mm × 250 mm) equipped with an Agilent ZORBAX SB-C18 guard column (5 µm, ID 4.6 mm × 12.5 mm). The mobile phase consisted of acetonitrile: methanol (9:1, v/v) (A) and water containing 0.3% formic acid (B). A gradient elution program was used: 0–13 min, 10–14% A; 13–25 min, 14–40% A; 25–27 min, 40–95% A; 27–30 min, 95% A. The flow rate was 0.8 ml/min. The column temperature was set at 35 °C and sample injector at 20 °C. The split ratio was 4:1. The mass spectrometer was operated in the positive ion mode with SRM scan. High purity nitrogen was used as the sheath (50 arb) and auxiliary (5 arb) gas. Other parameters were as follows:

spray voltage, 4.0 kV; capillary temperature, 350 °C; capillary voltage 208 V. The SRM parameters are listed in (Table S3).

Airways allergic inflammatory model

The experiment was carried out as described in literature (Xu et al., 2012). 110 mice were randomly divided into 11 groups ($n=10$) and given the following treatments separately, then, animals from group 3–12 were given the same process of modeling as OVA group; (1) Control: sham sensitization plus challenge with saline (intranasal [i.n.]); (2) OVA: sensitization with OVA via subcutaneous (s.c.) injection plus challenge OVA via i.n.; (3) DXM: dexamethasone (i.g.), 2 mg/kg, once daily; (4) TA-H-S: total alkaloids intragastrically (i.g.), 50 mg/kg, once daily; (5) TA-H-M: total alkaloids (i.g.), 16.7 mg/kg, 3 times daily; (6) TA-L-S: total alkaloids (i.g.), 25 mg/kg, once daily; (7) TA-L-M: total alkaloids (i.g.), 8.3 mg/kg, 3 times daily; (8) Sch (i.g.), 3 mg/kg, once daily; (9) Epi (i.g.), 3 mg/kg, once daily; (10) Val (i.g.), 3 mg/kg, once daily; (11) Pic (i.g.), 5 mg/kg, once daily. Briefly, on days 0 and 7, all groups of animals except the control saline group were sensitized by s.c. injection of 60 µg OVA emulsified with 2 mg of aluminium hydroxide gel in 0.2 ml saline. On days 15–21, mice were intra-gastrically administered with DXM, TA, Sch, Epi, Val, and Pic, respectively. All samples were dissolved in 0.5% (w/v) sodium carboxymethyl cellulose (CMC-Na). The animals in control and OVA group were administered with the same volume of solvent. The doses of four alkaloids were determined according to the content in TA. On days 18–20, all the groups except control group were challenged with 0.4% OVA (50 µl per mice) by intranasal administration after one hour treatment. An equal volume of intranasal saline was substituted for the control group. A schematic diagram of the treatment schedule is shown in Fig. 2.

Blood samples were collected via the orbital vein of mouse 48 h after OVA challenged. The serum samples were obtained by centrifuging blood samples at 4 °C, 4000 × g for 15 min, and serum samples were prepared and stored at −80 °C for the analysis of IgE, eotaxin (Neumann et al., 2013), SOD, and MDA (Lemos et al., 2014). The right lungs were ligated and the left lungs were lavaged with 1.5 ml autoclaved PBS for 3 times. The BALF was immediately centrifuged at 4 °C, 300 g for 10 min, and the supernatants were stored at −80 °C until required for the tests of IL-4, IL-10 (Stampfli et al., 1999), SOD, and MDA. 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). The right lung tissues were embedded in paraffin 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 (Ma et al., 2012; El-Agamy, 2011; Li et al., 2011).

Statistical analyses

All calibration and quantitation data were processed with Xcalibur 1.4 software (Thermo Fisher Scientific, CA, USA). Concentrations of four alkaloids were calculated by their calibration curves. In pharmacological studies, results are presented as mean ± 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 LSD (Least Significant Difference) or Kruskal Wallis analysis with Dunn post-test, in which nonparametric analyses were appropriate. Analyses were performed using Graphpad Prism Software (San Diego, Ca). A *P* value of less than 0.05 was considered as significant for all the analyses.

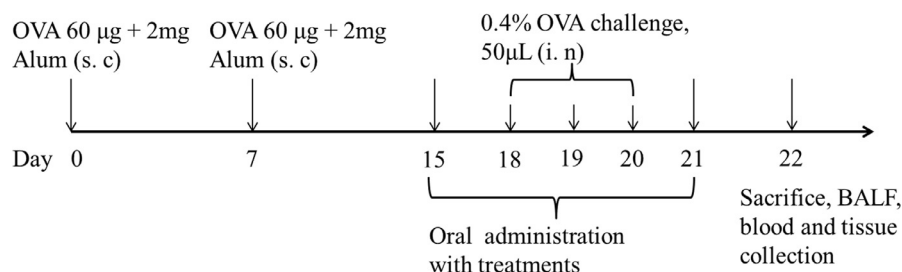


Fig. 2. Experimental protocol for the establishment of OVA-induced airways allergic inflammatory model and the treatment schedule.

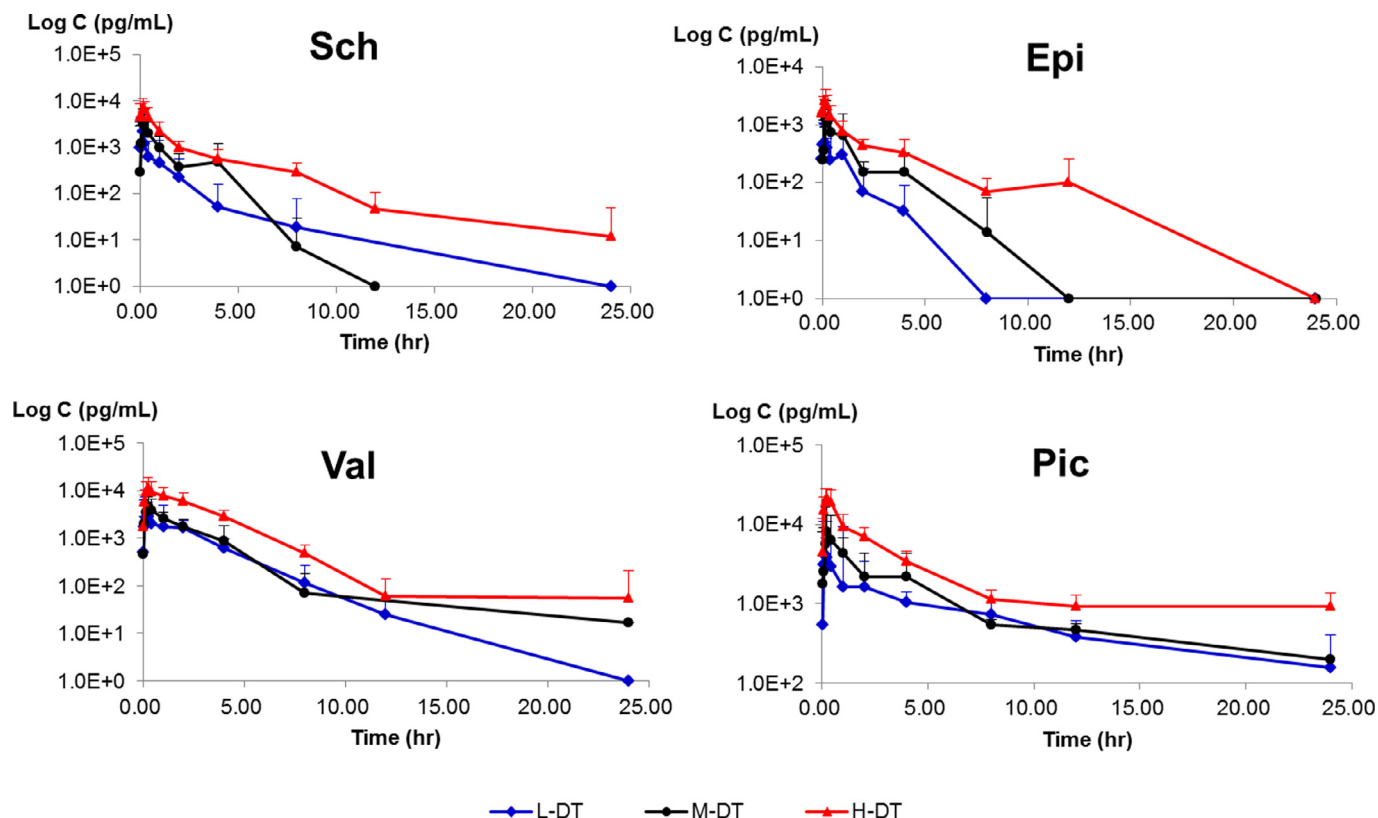


Fig. 3. Plasma concentration-time curves of four alkaloids after oral administration of total alkaloids at doses of 10, 25, and 50 mg/kg.

Results

Pharmacokinetics of total alkaloids

The LC-MS/MS method was fully validated according to the U.S. FDA guidance on bioanalytical method validation (CDER, 2001) (Fig. S1A, B and C). By using the SRM scans, the four alkaloids showed good linearity ($r^2 > 0.99$) in wide dynamic ranges (10^4 – 10^5 -fold) (Fig. S2 and S3). The lower limit of quantitation (LLOQ) was 0.050 ng/ml for Sch, 1.000 ng/ml for Pic, and 0.025 ng/ml each for Epi and Val. As shown in Table S4 and S5, the method showed good precision (RSD < 12.85%) and accuracy (88.29–106.42%). The samples were stable after 24 h storage in the sample tray or after 14 days storage at -80°C (given in Table S6, S7 and S8). Experimental details are provided in the supporting information.

This method was applied to the pharmacokinetics of Sch, Epi, Val, and Pic after oral administration of total alkaloids. The mean plasma concentration-time profiles ($n=10$) of these four analytes are presented in Fig. 3, and the main pharmacokinetic parameters of 3 administered groups are listed in (Tables 1–3). Results in (Fig. 3) indicated that $T_{\max}$ occurred within 10 min for Sch and Epi,

and 15 min for Val and Pic; then they eliminated from plasma after 24 h. As shown in (Tables 1–3), the $C_{\max}$ and AUC_{last} of four analytes increase were proportional to the dosage of TA and the elimination half-life ($T_{1/2\alpha}$) was short for four analytes. The pharmacokinetic properties indicated that Val and Pic acted rapidly while Sch and Epi were faster *in vivo*.

Observations on animal signs

In contrast to the control mice, obvious allergic symptoms were observed in all OVA-induced mice, including mouth breathing, wheezing, shaking, nose scratching, coughing and so forth. During administration period, the symptoms of treatment groups alleviated without dose-response dependence compared to the OVA group.

Effects of TA on allergen-induced inflammatory cell counting in BALF

Allergic asthma is characterized by inflammatory cell accumulation in the airway and lung. ICR mice were challenged with OVA and then sacrificed 48 h after the last sensitization. Lungs were lavaged at the end of experiment, and differential cells in BALF

Table 1

Pharmacokinetic parameters of four alkaloids in rat plasma after oral administration of total alkaloids at dose of 10 mg/kg (mean, n = 10).

Parameter	Units	Sch	Epi	Val	Pic
T _{max}	h	0.14	0.12	0.23	0.17
C _{max}	ng/ml	1.66	0.49	2.57	4.10
T _{1/2 α}	h	0.11	0.05	0.33	0.17
T _{1/2 β}	h	1.52	1.22	2.00	5.72
K ₀₁	1/h	9.77	12.89	19.07	14.69
K ₁₀	1/h	2.44	1.52	0.36	0.37
K ₁₂	1/h	3.05	7.22	0.09	2.54
K ₂₁	1/h	1.16	4.89	2.00	1.34
AUC _{last}	h*ng/ml	1.40	0.68	7.70	16.12
CL/F	ml/h/kg	2,506,179.70	1,116,876.60	332,312.50	268,026.14
V/F	ml/kg	2,710,937.20	1,088,604.60	38,868.48	1,358,401.10

Table 2

Pharmacokinetic parameters of four alkaloids in rat plasma after oral administration of total alkaloids at dose of 25 mg/kg (mean, n = 10).

Parameter	Units	Sch	Epi	Val	Pic
T _{max}	h	0.20	0.16	0.30	0.34
C _{max}	ng/ml	3.46	1.26	4.22	7.19
T _{1/2 α}	h	0.08	0.04	0.23	0.19
T _{1/2 β}	h	2.14	1.09	1.92	4.01
K ₀₁	1/h	8.24	16.31	7.89	3.67
K ₁₀	1/h	2.04	1.72	0.61	0.76
K ₁₂	1/h	5.21	9.35	0.98	2.31
K ₂₁	1/h	1.31	6.11	1.78	0.85
AUC _{last}	H × ng/ml	4.21	1.55	10.09	22.71
CL/F	ml/h/kg	1,699,863.40	1,215,763.70	634,446.10	475,496.58
V/F	ml/kg	3,312,333.50	1,081,863.50	571,928.09	1,693,220.00

Table 3

Pharmacokinetic parameters of four alkaloids in rat plasma after oral administration of total alkaloids at dose of 50 mg/kg (mean, n = 10).

Parameter	Units	Sch	Epi	Val	Pic
T _{max}	h	0.16	0.15	0.31	0.24
C _{max}	ng/ml	6.82	2.50	10.40	21.17
T _{1/2 α}	h	0.30	0.15	1.88	0.22
T _{1/2 β}	h	2.96	2.71	9.57	2.67
K ₀₁	1/h	14.40	11.93	12.93	8.60
K ₁₀	1/h	0.95	1.08	0.37	0.71
K ₁₂	1/h	1.01	2.74	0.00	1.52
K ₂₁	1/h	0.56	1.11	0.07	1.14
AUC _{last}	H × ng/ml	9.74	3.91	31.32	46.91
CL/F	ml/h/kg	2,772,464.30	951,884.63	409,897.67	460,435.60
V/F	ml/kg	1,553,156.20	2,187,033.70	125.11	868,137.55

were counted to investigate the effect of TA on inflammatory cells influx. Number of total leukocytes (Fig. 4A) and the percentage of eosinophils (Fig. 4B) in BALF increased in OVA sensitized mice, while they almost completely decreased by TA ($p < 0.05/0.01$); the inhibition with dose administrated 3 times a day was slightly better than once a day. Sch treatment had the similar inhibition ($p < 0.05$) compared with the OVA group while Epi inhibited only the eosinophils ($p < 0.05$), but Val and Pic exhibited inhibiting tendency ($p > 0.05$). The positive group (DXM) at 2 mg/kg reduced the number of inflammatory cells prominently ($p < 0.01$).

Effect of TA on IgE and eotaxin production in serum

Among immunological parameters, IgE is one of the most important factors related to immune-response; with high levels of serum IgE in allergic asthma (Robinson et al., 1992). IgE and eotaxin production in serum were analyzed by ELISA (Fig. 5); OVA significantly enhanced IgE (Fig. 5A) and eotaxin (Fig. 5B) production compared with the control group ($p < 0.01$) but in both treated 3 times and once a day, TA depressed IgE and eotaxin in serum notably ($p < 0.05/0.01$) and the action of treating for 3 times daily

was predominant. Besides, Sch could minimize the two observations remarkably ($p < 0.05$), with DXM groups ($p < 0.05/0.01$) as control. However, Epi, Val and Pic did not inhibit the expression of IgE and eotaxin ($p > 0.05$).

Effect of TA on IL-4 and IL-10 levels in BALF

Th2 (CD4+T helper cells 2) cytokines are highly expressed in allergic inflammation and asthma (Virchow et al., 1996). The production of Th2 cytokines including IL-4 and IL-10 were measured by ELISA. As indicated in (Fig. 6), the OVA administration induced a marked increase in the level of IL-4 (Fig. 6A) and a notable decrease in IL-10 (Fig. 6B) compared with control group ($p < 0.01$). TA administered 3 times or once daily reduced IL-4 level and enhanced IL-10 remarkably, in contrast to the OVA group ($p < 0.05/0.01$), and the reconciliation of administration 3 times daily was better than once without significance ($p > 0.05$). Sch, Epi, and Val exhibited a remarkable impact on IL-4 ($p < 0.05/0.01$). Furthermore, Sch and Epi also had an enhancing effect on IL-10 ($p < 0.05$), and DXM groups showed similar effects ($p < 0.05$).

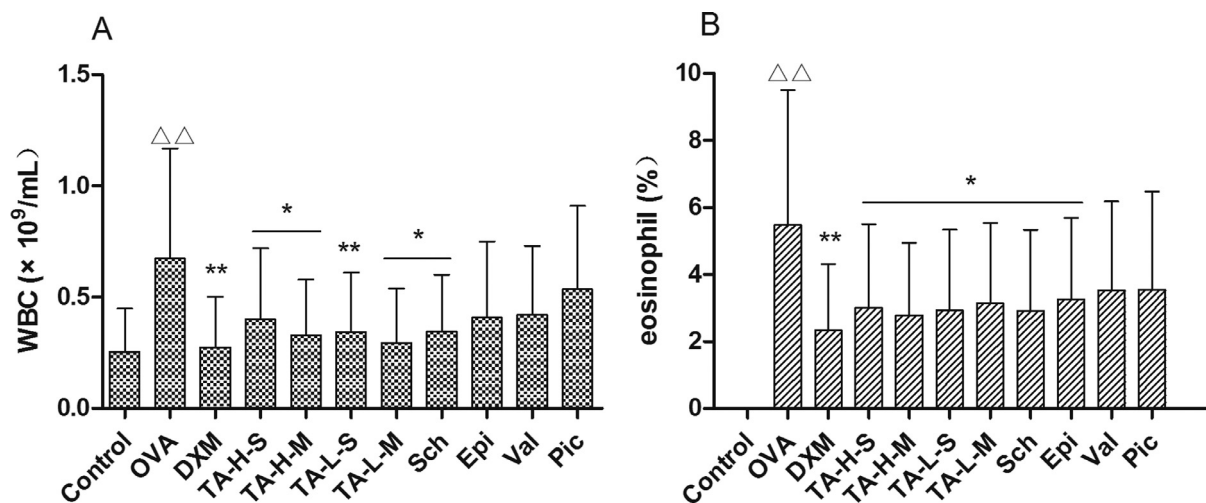


Fig. 4. The production of inflammatory cells in BALF after treatment. Data was shown as mean $\pm$ SEM values. $\Delta\Delta p < 0.01$ vs control, $*p < 0.05$, $**p < 0.01$ vs OVA ($n = 10$ mice per group). (A) the number of total leukocytes. (B) the percentage of eosinophils. WBC, white blood (cell) count.

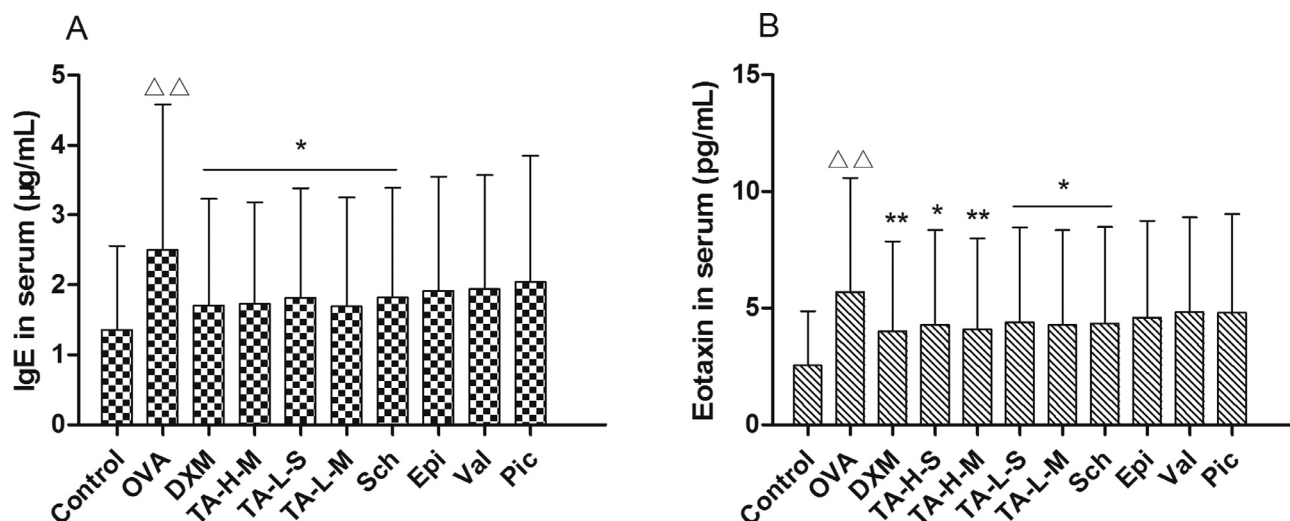


Fig. 5. The expression of immunoglobulin E (IgE; A) and eotaxin (B) in serum after treatment. Data was shown as mean $\pm$ SEM values. $\Delta\Delta p < 0.01$ vs control, $*p < 0.05$, $**p < 0.01$ vs OVA ($n = 10$ mice per group).

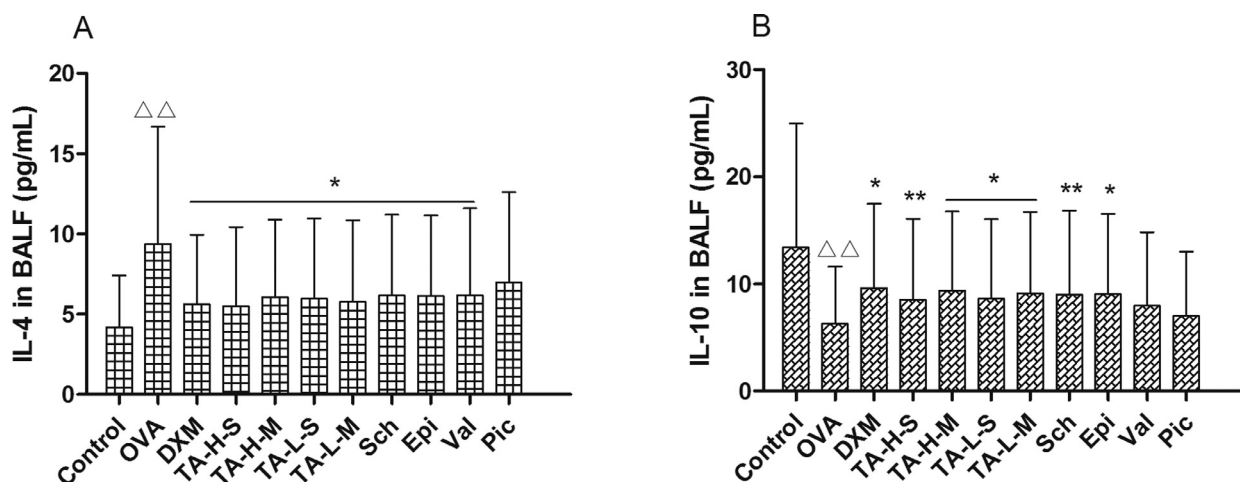


Fig. 6. The production of interleukin-4 (IL-4; A) and interleukin-10 (IL-10; B) in BALF after treatment. Data was shown as mean $\pm$ SEM values. $\Delta\Delta p < 0.01$ vs control, $*p < 0.05$, $**p < 0.01$ vs OVA ($n = 10$ mice per group).

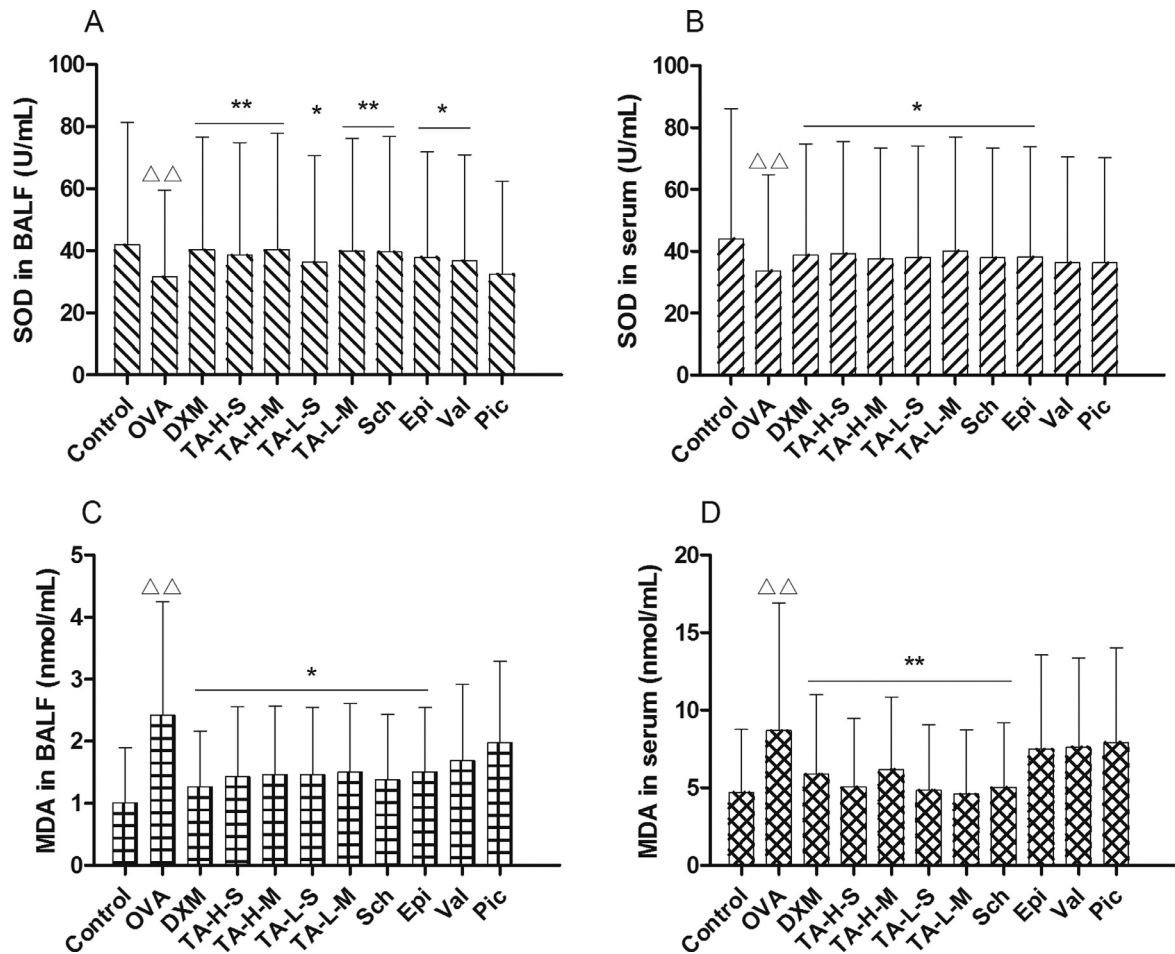


Fig. 7. The antioxidant parameters of superoxide dismutase (SOD; A: in BALF, B: in serum) and malondialdehyde (MDA; C: in BALF, D: in serum) after treatment. Data was shown as mean $\pm$ SEM values. $\Delta\Delta p < 0.01$ vs control, $*p < 0.05$, $**p < 0.01$ vs OVA ($n = 10$ mice per group).

Effect of TA on SOD and MDA activities in BALF and serum

Oxidative stress plays an important role in the development of OVA-induced asthma. SOD and MDA activities in serum and BALF were determined to evaluate the effects of TA on oxidative stress. As shown (Fig. 7), OVA group resulted in marked decline of SOD activities (Figs. 7A and B) and significant rise of MDA levels (Figs. 7C and D) compared with control group ($p < 0.01$). Encouragingly, all the groups of TA promoted SOD activities significantly ($p < 0.05/0.01$) and reduced MDA levels in the serum and BALF ($p < 0.05/0.01$). Sch and Epi exhibited a notable improvement on the two observations in BALF and serum ($p < 0.05/0.01$) while Val and Pic showed improving tendency ($p > 0.05$) in contrast to the OVA group. DXM also played important role in the two indices ($p < 0.05/0.01$).

Lung histopathology

The production of inflammatory cells in the lungs of mice was also investigated by histopathological studies. OVA provoked mice showed a marked infiltration of eosinophils and lymphocytes around peribronchial and perivascular spaces and alveolar walls compared to normal group (Fig. 8). Accordingly, the scores for total lung inflammation increased significantly (Table 4). Besides, submucosa widened, mucosal folds increased, the lumen narrowed, and the smooth muscles were also observed in OVA group. The results indicated that the infiltration of inflammatory cells reduced significantly in TA-treated mice compared to OVA challenged mice,

Table 4

The inflammatory scores of lung after challenged by OVA

Data are expressed as mean $\pm$ SEM. Statistical differences are represented as $\Delta\Delta p < 0.01$ vs control, $*p < 0.05$ vs OVA ($n = 10$ mice per group).

Control: sham sensitization plus challenge with saline (intranasal [i.n.]);

OVA: sensitization with OVA via a subcutaneous (s.c.) injection plus challenge OVA via i.n. DXM: dexamethasone, 2 mg/kg; orally administrated for 1 time a day.

TA-H-S: administrated TA for 1 time daily, 50 mg/kg;

TA-H-M: administrated TA for 3 times daily, 16.7 mg/kg;

TA-L-S: administrated TA for 1 time daily, 25 mg/kg;

TA-L-M: administrated TA for 3 times daily, 8.3 mg/kg;

Sch: scholaricine, 3 mg/kg;

Epi: 19-episclerol, 1 mg/kg;

Val: vallesamine, 3 mg/kg;

Pic: picric acid, 5 mg/kg.

Groups	Dose (mg/kg)	Scores (means $\pm$ SEM)
Control	–	1.00 $\pm$ 0.71
OVA	–	3.00 $\pm$ 1.22 $\Delta\Delta$
DXM	2.0	1.44 $\pm$ 1.01*
TA-H-S	50.0	1.78 $\pm$ 0.67*
TA-H-M	16.7	1.56 $\pm$ 0.88*
TA-L-S	25.0	1.67 $\pm$ 0.87*
TA-L-M	8.3	1.78 $\pm$ 0.97*
Sch	3.0	1.78 $\pm$ 0.67*
Epi	1.0	1.89 $\pm$ 1.27
Val	3.0	2.33 $\pm$ 1.32
Pic	5.0	2.78 $\pm$ 1.09

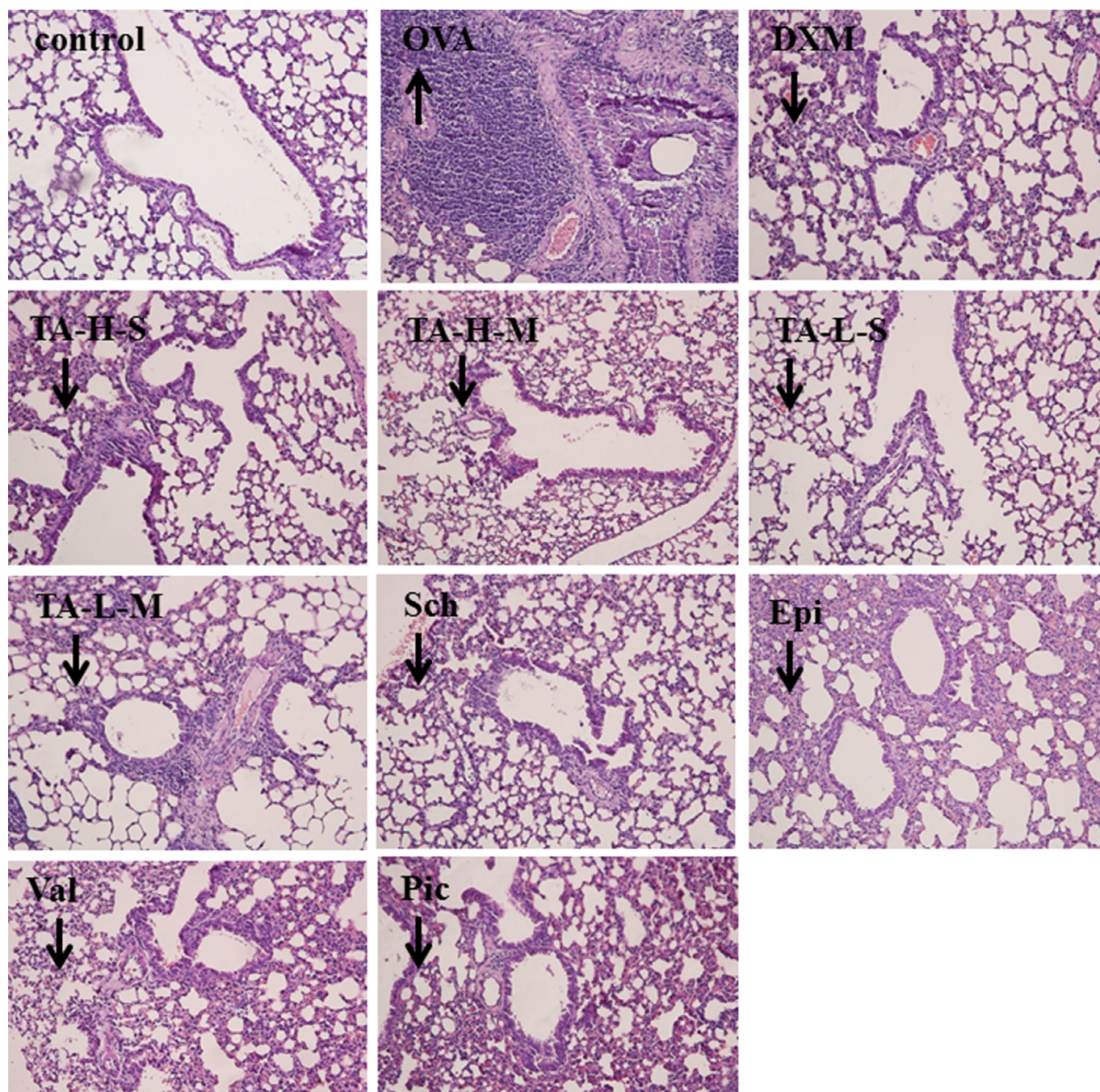


Fig. 8. Representative microscopic sections of tissue demonstrating lung histology. The lung sections were stained with H & E and examined by light microscopy. The upward ($\uparrow$) and downward ($\downarrow$) arrows in those pictures meant the increase or decrease in inflammation. Original magnification $\times 200$.

and treatment for 3 times daily was a little bit better than single dose daily. The four alkaloids (Sch, Epi, Val, and Pic) also prevented infiltration of inflammatory cells. DXM at 2 mg/kg treatment, used as reference drug, remarkably improved the pulmonary histologic changes.

Discussion

After preliminary testing, LC-MS/MS method was developed and validated for the simultaneous determination of Sch, Epi, Val, and Pic in rat plasma. This study established a reliable method for rapid screening of the main compounds of TA, and it provided substantial data that could be applied to further investigate ingredient

based pharmacodynamics of their anti-allergic effects. The method gave higher recovery, shorter analysis times, better separation, and retention of four analytes with no interference from endogenous plasma alkaloids. The pharmacokinetic profile of 4 major indole alkaloids (Sch, Epi, Val, and Pic) after oral administration of TA was determined in rats. Pharmacokinetic analyses indicated that TA at doses of 10, 25, and 50 mg/kg had a good oral exposure in rat, but the absorption half-life ($t_{1/2}$) was short. Results further indicated that Val and Pic acted rapidly while Sch and Epi were faster *in vivo*, suggesting that the best therapeutic efficacy in human may be achieved by taking TA 3 times a day.

In pharmacological investigation, an *in vivo* airways allergic inflammatory model was induced by aluminium hydroxide plus

ovalbumin sensitization and exposed to OVA via intranasal route for 3 days for provocation. Pathological slides, inflammatory cells, and cytokines data displayed that the model was successfully established. Asthma is a chronic inflammatory disease characterized by reversible airway obstruction, airway inflammation and bronchial hyper-responsiveness (Hansel and Barnes, 2001). Inflammatory cells and inflammatory cytokines production are the main characteristics of asthma. Particularly, the production and activation of eosinophils in the airways result into worse pulmonary inflammation and lung dysfunction in asthma patients (Jacobsen et al., 2007). Results in our investigation demonstrated that the number of leukocytes and eosinophils increased notably in OVA group compared with the control group, but they were decreased by TA. Furthermore, the group administered with multiple dosing was more effective with no dose-dependent manner, which might be due to reasonable blood drug concentration of TA administrated 3 times a day. Histopathological examination of the lungs further confirmed the inhibitory effects of TA on inflammation and recruitment of inflammatory cells, which was in accordance with the importance of inflammatory cells' secretion in asthma (Shimizu et al., 2003).

In a healthy individual, the ratio of TH1/TH2 is in fine balance, but when TH2 is predominant or TH1 decreases, the balance is disturbed and asthma occurs (Gore and Custovic, 2004). Pro-inflammatory cytokine IL-4 is mainly produced by Th2 cells (Brightling et al., 2002) and acts as an essential growth factor for TH2 cells. It converts the immunoglobulin class into immunoglobulin E (IgE) which plays a pivotal role in mediating allergic responses (Kelly-Welch et al., 2004). IL-10 plays a role of immunosuppression and anti-inflammation through inhibition of cytokine production by both TH1 and TH2 cells, hence, it is an attractive target to prevent both TH1 and TH2 responses (Coyle et al., 1996). In our investigation, TA, Sch, Epi, and Val down-regulated the secretion of IL-4 while TA, Sch, and Epi enhanced the production of IL-10 in BALF, which suggest the response of TH2 lymphocytes may be regulated by TA. Besides, the total IgE rise in serum was restrained by TA, most likely mediated through the inhibition of IL-4, which might be partly responsible for the dramatic reduction in pulmonary eosinophils. Eotaxin is a CC (cysteine chemo-attractant) chemokine with potent and specific chemotactic activity for eosinophils and contributes to the pathogenesis of asthma by specific production of eosinophils into the airways (Gonzalo et al., 1996). The high-expression of eotaxin in serum and airways of asthmatics has been verified (Lamkhieoued et al., 1997), hence, the elimination of eotaxin is a key to attenuate pulmonary eosinophilia after allergen challenge (Foster et al., 2001). In present study, eotaxin levels in the serum of TA-treated mice reduced significantly, and the group administrated with the dose 3 times daily was predominant.

Bronchial asthma has a close relationship with oxidative process, accompanied by oxygen free radicals, which significantly contribute to the inflammatory pathology (Nadeem et al., 2003). High levels of MDA and low SOD activity in BALF and serum of asthmatic patients were reported previously (Ruprai, 2011; Katsoulis et al., 2003). In the present investigation, the promotion effects of TA, Sch, and Epi were observed on oxidative stress parameters (SOD and MDA) both in sera and BALF, and the antioxidant activity of several doses daily was stronger than single dose daily. Hence, our model is consistent with the reported results.

Conclusion

Based on the prediction of pharmacokinetic parameters, the features of airways allergic inflammatory model induced by OVA in mice were improved remarkably by TA administrated 3 times a day, through the enhancement of blood drug concentration and

prolonged retention time. The pharmacological efficiency of TA was better than any of the single compound which might be due to synergistic effects of alkaloids. Moreover, scholaricine and vallesamine might be responsible for the treatment of allergic inflammation, mainly in total alkaloids.

Conflict of interest

We confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2017.02.002.

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Supporting Information for

Airways antiallergic effect and pharmacokinetics of alkaloids from *Alstonia scholaris*

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Supplementary Text

S1. Results of Method Validation for Pharmacokinetics

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S1.3. Precision accuracy

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S1.5. Stability

Figs. S1

Tables S1 to S8

S2. Alkaloids quantitative analysis

References

Supplementary Text

S1. Results of Method Validation for Pharmacokinetics

S1.1 Specificity

The LC-MS/MS analytical method was validated according to the U.S. FDA guidance on bioanalytical method validation^{S1}. Under the selected chromatographic conditions (Fig. S1), the baseline of four alkaloids and IS was separated. In ESIMS (positive ion detection mode), the molecular ion peak $[M+H]^+$ was mainly produced. No endogenous peaks were observed in blank plasma samples for 4 analytes.

S1.2 Calibration curve

The calibration curves (Table S2) covered wide dynamic ranges (10^4 – 10^5 -fold), and showed good linearity ($r^2 > 0.99$). The lower limit of quantitation (LLOQ) of Sch was 0.050 ng/ml; 1.000 ng/ml for Pic; 0.025 ng/ml for Epi and Val.

S1.3 Precision and accuracy

The precision and accuracy of the assay were evaluated by quality control (QC) samples at low (QC-L), medium (QC-M) and high (QC-H) concentrations on the same day (n = 5) and on 3 consecutive days. The measured concentrations were compared to the nominal concentrations, and the relative standard deviations (RSD) of 4 alkaloids were calculated. RSD values were not higher than 12.85% for QC-L, not higher than 8.22% for QC-M, and not higher than 11.02% for QC-H (Table S4), indicating acceptable variation of the method. As shown in (Table S5) the accuracy rates ranged from 88.29-106.42%.

S1.4 Matrix effects and extraction recovery

Matrix effects and extraction efficiency were examined by 3 groups of standard addition experiments. For each group, 3 concentrations were added. In group 1, analytes were added to plasma and then extracted as QC samples; in group 2, analytes were added into pre-extracted blank rat plasma; in group 3, analytes were dissolved in methanol. Absolute Recovery was calculated as:

$$AR (\%) = \frac{\text{Measured concentration of group 1}}{\text{Measured concentration of group 3}} \times 100\%$$

Extraction Recovery was calculated as:

$$ER (\%) = \frac{\text{Measured concentration of group 1}}{\text{Measured concentration of group 2}} \times 100\%$$

The AR and ER of 4 analytes were between 85-115%, and varied within $\pm 15\%$ at all the 3 concentrations (Table S6). The matrix effect was calculated as:

$$ME (\%) = \frac{\text{Measured concentration of group 2}}{\text{Measured concentration of group 3}} \times 100\%$$

As shown in (Table S7), matrix effects of blank plasma due to 4 analytes were related

to the concentration of QC, namely, 4 analytes had almost no matrix effect for QC-L; at QC-M, Sch and Epi had almost no matrix effect, Val and Pic had weak ionization enhancement; at QC-H, Sch had the ionization of weak inhibition, Epi had almost no matrix effect, Val and Pic had weak ionization enhancement.

S1.5. Stability

Stability was determined at 2 concentrations after 24h storage in the sample tray, and after 14-days storage at -80 °C. All the analytes were stable during analysis (RSD below 21.08% and 13.44% for QC-L and QC-H, respectively, as shown in Table S8). Results suggested plasma samples were stable during our experimental period.

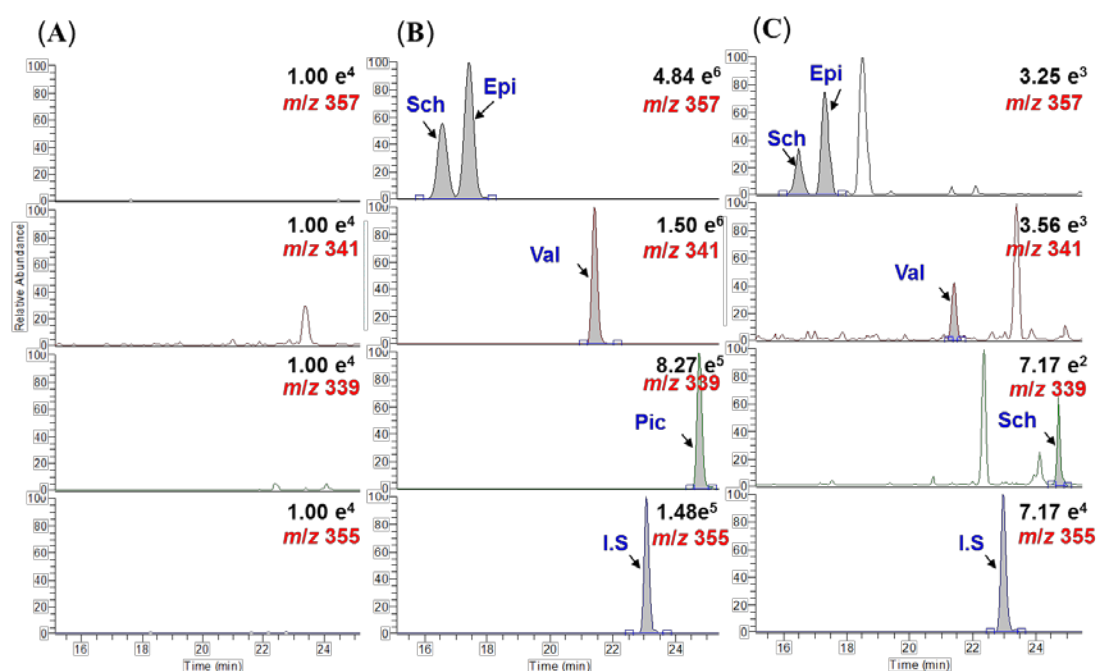


Fig. S1. Comparison of blank plasma (A), blank plasma spiked with 4 alkaloids and IS (B), and plasma obtained 15 minutes after administration (C).

Table S1. Final concentration of quality control samples

	Analytes			
	Sch	Epi	Val	Pic
QC-L (ng/ml)	0.227	0.114	0.114	4.545
QC-M (ng/ml)	227.000	113.500	113.500	4545.000
QC-H (ng/ml)	1135.000	568.000	568.000	22730.000

L: low dose; M: middle dose; H: high dose

Table S2. Regression data and range of quantification for 4 alkaloids

Analyte	Weighting Index	Equation	Correlation coefficient (R^2)	Range (ng/ml)
Sch	1/X	$Y = 0.0238271 + 0.137227 * X$	0.9910	0.050-5000
Epi	1/X	$Y = 0.0167316 + 0.297297 * X$	0.9953	0.025-2500
Val	1/X	$Y = 0.0057778 + 0.0332765 * X$	0.9957	0.025-2500
Pic	1/X	$Y = -0.00212676 + 0.00618072 * X$	0.9995	1.000-10000

Y, Peak area ratio of quantitative alkaloids with IS; X, Concentration of quantitative alkaloids

Table S3. Segmented SRM settings for total alkaloids

Analyte	Parent	Centre	CE	TLO
Sch	357.0	325.0	24.0	142.0
Epi	357.0	325.0	24.0	142.0
Val	341.0	282.0	17.0	63.0
Pic	339.0	296.0	17.0	129.0

CE, collision energy; TLO, tube lens offset

Table S4. Intraday and interday variations of 4 alkaloids

			Analytes (n = 5)			
			Sch	Epi	Val	Pic
QC-L (ng/ml)	intraday	NC	0.227	0.114	0.114	4.545
		M	0.262	0.125	0.112	3.967
		A (%)	115.52	109.95	98.86	87.28
		RSD (%)	1.42	6.50	10.36	12.85
	interday	MC	0.263	0.130	0.115	3.798
		A (%)	115.66	114.90	101.66	83.56
		RSD (%)	1.99	2.75	10.12	4.04
QC-M (ng/ml)	intraday	NC	227.000	113.500	113.500	4545.000
		MC	208.326	105.049	99.316	4408.763
		A (%)	91.77	92.55	87.50	97.00
		RSD (%)	8.22	8.00	5.81	7.88
	interday	MC	215.3933	108.9754	107.1624	4466.4360
		A (%)	94.89	96.01	94.42	98.27
		RSD (%)	5.53	6.55	7.09	2.74
QC-H (ng/ml)	intraday	NC	1135.000	568.000	568.000	22730.000
		MC	1062.503	536.524	526.577	22952.469
		A (%)	93.61	94.46	92.71	100.98
		RSD (%)	5.52	4.29	6.90	4.10
	interday	MC	1083.6705	543.9000	526.3035	22786.5949
		A (%)	95.48	95.76	92.66	100.25
		RSD (%)	6.91	9.14	11.02	7.19

NC, nominal concentration; MC, measured concentration; A, accuracy in %. RSD, Relative Standard Deviation in %.

Table S5. Precision of 4 alkaloids

		Analytes (n = 5)			
		Sch	Epi	Val	Pic
QC-L (ng/ml)	NC	0.227	0.114	0.114	4.545
	MC	0.233	0.121	0.114	4.297
	RR (%)	102.48	106.42	100.34	94.54
	RE (%)	2.48	6.42	0.34	-5.46
QC-M (ng/ml)	NC	227.000	113.500	113.500	4545.000
	MC	216.794	111.751	103.690	4012.675
	RR (%)	95.50	98.46	91.36	88.29
	RE (%)	-4.50	-1.54	-8.64	-11.71
QC-H (ng/ml)	NC	1135.000	568.000	568.000	22730.000
	MC	1034.573	533.523	525.313	22216.502
	RR (%)	91.15	93.93	92.48	97.74
	RE (%)	-8.85	-6.07	-7.52	-2.26

NC, nominal concentration; MC, measured concentration; $RR = MC/NC \times 100\%$; $RE = (RR - 1) \times 100\%$

Table S6. Absolute and extract recovery of 4 alkaloids

		Analytes (n = 5)			
		Sch	Epi	Val	Pic
QC-L (ng/ml)	Extract MC	0.256	0.125	0.116	4.203
	Standard MC	0.210	0.127	0.127	4.477
	Matrix MC	0.192	0.136	0.114	4.782
	AR (%)	112.37	99.10	96.31	93.89
	ER (%)	119.11	98.52	101.04	90.00
QC-M (ng/ml)	Extract MC	216.794	111.751	103.690	4012.675
	Standard MC	211.134	99.660	96.682	3955.331
	Matrix MC	198.005	105.674	115.743	4828.287
	AR (%)	103.54	112.25	106.14	101.46
	ER (%)	109.72	106.81	90.89	94.46
QC-H (ng/ml)	Extract MC	1008.900	513.906	500.617	21565.843
	Standard MC	1041.943	495.995	500.134	19189.227
	Matrix MC	945.063	499.824	577.878	23561.766
	AR (%)	96.87	103.80	100.41	112.45
	ER (%)	109.72	106.81	90.89	94.46

MC, measured concentration; Extract, normal quality control sample; Standard, matrix effect quality control sample; Matrix, extraction efficiency quality control sample; AR, Absolute Recovery; ER, Extract Recovery.

Table S7. Matrix effects of 4 alkaloids

		Analytes (n = 3)			
		Sch	Epi	Val	Pic
QC-L (ng/ml)	Matrix MC	0.195	0.134	0.123	4.076
	Standard MC	0.207	0.134	0.122	4.256
	MF	0.97	1.00	1.00	0.96
	SD	0.05	0.04	0.05	0.11
	RSD (%)	4.89	3.78	5.05	11.37
QC-M (ng/ml)	Matrix MC	196.726	105.183	112.597	4740.465
	Standard MC	208.630	99.503	97.965	3960.919
	MF	0.94	1.06	1.15	1.20
	SD	0.03	0.02	0.07	0.04
	RSD (%)	2.78	1.63	5.76	3.11
QC-H (ng/ml)	Matrix MC	924.035	492.769	571.345	23012.180
	Standard MC	1051.797	495.488	493.830	19172.923
	MF	0.88	0.99	1.16	1.20
	SD	0.04	0.05	0.06	0.07
	RSD (%)	4.95	4.90	4.86	5.42

MC, measured concentration; Standard, matrix effect quality control sample; Matrix, extraction efficiency quality control sample; MF, Matrix effect.

Table S8. Stability of 4 alkaloids

		Analytes (n = 3)			
		Sch	Epi	Val	Pic
QC-L (ng/ml)	NC	0.227	0.114	0.114	4.545
	0h MC	0.180	0.128	0.130	4.359
	24h MC	0.214	0.123	0.123	5.155
	ST RSD (%)	13.62	7.61	6.54	12.95
	FT MC	0.149	0.129	0.124	4.210
	FT RSD (%)	21.08	7.22	4.53	7.41
	LT MC	0.233	0.129	0.123	3.808
	LT RSD (%)	16.88	7.43	6.19	12.90
QC-H (ng/ml)	NC	1135.000	568.000	568.000	22730.000
	0h MC	1025.912	497.058	500.556	19315.510
	24h MC	1024.170	476.779	484.661	18338.257
	ST RSD (%)	3.79	6.57	6.90	3.27
	FT MC	1152.059	510.138	410.446	16564.927
	FT RSD (%)	7.14	4.20	11.51	8.44
	LT MC	1099.161	501.590	474.233	17669.751
	LT RSD (%)	6.39	6.55	13.44	11.11

NC, nominal concentration; MC, measured concentration; ST, short-time stability; FT, freeze and thaw stability; LT, long-time stability.

S2. Alkaloids quantitative analysis

S2.1. Sample preparation

The sample of total alkaloids was pulverized into fine powder. Then 1.0 mg of the total alkaloids was accurately weighed and extracted with 1.0 ml of 50% methanol in ultrasonic water bath for 30 min at 25 °C. The sample solution was filtered through a 0.22 µm membrane before use. A 10 µl-aliquot was injected for UHPLC/UV quantitative analysis.

S2.2. Preparation of standard solution

Four reference compounds, including DT₁ (scholarisine, 1.19 mg), DT₂ (19-episolarisine, 0.98 mg), DT₄ (vallesamine, 0.93 mg), and DT₅ (picrinine, 0.97 mg) were completely dissolved, respectively, in 30 % methanol (for DT₁), 50 % methanol

(for DT₂ and DT₅) or 100% methanol (for DT₄) to make their individual stock solutions (accur. 1 mg/ml for each analyte). Then 120 µl of stock solution of DT₁, 30 µl of stock solution of DT₂, 180 µl of stock solution of DT₄, and 270 µl of stock solution of DT₅ were well mixed to make the mixed stock solution. Then the mixed stock solution was further diluted with 50% methanol to obtain a series of standard working solutions for the establishment of calibration curves.

S2.3. UHPLC/UV quantitative analysis

The quantitative analysis was performed on an Agilent 1290 series UHPLC system (Agilent Technologies, Germany) comprised a quaternary pump, an auto-sampler, a column temperature controller and a PDA detector. All samples were separated on an ACQUITY UPLC[®] CSH column (2.1 × 100 mm, 1.7 µm) equipped with a Waters VanGuard pre-column (5 mm × 2.1 mm I.D., 1.8 µm). The mobile phase consisted of acetonitrile (A) and water containing 0.3% formic acid (v/v, B). The following linear elution gradient was used: 0-10 min, 4-16% A; 10-15 min, 16-20% A; 15-18 min, 20% A; 18-20min, 20-95% A. The flow rate was 0.20 ml/min. The column temperature was maintained at 45 °C. The sample tray temperature was maintained at 25 °C. The detection wavelength was set from 190 to 600 nm, and the samples were detected at 285 nm in accordance with the maximum absorption of the analytes.

S2.4. Results

All analytes showed good linearity ($r^2 \geq 0.9998$) within the test ranges (Fig. S2). The established UHPLC/UV method was used to determine four major compounds in the total alkaloids (TA). The typical UHPLC/UV chromatograms of the mixed standards and the TA are shown in Fig. S3. The contents of DT₁ (scholarisine), DT₂ (19-epischolarisine), DT₄ (vallesamine), and DT₅ (picrinine) were 71.78 mg/g, 18.61 mg/g, 64.20 mg/g, and 108.1 mg/g, respectively. It turned out that the four compounds accounted for 26.3% of the total alkaloids.

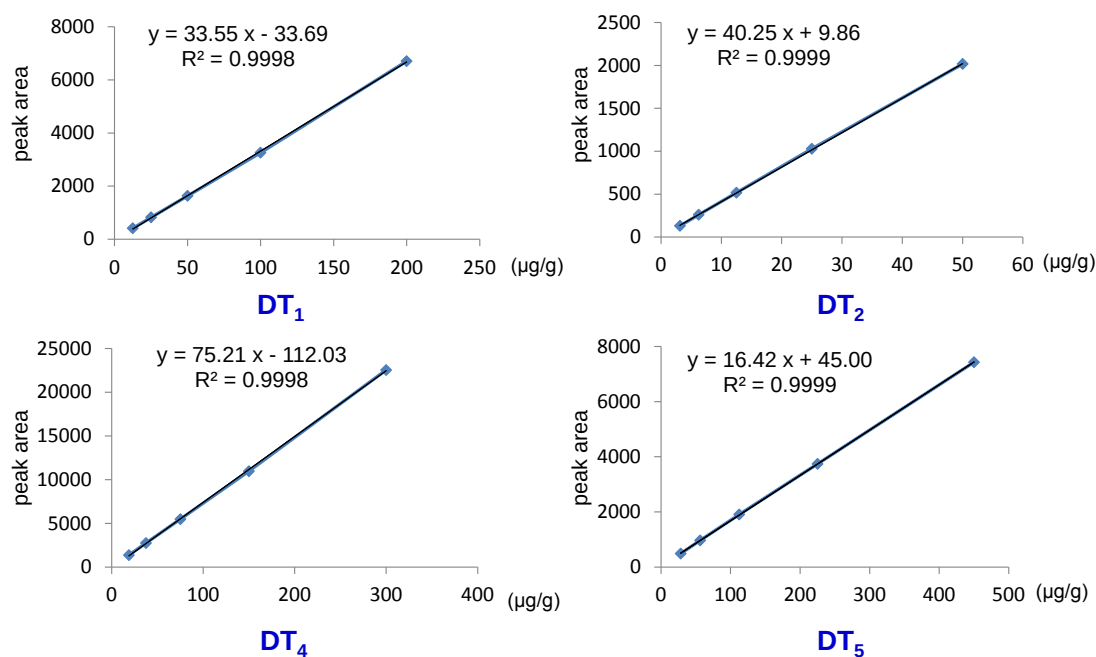


Fig. S2. Calibration curves of DT₁, DT₂, DT₄, and DT₅.

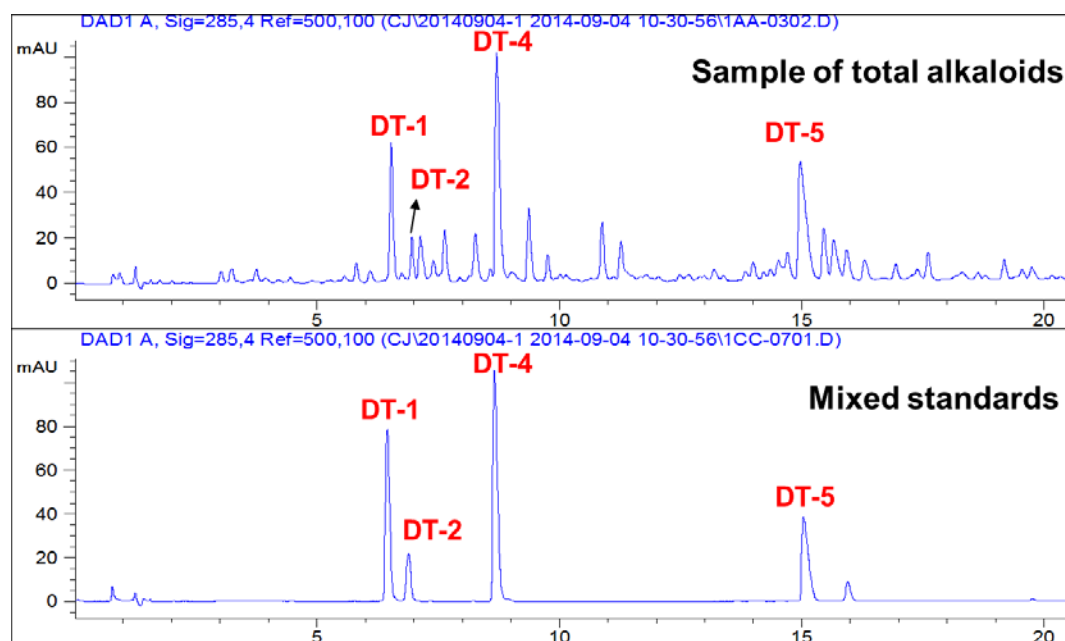


Fig. S3. UHPLC/UV chromatograms of mixed standards and sample of total alkaloids (285 nm).

References

(S1). Center for Drug Evaluation and Research. Guidance for Industry: Bioanalytical

Method Validation. <http://www.fda.gov/>.