



## Pharmacological evaluation of *Alstonia scholaris*: Anti-tussive, anti-asthmatic and expectorant activities

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### ABSTRACT

**Ethnopharmacological relevance:** *Alstonia scholaris* (Apocynaceae) was documented as an effective herb for the treatment of chronic respiratory diseases in “dai” ethnopharmacy historically, and its leaf crude extract, used for releasing tracheitis and cold symptom, was approved to be a commercial formulation by State Food and Drugs Administration of China (SFDA).

**Aim of the study:** The investigation evaluates the anti-tussive and anti-asthmatic activities of the ethanolic extract, fractions and main alkaloids of *Alstonia scholaris* leaf to provide experimental evidence for its traditional and modern clinical use. For our most interesting, is to reveal the active components for further new drug development.

**Materials and methods:** The leaf of *Alstonia scholaris* was extracted with ethanol and then separated into different fractions. Furthermore, alkaloids were isolated by phytochemical method. The anti-tussive activity was evaluated using three different models including ammonia or sulfur dioxide induced mice coughing, and citric acid induced guinea pigs coughing. The anti-asthmatic activity was investigated on guinea pigs bronchoconstriction induced by histamine. The expectorant activity was evaluated by volume of phenol red in mice's tracheas.

**Results:** The alkaloids fraction significantly inhibited mice's frequency of cough induced by ammonia, increased mice's latent period of cough induced by sulfur dioxide, and increased guinea pigs' latent period of cough and inhibited frequency of cough. Besides, the alkaloids fraction increased delitescence of convulsion, and tumble of guinea pigs in anti-asthmatic test, and enhanced tracheal phenol red output in expectorant evaluation. Moreover, the main alkaloid, picrinine exhibited anti-tussive and anti-asthmatic activities *in vivo*.

**Conclusions:** The alkaloids fraction was anti-tussive, anti-asthmatic and expectorant activities component of *Alstonia scholaris* leaf, and it may also be a valuable lead material for respiratory diseases drug development. Picrinine, the main anti-tussive and anti-asthmatic compound, could be applied in quality control of products from *Alstonia scholaris* leaf.

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### 1. Introduction

Coughing is a symptom of respiratory illness that prevents talking and causes chest and thorax pain (Irwing and Madison, 2000). Mucolytics, expectorants, anti-tussives, bronchodilators and glucocorticoids can be used to treat cough (Pérez et al., 2008). Presently available therapies to treat cough are limited for lack of effective and safe medications, so coughing remains among the most common complaints for which patients seek medical attention (Zhang et al., 2009). In the same way, there are increasing demands for the use of traditional medicines in the therapy of asthma, because of

little side effect compared to those of synthetic drugs to prevent and treat such chronic disease. In traditional Chinese medicines, many plants are recorded to treat respiratory complaints such as cough, asthma, bronchial affections, pneumonia and expectoration (Jiangsu New Medical College, 1977), which have been used for hundreds of years. However, they still cannot be accepted by most advanced countries as therapeutic agents, although many of today's new drugs come directly or indirectly from traditional medicines (Newman and Cragg, 2007). A major reason is lack of chemical and pharmacological investigation on them.

The leaf of *Alstonia scholaris* has been historically used in “dai” ethnopharmacy to treat chronic respiratory diseases in the Yunnan province of the People's Republic of China (Compiling Group of Yunnan Traditional Chinese Medicine, 1977). The leaf extract, developed as a commercially available traditional Chinese

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medicine, has also been prescribed in hospitals and sold over the counter in drug stores (Ministry of Public Health, People's Republic of China, 1997). The available clinical efficiency stimulated us to carry on the phytochemical and pharmacological research on this plant. In our previous chemical study on *Alstonia scholaris*, a series of monoterpenoid indole alkaloids, iridoids and terpenoids were isolated from different parts of plant (Cai et al., 2007, 2008a, 2008b; Du et al., 2007a, 2007b; Feng et al., 2008, 2009; Xu et al., 2009). The evaluation of the anti-tussive, anti-asthmatic and expectorant activities of different extracts and main alkaloids from *Alstonia scholaris* leaf is the subject of this paper.

## 2. Materials and methods

### 2.1. Plant materials

The leaves of *Alstonia scholaris* were collected in April 2006 in Simao of Yunnan Province, People's Republic of China, and identified by Dr. Chun-Xia Zeng, Key Laboratory of Biodiversity and Biogeography, Kunming Institute of Botany, the Chinese Academy of Sciences. A voucher specimen (Luo20060407) has been deposited in the herbarium of Kunming Institute of Botany Chinese Academy of Sciences (KUN).

### 2.2. Extract, fractions and alkaloids preparation

The dried and powdered leaves of *Alstonia scholaris* were extracted with EtOH under reflux conditions, and the solvent was evaporated *in vacuo* to afford the ethanolic extract. Part of the ethanolic extract was suspended with water and extracted with petroleum ether, EtOAc, successively. The other part of ethanolic extract was dissolved in 1% HCl, the residue was recognized as the non-alkaloid fraction, and the solution was subsequently basified using ammonia water to pH 9–10. The basic solution partitioned with EtOAc, afford the alkaloids fraction (EtOAc layer). The yields of the different fractions were expressed as the weight percentage of obtained extract in the total weight of plant material, specifically, 32%, 2.6%, 3.4%, 20.8%, 5.0%, and 1.0% for the ethanolic extract, petroleum ether fraction, EtOAc fraction, water fraction, non-alkaloid fraction, and alkaloids fraction respectively.

The alkaloids fraction was subjected to chromatography column on silica gel eluted with  $\text{CHCl}_3$ –MeOH (30:1–1:1) to afford 6 fractions (I–VI). Picrinine and vallesamine were isolated from fraction IV by column chromatography over silica gel ( $\text{CHCl}_3$ –acetone) (4:1–2:1), repeatedly. Scholaricine was isolated from fraction V by column chromatography over silica gel ( $\text{CHCl}_3$ – $\text{CH}_3\text{OH}$ ) (5:1–4:1).

### 2.3. Animals

ICR (Institute of Cancer Research) mice of either sex (19–24 g) and guinea pigs of either sex (280–345 g) were purchased from Kunming Medical College (licence number SYXK 2005-0001). All animals were housed at room temperature (20–25 °C) and constant humidity (40–70%) under a 12 h light–dark cycle in SPF (Specific Pathogen Free) grade laboratory. The animal study was performed according to the international rules considering animal experiments and the internationally accepted ethical principles for laboratory animal use and care.

### 2.4. Anti-tussive effects against ammonia induced coughing

ICR mice of either sex weighing 21–24 g were divided randomly, 10 mice per group. The negative control of animals was treated with distilled water orally, and the positive control was treated with codeine phosphate, the remaining groups treated were with test samples respectively. Anti-tussive activity was investigated on

a classical mouse cough model induced by ammonia liquor (Xu et al., 1991). Briefly, each mouse was placed in a 300 ml special glass chamber and exposed to 40  $\mu\text{l}$  25%  $\text{NH}_4\text{OH}$ . The cough frequency produced during 2 min exposure period was counted. In the second assay for alkaloids, cough frequency and latent period of cough were recorded.

### 2.5. Anti-tussive effects against sulfur dioxide induced coughing

ICR mice of either sex weighing 19–22 g were divided and treated as Section 2.4. The anti-tussive effects were measured according to literature (Miyagoshi et al., 1986). A burette containing concentrated sulfuric acid was fixed to a three necked flask containing aqueous saturated sodium hydrogen sulfite solution, and the acid was added to this solution to generate sulfur dioxide gas. After gastric perfusion 30 min, the mice were placed in a 300 ml special glass chamber and exposed for 4 ml sulfur dioxide. The latent period of cough was recorded for 2 min.

### 2.6. Anti-tussive effects against citric acid induced coughing

To screen the sensitivity, guinea pigs were placed in a glass chamber and sprayed with 33% citric acid (w/v) for 2 min (Chen, 1993). The period from the start to the onset of cough (latent period of cough) and frequency of cough was recorded. The frequency of cough between 10 and 30 were selected for further anti-tussive test. After 24 h recovery, the selected sensitive animals were randomly divided into seven groups ( $n=10$ ), and were individually placed into a transparent Perspex airtight chamber. At 45 min after oral treatment test samples, each animal was exposed to 1 M citric acid aerosols for 30 s with a flow rate of 1 ml/min. During the aerosol exposure, the animal was continuously monitored, and the latent period and frequency of cough were observed for 5 min.

### 2.7. The anti-asthmatic effects in guinea pigs

To screen the sensitivity, each guinea pig was sprayed with 2 ml of the mixture of 0.1% histamine and 2% acetylcholine chloride (1:1, v/v) (Xu et al., 1991). The time to onset of respiratory distress and tumble (preconvulsive time) was recorded. The guinea pigs with preconvulsive time in 90 s were selected. After 24 h recovery, the eligible guinea pigs were allotted randomly for test samples. All groups were administered once 45 min before, and each animal was exposed to the mixture of 0.1% histamine and 2% acetylcholine chloride aerosols for 20 s before the measurement of preconvulsive time. The delitescence of convulsion and tumble for each guinea pig within 6 min were observed. Guinea pig without convulsion and tumble was record as 360 s.

### 2.8. Expectorant test

The procedures were performed as described previously (Engler and Szelenyi, 1984). Male and female mice were randomly allotted and treated with a single dose 30 min before intraperitoneal injection of phenol red solution (5% in saline solution, w/v, 0.1 ml/10 g body weight). Mice were sacrificed by cervical dislocation 30 min after application of phenol red. After dissected free from adjacent organs, the trachea was removed from the thyroid cartilage to the main stem bronchi and put into 2 ml normal saline immediately. After ultrasonic for 5 min, 0.1 ml of 1 M NaOH solution was added to the saline and optical density of the mixture were measured at 546 nm using 722 UV-vis spectrophotometer.

**Table 1**

Effect of the extract and fractions on the ammonia induced cough in mice.

Group	Dose (mg/kg)	Treatment	Frequency of cough	Inhibition (%)
Control		ig	26.7 ± 3.5	–
Codeine phosphate	30	ig	11.9 ± 2.7**	55.4
Ethanol extract	1600	ig	23.3 ± 2.6	12.7
	3200	ig	17.9 ± 2.9	32.9
Petroleum ether fraction	1300	ig	21.5 ± 4.9	19.5
	2600	ig	28.1 ± 3.9	–5.2
EtOAc fraction	170	ig	23.8 ± 3.9	10.8
	340	ig	21.3 ± 3.3	20.2
Water fraction	1040	ig	25.0 ± 3.4	6.4
	2080	ig	20.9 ± 3.6	21.7
Alkaloids fraction	50	ig	18.5 ± 2.5	30.7
	100	ig	16.2 ± 2.8*	39.3
Non-alkaloid fraction	250	ig	23.9 ± 3.4	10.5
	500	ig	27.9 ± 4.5	–4.5

Values expressed as mean ± S.E.M. (*n* = 10). The doses of test samples are equal to 5/10 g of plant materials.\* *P* < 0.05 for comparison of treated groups with control.\*\* *P* < 0.01 for comparison of treated groups with control.**Table 2**

Effect of the extract and fractions on the sulfur dioxide induced cough in mice.

Group	Dose (mg/kg)	Treatment	Latent period of cough (s)	Increasing (%)
Control		ig	14.3 ± 1.5	–
Codeine phosphate	30	ig	26.2 ± 2.3**	83.2
Ethanol extract	1600	ig	14.6 ± 2.1	2.1
	3200	ig	24.3 ± 3.1*	69.9
Petroleum ether fraction	1300	ig	17.1 ± 2.1	19.6
	2600	ig	15.7 ± 1.9	9.8
EtOAc fraction	170	ig	14.4 ± 1.9	0.7
	340	ig	20.7 ± 2.4*	44.8
Water fraction	1040	ig	15.7 ± 2.2	9.8
	2080	ig	19.9 ± 1.9*	39.2
Alkaloids fraction	50	ig	21.4 ± 2.2*	49.7
	100	ig	23.2 ± 2.7*	62.2
Non-alkaloid fraction	250	ig	18.7 ± 2.6	30.7
	500	ig	16.6 ± 2.7	16.1

Values expressed as mean ± S.E.M. (*n* = 10). The doses of test samples are equal to 5/10 g of plant materials.\* *P* < 0.05 for comparison of treated groups with control.\*\* *P* < 0.01 for comparison of treated groups with control.**Table 3**

Effect of the extract and fractions on the citric acid induced cough guinea pigs.

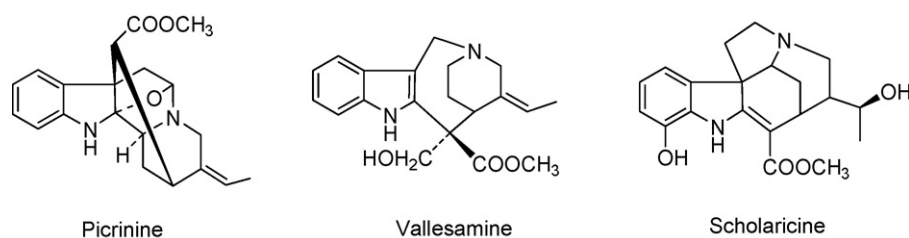
Group	Dose (mg/kg)	Treatment	Cough			
			Latent period (s)	Increasing (%)	Frequency	Inhibition (%)
Control	–	ig	48.9 ± 5.8	–	14.9 ± 1.5	–
Codeine phosphate	30	ig	253.8 ± 16.9**	419.0	1.2 ± 1.5**	91.9
Ethanol extract	3200	ig	117.6 ± 24.3*	140.5	8.0 ± 1.9*	46.3
Petroleum ether fraction	260	ig	51.1 ± 21.9	4.56	10.2 ± 1.6	31.8
EtOAc fraction	340	ig	76.3 ± 23.5	56.0	11.5 ± 1.6	22.8
Alkaloids fraction	100	ig	124.1 ± 28.6*	153.8	5.6 ± 1.1**	62.4
Non-alkaloid fraction	500	ig	91.4 ± 26.1	86.9	8.9 ± 1.1*	40.2

Values expressed as mean ± S.E.M. (*n* = 10). The doses of test samples are equal to 10 g of plant materials.\* *P* < 0.05 for comparison of treated groups with control.\*\* *P* < 0.01 for comparison of treated groups with control.**Table 4**

Effect of the extract and fractions on guinea pigs bronchoconstriction induced by mixture spraying histamine and acetylcholine chloride.

Group	Dose (mg/kg)	Treatment	Delitescence of convulsion and tumble (s)	Increasing (%)
Control	–	ig	189.3 ± 18.2	–
Aminophylline	100	ip	360.0 ± 0.0**	90.1
Ethanol extract	6400	ig	301.1 ± 23.1**	59.1
Petroleum ether fraction	520	ig	219.3 ± 25.8	16.1
EtOAc fraction	680	ig	323.1 ± 20.4**	70.7
Alkaloids fraction	200	ig	340.5 ± 9.1**	79.9
Non-alkaloid fraction	1000	ig	229.6 ± 44.5	21.3

Values expressed as mean ± S.E.M. (*n* = 8). The doses of test samples are equal to 20 g of plant materials.\*\* *P* < 0.01 for comparison of treated groups with control.



**Fig. 1.** Structures of picrinine, vallesamine and scholaricine.

**Table 5**

Effect of alkaloids on the ammonia liquor induced cough in mice.

Group	Dose (mg/kg)	Treatment	Cough			
			Latent period (s)	Increasing (%)	Frequency	Inhibition (%)
Control	–	ig	24.8 ± 3.2	–	12.6 ± 1.1	–
Codeine phosphate	30	ig	58.5 ± 8.5**	135.9	6.6 ± 1.3**	47.6
Alkaloids fraction	50	ig	41.2 ± 7.6*	66.1	9.3 ± 0.9*	26.2
	100	ig	39.7 ± 9.3	60.1	8.4 ± 1.6*	33.3
Picrinine	10	ig	43.5 ± 5.0**	75.4	7.7 ± 1.1**	38.9
	10	ip	39.4 ± 4.3*	58.9	9.6 ± 1.4	23.8
Vallesamine	8	ig	20.1 ± 2.1	–18.9	12.9 ± 1.4	–2.9
	8	ip	43.0 ± 5.5*	73.4	8.5 ± 1.0*	32.5
Scholaricine	5	ig	41.1 ± 7.7	65.7	8.3 ± 1.0**	34.1
	5	ip	37.6 ± 3.7*	51.6	9.9 ± 0.9	21.4

Values expressed as mean ± S.E.M. (n = 10).

\* P < 0.05 for comparison of treated groups with control.

\*\* P < 0.01 for comparison of treated groups with control.

**Table 6**

Effect of alkaloids on guinea pigs bronchoconstriction induced by mixture spraying histamine and acetylcholine chloride.

Group	Dose (mg/kg)	Treatment	Delitescence of convulsion and tumble (s)	Increasing (%)
Control	–	ig	141.6 ± 9.3	–
Aminophylline	100	ip	278.1 ± 30.9**	96.3
Alkaloids fraction	30	ig	209.6 ± 18.8*	48.0
	60	ig	257.5 ± 22.1**	81.8
Picrinine	10	ip	230.9 ± 23.0*	63.0

Values expressed as mean ± S.E.M. (n = 8).

\* P < 0.05 for comparison of treated groups with control.

\*\* P < 0.01 for comparison of treated groups with control.

## 2.9. Statistical analysis

The experimental results are expressed as mean ± standard error of mean (S.E.M.). Significance was evaluated using the Student's *t*-test (Woodson, 1987). Values of *p* < 0.05 imply significance of the pharmacological effects in the experiments.

## 3. Results

Both picrinine (Chatterjee et al., 1965) and scholaricine (Atta-Ur-Rahman et al., 1985) were obtained from *Alstonia scholaris*, and vallesamine (Walser and Carl, 1964) was obtained from *Vallesia dichroma* when they were isolated for the first time. Structures of them were identified by comparison spectral data with those

reported in literature (Fumiko et al., 1989; Atta-Ur-Rahman et al., 1985; Premila et al., 1984).

The effects of the different extracts of *Alstonia scholaris* leaf on the ammonia liquor induced cough in mice are shown in Table 1. The ethanolic extract (3.2 g/kg) and the alkaloids fraction (50 mg/kg) showed the trend of anti-tussive. At doses of 100 mg/kg, the alkaloids fraction inhibited frequency of cough by 39.3%, which are less active comparison with effect produced by the anti-tussive agent codeine phosphate (55.4%).

The effects of the different extracts on the sulfur dioxide induced cough in mice are shown in Table 2. The ethanolic extract, EtOAc fraction, water fraction and alkaloids fraction exhibited anti-tussive effect. Comparing with control group, there were significant differences in the ethanolic extract (3.2 g/kg), the alkaloids fraction

**Table 7**

Effect of the alkaloids fraction on the tracheal phenol red output in mice.

Group	Dose (mg/kg)	Treatment	OD (546 nm)	Concentration of phenol red (μg/ml)	Increasing (%)
Control	–	ig	0.058 ± 0.012	0.33 ± 0.07	–
Ammonium chloride	1500	ig	0.119 ± 0.014**	0.70 ± 0.09**	112.7
Alkaloids fraction	30	ig	0.106 ± 0.010**	0.62 ± 0.06**	88.6
	60	ig	0.141 ± 0.012**	0.83 ± 0.07**	152.1
	120	ig	0.133 ± 0.013**	0.79 ± 0.08**	138.6

Values expressed as mean ± S.E.M. (n = 10).

\*\* P < 0.01 for comparison of treated groups with control.

(100 mg/kg), and codeine phosphate (30 mg/kg) groups, latent period of cough of which increased by 69.9, 62.2 and 83.2%, respectively.

The effects of the different extracts on the citric acid induced cough in guinea pigs are shown in Table 3. Comparing with control group, there were significant difference in the ethanolic extract (3.2 g/kg), the alkaloids fraction (100 mg/kg), and codeine phosphate (30 mg/kg) groups, latent period of cough of which increased by 140.5, 153.8 and 419.0%, respectively. At above mentioned doses, the ethanolic extract, the alkaloids fraction, and codeine phosphate inhibited frequency of cough by 46.3, 62.4, and 91.9%, respectively.

The anti-asthmatic effects in guinea pigs are shown in Table 4. Comparing with control group, there were significant difference in the ethanolic extract (6.4 g/kg), the EtOAc fraction (680 mg/kg), and the alkaloids fraction (200 mg/kg) groups increased delitescence of convulsion and tumble of guinea pigs by 59.1, 70.7, and 79.9%, respectively, roughly comparable to aminophylline group (90.1%).

Above data exhibited that only the alkaloids fraction possessed anti-tussive and anti-asthmatic effects significantly in four *in vivo* evaluations. Then we focus further evaluations on the alkaloids fraction and three main alkaloids, picrinine, vallesamine, and scholaricine (Fig. 1). The ammonia liquor inducing cough in mice was used to investigate effects of the alkaloids fraction, three main alkaloids. Cough frequency and latent period of cough were recorded in Table 5. The alkaloids fraction treated by intragastrical at two doses (50, 100 mg/kg), picrinine treated by either intragastrical or intraperitoneal, vallesamine and scholaricine treated by intraperitonea increased latent period of cough in mice significantly comparison to control. It is noteworthy, picrinine (10 mg/kg) and scholaricine (5 mg/kg) treated by intragastrical inhibited frequency of cough by 38.9, and 34.1%, respectively, roughly comparable to codeine phosphate (47.6%).

The anti-asthmatic effect for the alkaloids fraction and picrinine were evaluated (Table 6). Both the alkaloids fraction and picrinine exhibited significant anti-asthmatic active, comparing with control group. At dose of 60 mg/kg, the alkaloids fraction group increased delitescence of convulsion and tumble of guinea pigs by 81.8%, roughly comparable to aminophylline group (96.3%).

The results of expectorant test are shown in Table 7. Both ammonium chloride and the alkaloids fraction could enhance tracheal phenol red output after administration, markedly. At doses of 30, 60, 120 mg/kg, the alkaloids fraction increased phenol red output by 88.6, 152.1, and 138.6%, respectively.

#### 4. Discussion

*Alstonia scholaris*, a 20–40 m high tree in family Apocynaceae, is widely distributed in the tropical regions of Africa and Asia (Li et al., 1995). The different parts of the plant exhibited anticancer (Jageti and Baliga, 2006) and antibacterial (Khan et al., 2003) activities. In addition, the ethanol extract of *Alstonia scholaris* leaves induced pronounced bronchodilatory activity in anaesthetized rats (Channa et al., 2005). The leaves of *Alstonia scholaris* have been historically used in “dai” ethnopharmacy to treat chronic respiratory diseases for several hundred years. The traditional knowledge and available clinical efficiency led us to evaluate anti-tussive, anti-asthmatic and expectorant effects of *Alstonia scholaris* leaf. In the present study, the ethanol extract from *Alstonia scholaris* was separated by two ways independently. First, petroleum ether fraction, EtOAc fraction and water fraction were prepared by liquid–liquid partition based on components polarity. Second, non-alkaloid fraction and alkaloids fraction were produced by acid and base processing. Through the pharmacological evaluation on ammonia and sulfur dioxide induced mice coughing, and citric acid induced guinea pigs

coughing, together with histamine induced guinea pigs asthma, respectively, the ethanolic extract and the EtOAc fraction were active on some of assays. To our satisfactory, the alkaloids fraction appeared to be the most active part in three cough and the asthma relieving models (Tables 1–4).

According to first evaluation, we paid much attention to the alkaloids fraction. Then, three major alkaloids, picrinine, vallesamine, and scholaricine (Fig. 1) were isolated from it by column chromatography over silica gel, repeatedly, with contents of 0.1, 0.08, and 0.05%, respectively. The alkaloids fraction at two doses (50/100 mg/kg), equal to 5/10 g plant materials, treated by intragastrical, and picrinine (10 mg/kg), vallesamine (8 mg/kg) and scholaricine (5 mg/kg), equal to 10 g plant materials, treated both by intragastrical and intraperitoneal, respectively, in the ammonia liquor inducing cough model exhibited their efficiency (Table 5). As we anticipated, the three main alkaloids either inhibited frequency or increased latent period of cough, which also supported anti-tussive effect of the alkaloids fraction. *In vivo* evaluation with the alkaloids fraction and picrinine on the bronchoconstriction induced by mixed spray of histamine and acetylcholine chloride in guinea pigs, strongly supported anti-asthmatic effect of them (Table 6). Picrinine, an effective alkaloid both in anti-tussive and anti-asthmatic evaluations, could be used in quality control of *Alstonia scholaris* in future. Additionally, the alkaloids fraction also showed significant expectorant effect *in vivo* (Table 7). The results obtained not only supported for the use of *Alstonia scholaris* in the treatment of respiratory diseases, but also indicated that the alkaloids fraction of *Alstonia scholaris* might be a refined formulation in further new drug development.

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