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Pharmacological evaluation of *Alstonia scholaris*: Anti-inflammatory and analgesic effects

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ABSTRACT

Ethnopharmacological relevance: Alstonia scholaris (Apocynaceae) has been historically used in "dai" ethnopharmacy to treat chronic respiratory diseases. The leaf extract, developed as a commercially available traditional Chinese medicine, used to release tracheitis and cold symptom, has also been prescribed in hospitals and sold over the counter in drug stores.
Aim of the study: The investigation evaluated the anti-inflammatory and analgesic activities of the ethanolic extract, fractions and main alkaloids of *Alstonia scholaris* leaf to provide experimental evidence for its traditional and modern clinical use. Besides, to discover the active fraction and components for further better use in Chinese medicine is hopeful.
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 analgesic activities were investigated using acetic acid-induced writhing, hot-plate and formalin tests in mice. The anti-inflammatory activities were carried out *in vivo* and *in vitro*, including xylene-induced ear edema and carrageenan-induced air pouch formation in mice, and COX-1, -2 and 5-LOX inhibition.
Results: It has been exhibited that the EtOAc and alkaloid fractions reduced acetic acid-induced writhing response in mice, significantly. The ethanolic extract, EtOAc and alkaloid fractions remarkably inhibited xylene-induced ear edema. Further investigation was focused on the alkaloids fraction and three main alkaloids isolated from the alkaloids fraction, in different animal models. Alkaloids reduced acetic acid-induced writhing response, and xylene-induced ear edema in mice. In the hot-plate test, alkaloids did not increase the latency period of mice obviously. In the formalin test, alkaloids did not inhibit the licking time in first phase, but significantly inhibited the licking time in second phase of mice. Alkaloids increased significantly SOD activity and decreased levels of NO, PGE2 and MDA significantly, in air pouch mice model. Moreover, some alkaloids isolated from the leaf of *Alstonia scholaris* exhibited inhibition of COX-1, COX-2 and 5-LOX *in vitro* anti-inflammatory assay, which supported alkaloids as the bioactive fraction.
Conclusions: The alkaloids fraction of *Alstonia scholaris* leaf, three main alkaloids, picrinine, vallesamine and scholaricine, may produce the anti-inflammatory and analgesic effect peripherally based on several *in vivo* assays. In *in vitro* tests, alkaloids exhibited inhibition of inflammatory mediators (COX-1, COX-2 and 5-LOX), which is accordant with results on animal models. Besides, COX-2/5-LOX dual inhibitors found in the experiment, such as 16-formyl-5α-methoxystrictamine, picralinal, and tubotaiwine might be valuable for further attention.

1. Introduction

The leaves of *Alstonia scholaris* (L.) R. Br. (Apocynaceae) have been historically used in "dai" ethnopharmacy to treat chronic respiratory diseases in the Yunnan Province PR China (Compiling Group of Yunnan Traditional Chinese Medicine, 1977). The leaf extract, developed as a commercially available traditional Chi-

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Table 1
Effect of the extract and fractions on acetic acid-induced writhing response.

Group	Dose (mg/kg)	Treatment	Number of writhing	Inhibition ratio (%)
Control	–	ig	33.1 ± 3.2	–
Aspirin	200	ig	9.3 ± 3.5**	71.9
Ethanolic extract	3200	ig	28.4 ± 3.6	14.2
Petroleum ether fraction	260	ig	28.7 ± 5.7	14.2
EtOAc fraction	340	ig	21.9 ± 3.6*	33.8
Water fraction	2080	ig	23.3 ± 3.9	29.6
Alkaloids fraction	100	ig	20.0 ± 2.0**	39.6
Non-alkaloid fraction	500	ig	28.1 ± 3.9	15.1

Values expressed as mean ± S.E.M. ($n = 10$).
The doses of test samples are equal to 10 g of plant materials.
* $p < 0.05$ compared with control.
** $p < 0.01$ compared with control.

nese medicine, used to release tracheitis and cold symptom, has also been prescribed in hospitals and sold over the counter in drug stores (Ministry of Public Health, People's Republic of China, 1997). Allergic asthma is a chronic inflammatory disease characterized by eosinophilic airway inflammation, mucus hyper-secretion, and bronchial hyper-responsiveness (Busse and Lemanske, 2001). The traditional and clinical uses are likely related to the anti-inflammatory and analgesic actions. The available clinical efficiency stimulated us to investigate the anti-inflammatory components from this plant. For this purpose, intensive phytochemical investigations were carried out. As a result, a series of monoterpenoids indole alkaloids, iridoids and terpenoids were isolated from different parts of plant (Cai et al., 2007, 2008a,b; Du et al., 2007a,b; Feng et al., 2008, 2009; Xu et al., 2009b). This paper focuses on the evaluation of the anti-inflammatory and analgesic activities in different animal models of extracts, fractions and main alkaloids from the leaf of *Alstonia scholaris*. Moreover, the *in vitro* anti-inflammatory evaluation of alkaloids from *Alstonia scholaris* against COX-1, -2 and 5-LOX was also reported.

2. Materials and methods

2.1. Plant materials

The leaves of *Alstonia scholaris* (L.) R. Br. 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, the Chinese Academy of Sciences (KUN).

2.2. Extracts and fractions 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 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 non-alkaloid fraction, and the solution was subsequently basified using ammonia water to pH 9–10. The basic solution partitioned with EtOAc, afford 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 ethanolic extract, the petroleum ether fraction, EtOAc fraction, water fraction, non-alkaloid fraction, and alkaloids fraction, respectively.

2.3. Alkaloids preparation

The alkaloids fraction was subjected to chromatography column on silica gel eluted with $CHCl_3$–MeOH (30:1–1:1) to afford 6 fractions (I–VI). Akuammidine, leuconoxine, isogentialutine, picralinal and 5-methoxylstrictamine were isolated from fraction II by column chromatography over silica gel (petroleum ether–EtOAc) and RP_{18} (H_2O–CH_3OH), repeatedly. Cantleyine, corypalmine, strictamine, vallesiachotamine, scholarisines B and E were isolated from fraction III by column chromatography over silica gel (petroleum ether–Acetone) and RP_{18} (H_2O–CH_3OH), repeatedly. Crebanine, dicentrine, discretamine, picrinine, 3-epi-dihydrocorymine 17-acetate, 16-formyl-5α-methoxystrictamine, stephanine and salutaridine were isolated from fraction IV by column chromatography over silica gel ($CHCl_3$–Acetone) and RP_{18} (H_2O–CH_3OH), repeatedly. Nareline, scholaricine and tubotaiwine were isolated from fraction V by column chromatography over silica gel ($CHCl_3$–CH_3OH), repeatedly. Echitamine, 19-epi-scholaricine, 12-hydroxy-echitamidine N^b-oxide, N(4)-demethylechitamine, and strictosamine were isolated from fraction VI by column chromatography over silica gel ($CHCl_3$–CH_3OH) and RP_{18} (H_2O–CH_3OH), repeatedly.

2.4. Animals

ICR mice of either sex (18–22 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 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.

2.5. Chemicals

Carrageenan was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Nitric oxide (NO), malondialdehyde (MDA), superoxide dismutase (SOD) kits were purchased from Nanjing Jiancheng Bioengineering Institute (China). All other reagents were of the highest commercial grade available.

2.6. Acetic acid-induced writhing response in mice

The writhing test was carried out as described in literature (Nakamura et al., 1986). Each mouse was administered with aspirin, test samples or vehicle 30 min before an intraperitoneal injection of 0.6% acetic acid at 0.1 ml/10 g bodyweight. The number of stretching or writhing was recorded between 5 min and 20 min after acetic acid injection.

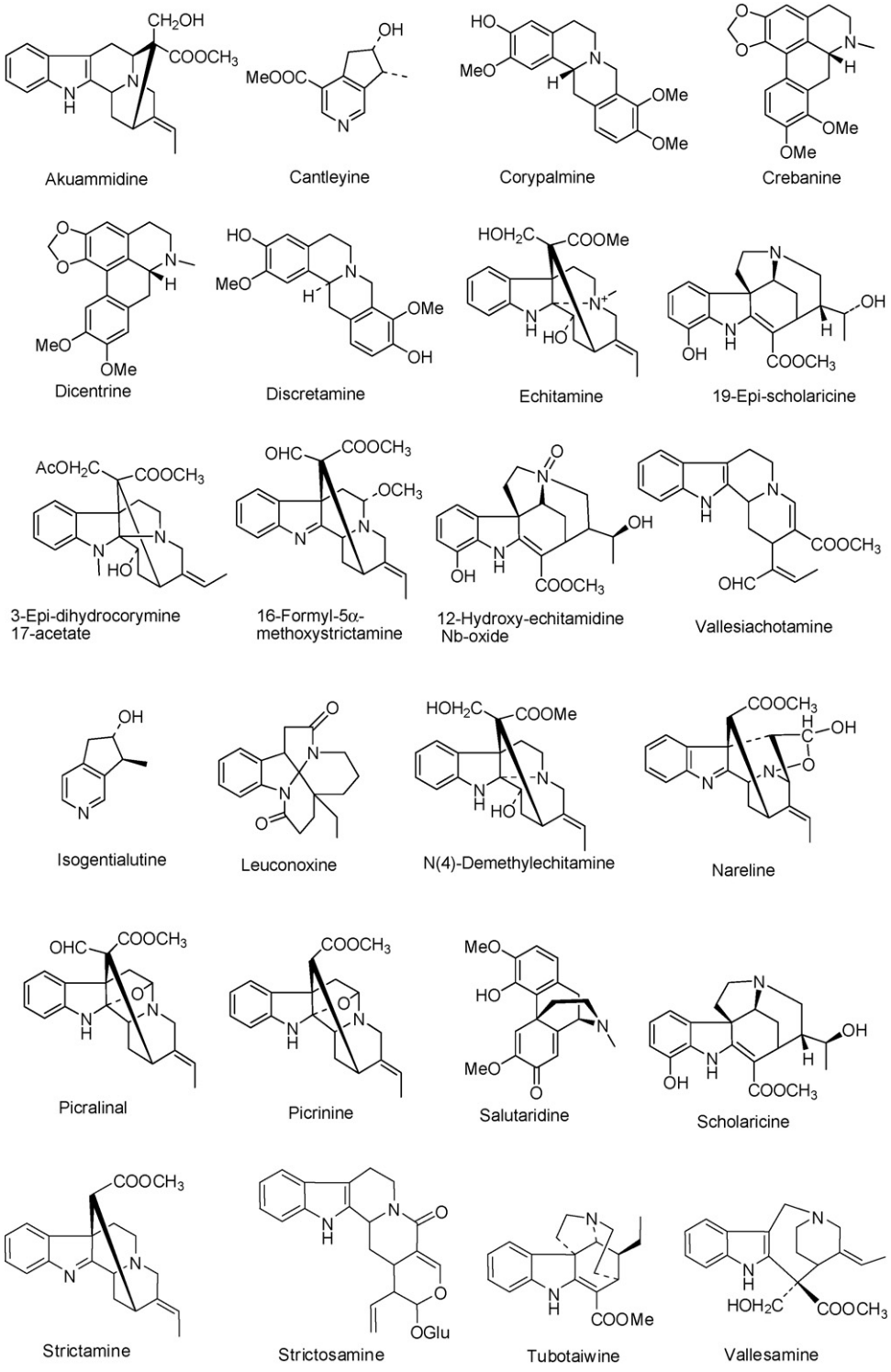

Fig. 1. Structures of alkaloids from *Alstonia scholaris*.

2.7. Hot-plate latent pain response test in mice

This test was performed according to the method previously reported (Lanhers et al., 1991). Each female mouse was placed on a $55 \pm 1\,^{\circ}C$ hot-plate to observe its pain responses. The reaction consisted of paw licking and jumping. The time in seconds between the platform and reaction was recorded as the response latency. The mice exhibiting latency time greater than 30 s or less than 5 s were excluded. The latency time was determined at 30 min and 60 min after administration of the test samples, aspirin, hydrochloric morphine, and vehicle. If the reaction time is more than 60 s, the latency time will be recorded as 60 s.

2.8. Formalin test

This test was based on the method previously reported (Lu et al., 2007). Ten microliters of 1% formalin were injected subcutaneously into the right hind paw of mice. The time spent in licking and biting responses of the injected paw was taken as an indicator of pain response. Responses were measured for 5 min after

Table 2
Effect of alkaloids on acetic acid-induced writhing response.

Group	Dose (mg/kg)	Treatment	Number of writhing	Inhibition ratio (%)
Control (1%CMC-Na)	–	ig	37.4 ± 4.2	–
Aspirin	200	ig	16.0 ± 4.5**	57.2
Alkaloids fraction	10	ig	19.3 ± 1.7**	48.4
	20	ig	22.4 ± 3.9*	40.1
	40	ig	23.7 ± 3.1*	36.6
	80	ig	18.9 ± 2.2**	49.5
Picrinine	10	ig	21.7 ± 3.7*	41.9
	10	ip	16.8 ± 5.1**	55.1
Vallesamine	8	ig	23.0 ± 4.6*	38.5
	8	ip	14.7 ± 5.0**	60.7
Scholaricine	5	ig	18.7 ± 5.0*	50.0
	5	ip	14.9 ± 3.8**	60.2

Values expressed as mean ± S.E.M. (n = 10).
* $p < 0.05$ compared with control.
** $p < 0.01$ compared with control.

formalin injection (phase I) and 15–30 min after formalin injection (phase II). The test samples, aspirin, hydrochloric morphine, and vehicle (1%CMC-Na) were administered 30 min before the formalin injection.

2.9. Xylene-induced ear edema in mice

A previously described procedure was followed (Chen, 1993). Briefly each mouse was given aspirin, test samples or vehicle 30 min before the induction of ear edema by topical application of 50 μl xylene on both surfaces of the right ear. The left ear served as a control. Mice were sacrificed by cervical dislocation 1 h after xylene application. Ear disks of 8.0 mm in diameter were punched out and weighed. The extent of edema was evaluated by the weight difference between the right and the left ear disks of the same animal.

2.10. Carrageenan-induced air pouch formation in mice

Air pouches were produced in male ICR mice according to a modification of the procedure of Sin and Wong (1992). To generate the air pouch, 5 ml of sterile air was injected s.c. into the back of mice on day 1, followed by two injections of 4 ml of sterile air each 3 days later (days 4 and 7). On day 8, 1 ml of a 1% (w/v) carrageenan solution or saline was injected into each cavity. The animals were sacrificed 12 h after the carrageenan injection, and serum was selected from eye bold of mice for MDA measurement. Pouches were washed with 1 ml of sterile phosphate-buffered saline (PBS, composition mmol/l: NaCl 137, KCl 2.7, phosphaten buffer 10) containing heparin (5 IU/ml), exudates were collected, and the levels of PGE2, NO, and SOD in exudates were measured as described below. The animals were treated orally with the test samples 30 min prior to carrageenan or saline injection.

2.11. MDA, NO, SOD, and PGE2 assay

The levels of malondialdehyde (MDA), nitric oxide (NO), superoxide dismutase (SOD) were determined using the MDA, NO and SOD kits (Nanjing Jiancheng Bioengineering Institute), according to the manufacturer's instructions. All samples were assayed in triplicate. The measurement of PGE2 in the exudates was performed according to the chemical method previously described by Wu (1991).

2.12. In vitro anti-inflammatory assay

The anti-inflammatory activity was performed identical to the literature (Tan et al., 2009). Briefly, the reaction system was incubated for 5 min at 25 °C, by sequential addition of the buffer, heme, test compounds, and COX-1 or COX-2 into the system followed by mixing with N,N,N′,N′-tetramethyl-*p*-phenylenediamine (TMPD)

Table 3
Effects of alkaloids on hot-plate test in mice.

Group	Dose (mg/kg)	Treatment	Latency period (s)		
			0 min	30 min	60 min
Control	–	ig	21.5 ± 1.7	30.5 ± 2.8	28.6 ± 3.0
Hydrochloric morphine	10	ip	20.9 ± 1.7	55.6 ± 3.0**	42.1 ± 3.9*
Aspirin	200	ig	21.8 ± 2.0	28.3 ± 4.7	21.7 ± 4.3
Alkaloids fraction	20	ig	20.5 ± 2.4	31.9 ± 5.9	22.7 ± 2.1
	40	ig	21.6 ± 2.3	25.7 ± 2.7	25.4 ± 3.2
	80	ig	20.6 ± 1.5	32.4 ± 5.0	30.1 ± 4.9
Picrinine	10	ig	21.3 ± 1.5	30.4 ± 4.2	22.1 ± 4.1
	10	ip	21.2 ± 1.4	38.6 ± 5.5	31.3 ± 6.5
Vallesamine	8	ig	20.9 ± 2.0	28.1 ± 4.8	25.5 ± 5.0
	8	ip	21.3 ± 1.9	30.3 ± 4.2	22.9 ± 3.4
Scholaricine	5	ig	21.2 ± 1.6	40.0 ± 4.2	33.8 ± 5.5
	5	ip	21.9 ± 2.2	35.0 ± 6.1	24.7 ± 4.7

Values expressed as mean ± S.E.M. (n = 10).
* $p < 0.05$ vs. the control group.
** $p < 0.01$ vs. the control group.

Table 4
Effects of alkaloids on formalin test in mice.

Group	Dose (mg/kg)	Treatment	Licking time (s)			
			Phase I (s)	Inhibition ratio (%)	Phase II (s)	Inhibition ratio (%)
Control (1%CMC-Na)	–	ip	43.0 ± 5.1	–	94.6 ± 14.6	–
Hydrochloric morphine	10	ip	5.4 ± 2.2**	87.4	19.3 ± 10.2**	79.6
Aspirin	200	ig	32.1 ± 7.1	25.3	30.7 ± 7.8**	67.6
Alkaloids fraction	20	ig	40.7 ± 11.6	5.4	42.8 ± 11.9*	54.8
	40	ig	41.0 ± 6.1	4.5	46.0 ± 14.9*	51.3
	80	ig	30.2 ± 8.3	29.8	44.9 ± 9.6*	52.5
Picrinine	10	ig	38.0 ± 8.8	11.6	48.4 ± 8.3*	48.8
	10	ip	43.1 ± 9.6	−0.2	41.5 ± 7.9**	56.1
Vallesamine	8	ig	27.6 ± 6.3	35.8	39.2 ± 10.7 **	58.6
	8	ip	32.1 ± 5.8	25.4	38.5 ± 5.2**	59.3
Scholaricine	5	ig	48.0 ± 7.9	−11.5	49.9 ± 12.2*	47.3
	5	ip	52.9 ± 15.8	−22.9	35.2 ± 6.1**	62.7

Values expressed as mean ± S.E.M. ($n = 10$).
* $p < 0.05$ vs. the control group.
** $p < 0.01$ vs. the control group.

Table 5
Effect of the extract and fractions on xylene-induced ear edema.

Group	Dose (mg/kg)	Treatment	Weight of ear (mg)	Inhibition ratio (%)
Control	–	ig	14.43 ± 0.94	–
Aspirin	200	ig	4.80 ± 0.90**	66.7
Ethanolic extract	3200	ig	7.52 ± 1.19**	47.9
Petroleum ether fraction	260	ig	12.21 ± 1.58	15.2
EtOAc fraction	340	ig	8.88 ± 1.51**	38.5
Water fraction	2080	ig	9.49 ± 1.77*	34.2
Alkaloids fraction	100	ig	5.65 ± 1.32**	60.9
Non-alkaloid fraction	500	ig	10.22 ± 1.46*	29.1

Values expressed as mean ± S.E.M. ($n = 10$).
The doses of test samples are equal to 10 g of plant materials.
* $p < 0.05$ vs. the control group.
** $p < 0.01$ vs. the control group.

and arachidonic acid, and soft agitation for several seconds. The absorbance value was recorded at a wavelength of 590 nm after another 15 min of incubation at 25 °C. The performance of the assay was checked using SC-560 and NS-398 as positive controls, which gave the inhibition of COX-1 (61.3%) and COX-2 (97.1%), respectively. Different from the method mentioned above, the reaction system was added to the assay buffer with 5-LOX in the presence of the colorimetric substrate and test compounds and then incubated for a period of 5 min at 25 °C. After the completion of the reaction, the chromogen was added, and the plate was shaken softly for a few seconds. A further period of 5 min incubation was performed at 25 °C. The inhibitory effect against 5-LOX was determined by measuring the absorbance at a wavelength of 500 nm. The performance of the assay was checked using zileuton as a positive control, which led to the inhibition of 83.05%. Due to limited quantity of enzyme and alkaloids, all compounds were tested at single concentration, 100 μM.

2.13. Statistical analysis

The experimental results are expressed as mean ± standard error of mean (S.E.M.). Significance was evaluated using the

Table 6
Effect of alkaloids on xylene-induced ear edema.

Group	Dose (mg/kg)	Treatment	Weight of ear (mg)	Inhibition ratio (%)
Control (1%CMC-Na)	–	ig	17.05 ± 2.32	–
Aspirin	200	ig	9.25 ± 1.62*	45.7
Alkaloids fraction	50	ig	9.20 ± 1.50*	46.0
	100	ig	10.03 ± 2.33 *	41.2
Picrinine	10	ig	9.91 ± 1.62*	41.9
	10	ip	9.97 ± 1.55*	41.5
Vallesamine	8	ig	14.12 ± 1.80	17.2
	8	ip	10.18 ± 2.09*	40.3
Scholaricine	5	ig	9.89 ± 1.83*	42.0
	5	ip	14.14 ± 1.69	17.1

Values expressed as mean ± S.E.M. ($n = 10$).
* $p < 0.05$ vs. the control group.

Table 7
Effect of alkaloids on levels of NO, PGE2, SOD and MDA in the air pouch mice induced by carrageenan.

Group	Dose (mg/kg)	Treatment	PGE2 (OD)	NO (μmol/L)	SOD (U/ml)	MDA (nmol/L)
Control (saline)	–	ig	0.076 ± 0.013	6.36 ± 4.21	5.71 ± 2.09	17.53 ± 7.05
Inflammation model (carrageenan-induced)	–	ig	0.202 ± 0.051$^{\Delta\Delta}$	31.18 ± 8.22$^{\Delta\Delta}$	3.49 ± 1.33$^{\Delta\Delta}$	31.71 ± 11.50$^{\Delta\Delta}$
Aspirin	200	ig	0.135 ± 0.051*	19.59 ± 10.34*	5.85 ± 2.62*	20.83 ± 4.22*
Alkaloids fraction	10	ig	0.148 ± 0.033**	18.51 ± 11.09**	5.27 ± 1.87*	21.65 ± 5.82*
	20	ig	0.127 ± 0.041**	20.91 ± 8.22*	4.95 ± 1.25*	18.20 ± 2.72**
	80	ig	0.132 ± 0.045**	15.21 ± 6.83**	5.48 ± 2.21*	17.25 ± 3.18**
Picrinine	10	ig	0.131 ± 0.041**	15.54 ± 12.25**	6.28 ± 2.41**	18.46 ± 3.37**
Vallesamine	8	ig	0.141 ± 0.069*	19.09 ± 13.89*	6.67 ± 3.39*	18.80 ± 5.62**
Scholaricine	5	ig	0.147 ± 0.059*	14.55 ± 9.16**	5.80 ± 2.14**	20.77 ± 7.70*

Values expressed as mean ± S.E.M. (n = 11).
$^{\Delta\Delta}$ $p < 0.01$ vs. the control group.
* $p < 0.05$ vs. the model group.
** $p < 0.01$ vs. the model group.

student's t-test. Values of $p < 0.05$ imply significance of the pharmacological effects in the experiments.

3. Results

3.1. Acetic acid-induced writhing response in mice

Results in Table 1 demonstrated that aspirin at 200 mg/kg, and the alkaloids fraction at 100 mg/kg, significantly inhibited the writhing response by 71.9% and 39.6%, respectively. The results hint that the moderate polarity alkaloids might be bioactive fraction in this animal model. Then we performed another test, in which the mice were administrated by the alkaloids fraction, picrinine, vallesamine, scholaricine (Fig. 1). Results shown in Table 2 indicated that alkaloids fraction treated by intragastrical at four doses (10, 20, 40, 80 mg/kg), three alkaloids treated by either intragastrical or intraperitoneal inhibited mice writhing reflex markedly, roughly comparable to aspirin at 200 mg/kg. Moreover, picrinine, vallesamine, scholaricine administrated by intraperitoneal exhibited higher inhibition than by intragastrical at same dose.

3.2. Hot-plate latent pain response test in mice

The results showed that both at 30 min and 60 min, alkaloids fraction treated by intragastrical at three doses (20, 40, 80 mg/kg), three alkaloids treated by either intragastrical or intraperitoneal, or aspirin by intragastrical did not increase the latency time of mice significantly, except for hydrochloric morphine group (Table 3).

3.3. Formalin test

In the first phase, alkaloids fraction treated by intragastrical at three doses (20, 40, 80 mg/kg), three alkaloids treated by either intragastrical or intraperitoneal, and aspirin by intragastrical did not inhibited mice licking time compared to the control, except for hydrochloric morphine group (Table 4). In the second phase, all groups significantly reduced nociception at different doses and administrated.

3.4. Xylene-induced ear edema in mice

As shown in Table 5, test samples exhibited inhibitory effect on xylene-induced ear edema except for petroleum ether fraction. Particularly, the potency of 100 mg/kg alkaloids fraction inhibited ear edema in mice by 60.9%, comparable to that of 200 mg/kg aspirin (66.7%). Then we pay much attention on the alkaloids fraction and three main alkaloids. Further test in same model was carried out, in which the mice were administrated by the alkaloids fraction, picrinine, vallesamine, scholaricine. Results shown in Table 6 indicated that alkaloids fraction treated by intragastrical at two doses (50, 100 mg/kg), picrinine treated by either intragastrical or intraperitoneal, vallesamine treated by intraperitoneal, scholaricine by intragastrical inhibited xylene-induced ear edema in mice significantly, comparable to aspirin at 200 mg/kg.

3.5. Carrageenan-induced air pouch formation in mice

As shown in Table 7, alkaloids fraction at three doses (10, 20, 80 mg/kg), picrinine (10 mg/kg), vallesamine (8 mg/kg), and scholaricine (5 mg/kg) treated by intragastrical increased SOD activity significantly, and decreased levels of NO, PGE2 and MDA significantly, in mice air pouch model, roughly comparable to aspirin at 200 mg/kg.

Table 8
In vitro evaluation of anti-inflammatory activity of alkaloids.[a].

Compounds	COX-1	COX-2	5-LOX
Alkaloids fraction	54.3	63.3	57.3
Akuammidine	<0	67.1	76.5
Cantleyine	29.8	45.8	44.9
Corypalmine	<0	<0	19.0
Crebanine	53.3	90.7	38.4
Dicentrine	33.0	67.0	13.3
Discretamine	13.5	89.8	<0
Echitamine	<0	<0	<0
19-Epi-scholaricine	10.3	47.5	43.2
3-Epi-dihydrocorymine 17-acetate	13.5	50.6	31.3
16-Formyl-5α-methoxystrictamine	45.8	95.7	79.9
12-Hydroxy-echitamidine N^{b}-oxide	13.1	7.9	67.5
Isogentialutine	43.1	95.9	<0
Leuconoxine	<0	88.8	<0
5-Methoxylstrictamine	<0	<0	<0
N(4)-Demethylechitamine	14.7	27.1	30.1
Nareline	14.1	37.2	<0
Picralinal	44.7	96.4	79.5
Picrinine	<0	16.6	79.2
Salutaridine	27.7	48.2	<0
Scholaricine	44.7	92.0	31.0
Scholarisine B	13.8	37.6	52.2
Scholarisine E	<0	20.8	27.9
Scholarisine G	38.5	91.1	57.3
Stephanine	68.3	90.0	20.8
Strictamine	47.0	95.6	<0
Strictosamine	32.8	<0	<0
Tubotaiwine	60.8	90.4	69.5
Vallesamine	49.4	63.7	60.8
Vallesiachotamine	5.1	41.9	<0
SC-560	61.3		
NS-398		97.1	
Zileuton			83.1

[a] Percent inhibition (all compounds concentration: 100 μM, alkaloids fraction concentration: 300 μg/ml).

3.6. In vitro anti-inflammatory assay

The *in vitro* anti-inflammatory effects of alkaloids from *Alstonia scholaris* (Fig. 1) are shown in Table 8. The percentage inhibition of stephanine and tubotaiwine to COX-1 were 68.3% and 60.8%, respectively. It is noteworthy that compounds crebanine, discretamine, 16-formyl-5α-methoxystrictamine, isogentialutine, leuconoxine, picralinal, scholaricine, scholarisine G, and strictamine showed high inhibition on COX-2 (>85%) and low inhibition on COX-1 (<50%), which can be regarded as selective inhibitors of COX-2. Moreover, akuammidine, 16-formyl-5α-methoxystrictamine, picralinal, picrinine inhibited 5-LOX by 76.5%, 79.9%, 79.5% and 79.2%, respectively, roughly comparable to positive control zileuton (83.1%).

4. Discussion and conclusion

The different parts of *Alstonia scholaris* exhibited anticancer (Jagetia and Baliga, 2006), antibacterial (Khan et al., 2003) and bronchodilatory activities (Channa et al., 2005). However, the traditional and clinical use of *Alstonia scholaris* in China related to anti-inflammatory and analgesic actions and the mechanisms have not been investigated previously. In the present study, the ethanol extract, the petroleum ether fraction, the EtOAc fraction, the water fraction, the non-alkaloid fraction and the alkaloids fraction were prepared from *Alstonia scholaris* leaf for pharmacological screening. On the basis of the pharmacological evaluation of acetic acid-induced writhing response and xylene-induced ear edema in mice, the alkaloids fraction appeared to be the most active part in both animal models (Tables 1 and 5). Then, intensive investigation of anti-inflammatory, analgesic actions and mechanisms were carried out on the alkaloids fraction and main alkaloids from *Alstonia scholaris* leaves.

The analgesic activities were evaluated using three animal models. Acetic acid-induced writhing response was selected to observe peripheral analgesic effects, while the hot-plate test was selected to investigate central analgesic activity. Furthermore, the formalin test is employed to investigate both peripheral and central mechanisms (Tjolsen et al., 1992). In the acetic acid-induced writhing test (Table 2), the alkaloids fraction, picrinine, vallesamine, scholaricine demonstrated significant analgesic effects. Acetic acid caused an increase in the peritoneal fluids of PGE2, serotonin, and histamine (Deraedt et al., 1980), so the results suggested that the alkaloids have a significant inhibitory activity in inflammation pain, and this activity may be related with the suppression of synthesis and/or release of endogenous pro-inflammatory substances. In the hot-plate test, negative effect indicated that the alkaloids were not central analgesic mediated through inhibition of central pain receptors (Table 3). The formalin test consists of two different phases: the first phase measures direct chemical stimulation of nociceptors, whereas the second phase is dependent on peripheral inflammation and changes in central processing. Previous studies demonstrated that bradykinin participate in the first phase, whereas histamine, serotonin, PGs, NO and bradykinin were involved in the second phase of the formalin test (Tjolsen et al., 1992). The experimental results showed that alkaloids produced a significant inhibitory effect, but only during the second phase of the formalin test (Table 4). All experimental results from three animal models indicated that the alkaloids might produce the analgesic effect peripherally, not through a central way.

The inhibition activity of the alkaloids in inflammation pain encouraged us to evaluate their anti-inflammatory activity. Xylene-induced ear edema in mice reflected the edematization during the early stages of acute inflammation (Vogel and Vogel, 1997). The experimental results indicated that the alkaloids fraction, picrinine, vallesamine and scholaricine have significant anti-inflammatory effect (Table 6). Meanwhile, it is odd that vallesamine treated by intragastrical, scholaricine by intraperitoneal did not inhibit xylene-induced ear edema significantly. The carrageenan-induced air pouch model is known to be an excellent acute inflammatory model in which various biochemical parameters in the exudate involved in the inflammatory response can be easily detected. The results showed (Table 7) that all the test samples significantly decreased the level of PGE2 in the exudates, thereby suggesting alkaloids interference with the cyclooxygenase pathways of arachidonic acid metabolism. Nitric oxide (NO) is one of well-known pro-inflammatory mediators in the pathogenesis of inflammation (Nanthan, 1996). The reaction of NO with superoxide anion forms peroxynitrite, a potent cytotoxic oxidant eliciting lipid peroxidation and cellular damage (Rubbo et al., 1994). MDA, an indicator of lipid peroxidation, and SOD were also measured for evaluating the ability to scavenge radicals. The test samples reduced NO production, lowered the MDA levels of serum and increased the levels of SOD in exudates (Table 7), which implied that the alkaloids could inhibit the lipid peroxidation and scavenge radicals by enhancing the activities of the antioxidant enzymes.

The development of inflammatory may be accompanied by increased production of prostaglandins and leukotrienes from arachidonic acid. COX-1 and COX-2 are responsible for the production of prostaglandins and LOX for leukotrienes. Inhibition of cyclooxygenase by nonsteroidal anti-inflammatory drugs and selective COX-2 inhibitors reduces the levels of prostaglandins, resulting in a reduction in pain and inflammation. However, this inhibition can cause alternative processing of arachidonic acid via the 5-lipoxygenase (5-LOX) pathway, resulting in increased production of pro-inflammatory and gastrotoxic leukotrienes. The dual inhibitors of COX and 5-LOX decrease the production of both leukotrienes and prostaglandins, and as such, they should theoretically display enhanced anti-inflammatory effects and decreased cardiovascular side effects caused by some selective COX-2 inhibitors, such as rofecoxib and valdecoxib (Xu et al., 2009a). The *in vitro* anti-inflammatory activity of alkaloids (Fig. 1) against COX-1, -2 and 5-LOX is shown in Table 8. The percentage inhibition of the alkaloids fraction to COX-1, -2 and 5-LOX was 54.3%, 63.3%, and 57.3%, respectively, which also supported the *in vivo* results. Besides, COX-2/5-LOX dual inhibitors found in this experiment, such as 16-formyl-5α-methoxystrictamine, picralinal, and tubotaiwine might be valuable for further attention.

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