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Identification of Potential Biomarkers from *Aconitum carmichaelii*, a Traditional Chinese Medicine, Using a Metabolomic Approach

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Bibliography

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ABSTRACT

Despite their well-known toxicity, *Aconitum* species are important traditional medicines worldwide. *Aconitum carmichaelii*, known in Chinese as 附子 (fuzi), is an officially recognized traditional Chinese medicine with characteristic analgesic and anti-inflammatory activities, whose principal pharmacological ingredients are considered as aconitine-type diterpene alkaloids. Notwithstanding the long-recorded use of *A. carmichaelii* in traditional Chinese medicine, no single-entity aconitum alkaloid drug has been developed for clinical use. UPLC-Q-TOF-MS was used to investigate the marker compounds that can be used to differentiate *A. carmichaelii* from seven other *Aconitum* species collected in Yunnan Province. Nontargeted principle component analysis scores plots found that all the tested *Aconitum* species clustered into three distinct groups, and *A. carmichaelii* was significantly different chemically than the other seven species. Furthermore, the primary and lateral roots of *A. carmichaelii* also showed significant differences. Using orthogonal partial least squares discriminate analysis analysis, eight marker compounds were identified, including 14-acetylkarakoline, aconitine, carmichaeline, fuzi-

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line, hypaconitine, mesaconitine, neoline, and talatisamine. Four of these aconitum alkaloids, fuziline, hypaconitine, mesaconitine, and neoline, showed significant analgesic activity in a dose-dependent manner compared to the negative and positive controls. However, hypaconitine, mesaconitine, and neoline exhibited significant acute toxicity activity, while fuziline showed no acute toxicity in mice, suggesting the relative safety of this alkaloid. This study provides a good example of how to differentiate an authentic medicinal plant from common adulterants using a metabolomics approach, and to identify compounds that may be developed into new drugs.

Introduction

Aconitum, a large genus of the Ranunculaceae family, consists of approximately 400 species distributed in the temperate regions of the northern hemisphere, with 211 species in China [1, 2]. The roots of *Aconitum* are used in traditional Chinese medicine (TCM) to treat various diseases, such as fainting, rheumatic fever, painful joints, gastroenteritis, diarrhea, edema, bronchial asthma, and some endocrinal disorders like irregular menstruation [3]. Of the 211 known species of the genus that grows in China, *Aconitum carmichaelii* is one of two species officially recorded as an aconite in the Chinese Pharmacopoeia [1]. The primary root of *A. carmichaelii* is known as chuanwu (川乌), whereas the lateral root is called fuzi (附子) in TCM [4]. However, the high toxicity risk and narrow therapeutic range limit the medicinal application on a larger scope [5]. More than 1500 diterpene alkaloids have been isolated from *Aconitum*, but only lappaconitine, 3-acetylaconitine, and bulleyaconitine A have been clinically used as analgesics in China [6]. Since the therapeutic window of diterpene alkaloids is narrow, it is critical to provide a method for standardization of these compounds to ensure their safe use. The quality control of this well-known and widely used TCM is an important public health issue that needs to be addressed using modern phytochemical methods [7]. Therefore, the development of a rapid and sensitive method to assess the aconitum alkaloids in *Aconitum*, both qualitatively and quantitatively, is useful to ensure their safety and effectiveness in the area of clinical drug use.

Many methods for the determination of aconitum alkaloids have been reported. UPLC-Q-TOF-HDMS combined with pattern recognition methods and pathway analysis was used to investigate comprehensive metabolomic characters of the *Aconitum* crude extract and its processed products, revealing the significant differences in metabolic profiles and changes of metabolite biomarkers of interest between the crude extract and processed preparations [8]. Moreover, a recently published profiling approach was applied successfully to evaluate the chemical constitution between co-decoction and mixed decoction of *Radix aconiti* and *Pinellia praeparata* using UPLC coupled with UPLC-Q-TOF-MS [9]. Hence, UPLC coupled with Q-TOF-MS provides a method with efficient separation and good sensitivity, and also allows for the identification of the fragmentation pathways of metabolites [10].

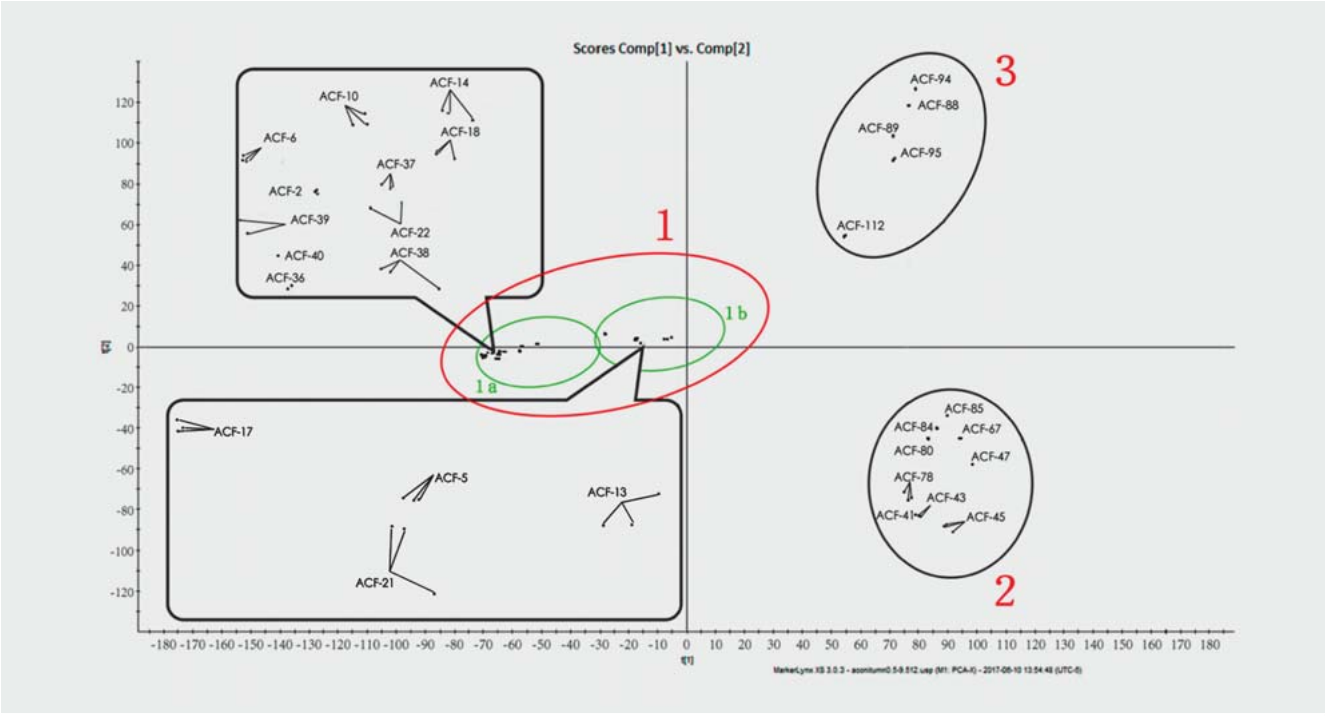
A large number of *Aconitum* species in the rural markets of Yunnan Province, including *A. carmichaelii, Aconitum bulleyanum, Aconitum fengii, Aconitum ouvrardianum,* and *Aconitum transsectum* have been sold interchangeably as *Aconitum*. Making the matter worse, adulterations and substitutions of the original species usually cannot be correctly identified or distinguished by conventional methods [11]. In this paper, a UPLC-Q-TOF-MS coupled with bioactivity testing and chemometrics was used to compare *A. carmichaelii* with seven other *Aconitum* species (*A. bulleyanum, A. carmichaelii, A. fengii, Aconitum iochanicum, A. ouvrardianum, Aconitum pukeense, A. transsectum,* and *Aconitum weixiense*) from Yunnan Province to determine if marker compounds could be identified that have analgesic activity, but lower toxicity than common aconitum alkaloids. This study provides a good example of how to differentiate a genuine medicinal plant from common adulterants using a metabolomics approach.

Results

Principal component analysis (PCA) is a nontargeted statistical method used to define nonobvious differences between samples [12]. To assess the differences in the metabolite compositions of different root parts of the *Aconitum* genus, a nontargeted metabolite profiling of primary and lateral root extracts from different collections were conducted and analyzed by high-resolution UPLC-QTOF-MS with full scan analysis. As shown in ▶ **Fig. 1**, 29 samples (each sample injected three times) clustered into three groups in nontargeted PCA scores plotted are observed for the positive mode. There is a significant difference between *A. carmichaelii* and the seven other *Aconitum* species. Primary and lateral roots also showed significant differences within the *A. carmichaelii*.

From the 3D plots, the retention times, *m/z* value of mass fragmental ions, and their intensities were used to compare the phenotypic differences of the eight *Aconitum* species using PCA, an unsupervised and therefore unbiased technique for multivariate analysis. Processed data displayed a clear differentiation of two clusters, including a group of *A. carmichaelii* and another one consisting of the seven other *Aconitum* species (▶ **Fig. 2**).

Processed data displayed a clear differentiation of three clusters of the eight species of *Aconitum*, suggesting that there may be characteristic compounds or higher levels of certain compounds in *A. carmichaelii* that can differentiate this important species from the others. Previous studies using PCA combined with orthogonal partial least squares discriminate analysis (OPLS-DA) were useful to distinguish chemical patterns and reveal marker compounds from different extracts [12–14]. In this study, OPLS-DA along with S-plots was used to look for the potential marker compounds in the metabolite profiles of the TCM plant. Eight ions may be useful markers for compounds to chemically distinguish *A. carmichaelii* from the seven other species. Based on the comparison of their retention time, exact mass generated from UPLC-Q-TOF-MS in positive mode, fragmentation mass data, co-injection experiments [14], and the spectroscopic data (^{1}H, ^{13}C NMR and MS) of the standard alkaloids used in the study [15, 16], the eight marker compounds were identified as 14-acetylkarakoline, aconitine, carmichaeline, fuziline, hypaconitine, mesaconitine, neoline, and talatisamine (▶ **Table 1** and **Figs. 3** and **4**).



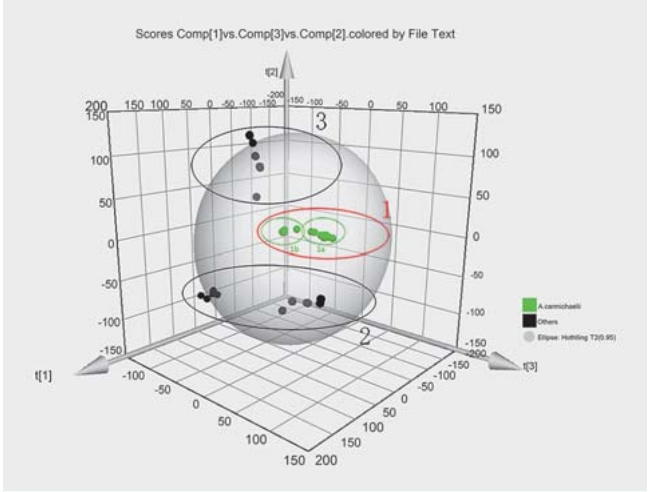
▶ **Fig. 1** 2D PCA plots of the roots of eight species of *Aconitum* (positive mode, time rang: 0.5–9.5 min, each sample injected three times). **1** Roots of *A. carmichaelii* in total, **1a** Lateral root of *A. carmichaelii*, **1b** Primary root of *A. carmichaelii*. **2** Primary root of *A. bulleyanum*, lateral root of *A. pukeense*, lateral root of *A. ouvrardianum*, lateral and primary roots of *A. iochanicum*, lateral and primary roots of *A. transsectum*. **3** Lateral and fibrous roots of *A. fengii*, lateral and primary roots of *A. weixiense*, and fibrous root of *A. bulleyanum*.

The four alkaloids, fuziline, hypaconitine, mesaconitine, and neoline, showed obvious analgesic activity in different tested doses in a dose-dependent manner compared to the negative and positive controls. The best analgesic effects for fuziline, hypaconitine, mesaconitine, and neoline were achieved in dosages of 400, 4, 2, and 100 mg/kg, respectively (▶ **Table 2**). However, hypaconitine, mesaconitine, and neoline showed significant acute toxicity, with LD_{50}s of 12.8, 6.41, and 267.95 mg/kg, respectively (▶ **Table 3**). In contrast, fuziline showed no acute toxicity in mice (▶ **Table 3**), indicating that this aconitum alkaloid is safer than others.

Discussion

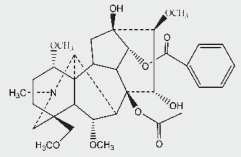
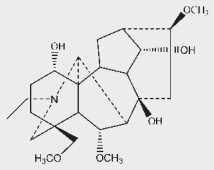
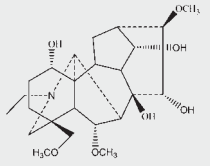
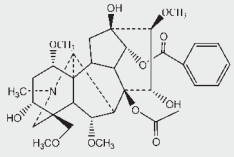
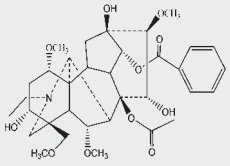
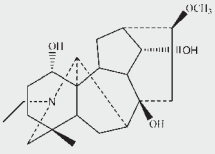
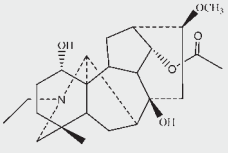
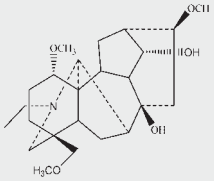
A. carmichaelii is one of two species officially recognized in the Chinese Pharmacopeia. It is also a well-known poisonous plant with analgesic and anti-inflammatory activities. The roots of *Aconitum* are mainly used as a medicinal plant in TCM and also as a root vegetable in the Qinling Mountains (Shaanxi, China) [17]. Until now, no low-toxic and active compound has been isolated from either of these roots. Furthermore, the clinical efficiency of certain TCMs containing *A. carmichaelii* may be compromised due to adulterations and substitutions. Therefore, this study aimed at identifying biomarkers from *A. carmichaelii* to differentiate it from other closely related species, and provide a basis for quality control for this important but potentially toxic medicinal plant.

Our results showed that *A. carmichaelii* is chemically distinct from the seven other *Aconitum* species that are known adulterants



▶ **Fig. 2** 3D PCA plots of the roots of eight species of *Aconitum* (positive mode, time rang: 0.5–9.5 min, each sample injected three times). **1** Roots of *A. carmichaelii* in total, **1a** Lateral root of *A. carmichaelii*, **1b** Primary root of *A. carmichaelii*. **2** Primary root of *A. bulleyanum*, lateral root of *A. pukeense*, lateral root of *A. ouvrardianum*, lateral and primary roots of *A. iochanicum*, lateral and primary roots of *A. transsectum*. **3** Lateral and fibrous roots of *A. fengii*, lateral and primary roots of *Aconitum weixiense*, and fibrous root of *A. bulleyanum*.

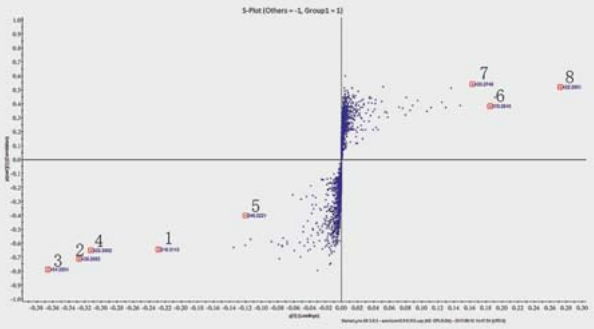
▶ **Table 1** The potential biomarkers identified from *Aconitum* roots.

#	Compound	Formula	t_R	Observed $[M + H]^+$	Calculated $[M + H]^+$	Major ions	Molecular structure
1	Hypaconitine [14]	$C_{33}H_{45}NO_{10}$	6.67	616.3113	616.3043	616 $[M + H]^+$ 584 $[M + H\text{-}CH_4O]^+$ 556 $[M + H\text{-}C_2H_4O_2]^+$ 524 $[M + H\text{-}C_3H_8O_3]^+$ 494 $[M + H\text{-}C_4H_{10}O_4]^+$ 105 $[M + H\text{-}C_{26}H_{41}NO_9]^+$	
2	Neoline [14]	$C_{24}H_{39}NO_6$	0.75	438.2851	438.2852	438 $[M + H]^+$ 420 $[M + H\text{-}H_2O]^+$ 402 $[M + H\text{-}H_4O_2]^+$ 388 $[M + H\text{-}CH_6O_2]^+$ 370 $[M + H\text{-}CH_8O_3]^+$ 342 $[M + H\text{-}C_3H_{12}O_3]^+$	
3	Fuziline [14]	$C_{24}H_{39}NO_7$	0.68	454.2801	454.2801	454 $[M + H]^+$ 436 $[M + H\text{-}H_2O]^+$ 422 $[M + H\text{-}CH_4O]^+$ 408 $[M + H\text{-}C_2H_6O]^+$ 404 $[M + H\text{-}CH_6O_2]^+$ 374 $[M + H\text{-}C_2H_8O_3]^+$ 344 $[M + H\text{-}C_4H_{14}O_3]^+$	
4	Mesaconitine [14]	$C_{33}H_{45}NO_{11}$	5.67	632.3062	632.3062	632 $[M + H]^+$ 602 $[M + H\text{-}CH_2O]^+$ 572 $[M + H\text{-}C_2H_4O_2]^+$ 540 $[M + H\text{-}C_3H_8O_3]^+$ 512 $[M + H\text{-}C_4H_8O_4]^+$ 496 $[M + H\text{-}C_5H_{12}O_4]^+$ 390 $[M + H\text{-}C_{11}H_{14}O_6]^+$ 105 $[M + H\text{-}C_{26}H_{41}NO_{10}]^+$	
5	Aconitine [14]	$C_{34}H_{47}NO_{11}$	6.78	646.3219	646.3221	646 $[M + H]^+$ 586 $[M + H\text{-}C_2H_4O_2]^+$ 556 $[M + H\text{-}C_3H_6O_3]^+$ 522 $[M + H\text{-}C_4H_{12}O_4]^+$ 404 $[M + H\text{-}C_{11}H_{14}O_6]^+$ 105 $[M + H\text{-}C_{27}H_{43}NO_{10}]^+$	
6	Carmichaeline [14]	$C_{22}H_{35}NO_4$	1.35	378.2641	378.2641	378 $[M + H]^+$ 360 $[M + H\text{-}H_2O]^+$ 342 $[M + H\text{-}H_4O_2]^+$ 328 $[M + H\text{-}C_2H_{10}O]^+$	
7	14-Acetylkara-koline [14]	$C_{24}H_{37}NO_5$	1.06	420.2745	420.2745	420 $[M + H]^+$ 402 $[M + H\text{-}H_2O]^+$ 388 $[M + H\text{-}CH_4O]^+$ 370 $[M + H\text{-}CH_6O_2]^+$ 356 $[M + H\text{-}C_2H_8O_2]^+$ 340 $[M + H\text{-}C_2H_8O_3]^+$ 328 $[M + H\text{-}C_4H_{12}O_2]^+$	
8	Talatisamine [14]	$C_{24}H_{39}NO_5$	0.87	422.2902	422.2902	422 $[M + H]^+$ 390 $[M + H\text{-}CH_4O]^+$ 358 $[M + H\text{-}C_2H_8O_2]^+$ 344 $[M + H\text{-}C_3H_{10}O_2]^+$ 326 $[M + H\text{-}C_3H_{12}O_3]^+$	

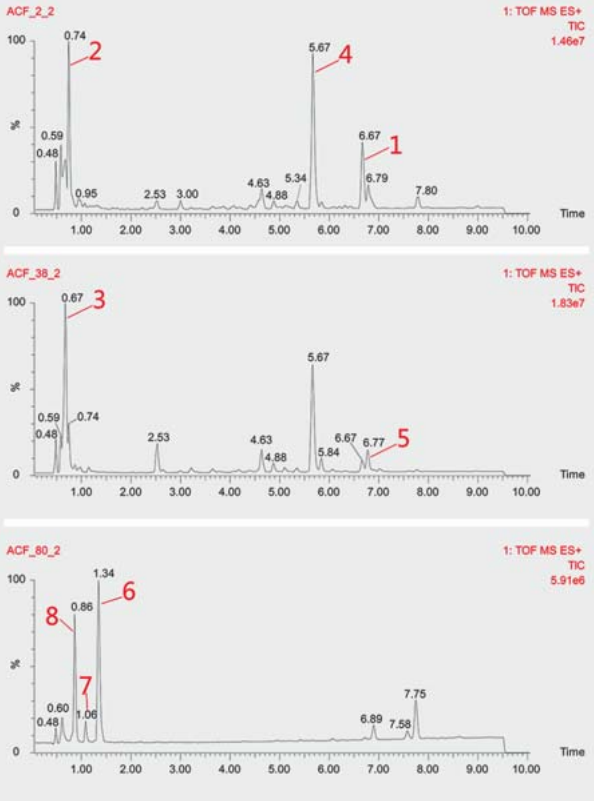
(▶ **Figs. 1** and **2**), suggesting the uniqueness of this medicinal species. To date, 75 aconitine skeleton-based C_{19}- diterpenoid alkaloids and 18 C_{20}-diterpenoid alkaloids have been identified from the roots of *A. carmichaelii* [18], among which the principal pharmacological ingredients of fuzi are considered as aconitine-type C_{19}-diterpenoid alkaloids [4, 18]. Eight potential biomarkers were discovered including five C_{19}-diterpenoid alkaloids, 14-acetylkarakoline, aconitine, hypaconitine, mesaconitine, and talatisamine, and three C_{18}-diterpenoid alkaloids, carmichaeline, fuziline, and neoline, some of which may correlate to the active constituents of *A. carmichaelii*. Four alkaloids, fuziline, hypaconitine, mesaconitine, and neoline, from the roots of *A. carmichaelii* were selected to test their analgesic activity and acute toxicity and all displayed obvious analgesic activity (▶ **Table 2**). However, the acute toxicity of hypaconitine, mesaconitine, and neoline (▶ **Table 3**) made them a less attractive target for the development of a new analgesic drug. Fuziline alone was found to be both an effective analgesic as well as possessing lower toxicity, with a maximum tolerated dose (MTD) of 1000 mg/kg (▶ **Table 3**). Thus, fuziline may be a candidate for development into a new analgesic drug.

Different root tubers have different names and different medical uses in TCM. The primary root of *A. carmichaelii* is known as chuanwu (川乌), whereas the processed lateral root is called fuzi (附子) [4]. From ▶ **Fig. 1**, Group 1 was divided into two subparts including the primary roots and lateral roots of *A. carmichaelii*, suggesting that there should be different chemical constitutes in the two kinds of roots [19, 20].

In clinical practice, knowledge of herbal toxicity is often based upon the training and experience of TCM practitioners, passed on for more than 1000 years. However, there is a lack of sophisticated assays to assess the toxicity of TCM routinely. Therefore, to augment traditional knowledge, it is important to carry out state-of-the-art toxicological analysis on *Aconitum* to improve its quality and safety.



▶ **Fig. 3** Identification of marker compounds from S-plot analysis of eight *Aconitum* species. (*A. carmichaelii* = − 1; 7 other *Aconitum* species = 1)



▶ **Fig. 4** Eight marker compounds identified from LC-MS chromatograms of *A. carmichaelii* (top two chromatograms) and *A. transsectum* (bottom chromatogram) including 14-acetylkarakoline (**7**), aconitine (**5**), carmichaeline (**6**), fuziline (**3**), hypaconitine (**1**), mesaconitine (**4**), neoline (**2**), and talatisamine (**8**).

Materials and Methods

Chemicals

UPLC-MS grade acetonitrile, methanol, water (J. T. Baker), and formic acid (Sigma) were used for UPLC-TOF-MS analysis. Guaranteed reagent grade methanol was from EMD Chemicals. The 11 standard compounds (aconitine, aconosine, andyunaconitine, benzoylmesaconitine, bulleyaconitine A, fuziline, hypaconitine, karakoline, mesaconitine, neoline, and songorine, purity ≥ 98.0 % by HPLC-PDA and ^{1}H-NMR) were isolated and purified from *Aconitum* spp. in our lab [15, 16, 21].

Sample materials

Twenty-nine samples from eight *Aconitum* species (*A. bulleyanum* Diels, *A. carmichaelii* Debeaux, *A. fengii* W. T. Wang, *A. iochanicum* Ulbr., *A. ouvrardianum* Hand.-Mazz., *A. pukeense* W. T. Wang, *A. transsectum* Diels, and *A. weixiense* W. T. Wang) were collected in China from 2012 to 2015. The collection locations for all the *Aconitum* samples are shown in **Table S1**, Supporting Information. The specimens were identified by Professor Qin-Er Yang, South China Botanical Garden, Chinese Academy of Sciences. Voucher specimens of *Aconitum* samples used in this study were deposited at the Herbarium of Yunnan University, China.

▶ **Table 2** Effect of hypaconitine, neoline, fuziline, and mesaconitine on the analgesic activity caused by acetic acid.

Selected marker compounds	Treatments	Dose (mg/kg)	Writhing numbers	Pain suppression rate (%)
Hypaconitine	H_2O	–	33.9 ± 13.8	–
	Pethidine hydrochloride	40	0***	100
	Acetyl salicylic acid	200	3.1 ± 3.2***	91.11
	Hypaconitine	4	2.8 ± 4.5***	92.88
	Hypaconitine	2	23.6 ± 10.9***	39.95
	Hypaconitine	1	24.5 ± 9.8*	37.66
	Hypaconitine	0.5	27.6 ± 11.5	29.77
Neoline	H_2O	–	33.9 ± 8.0	–
	Pethidine hydrochloride	40	0***	99.41
	Acetyl salicylic acid	200	2.9 ± 4.0***	91.45
	Neoline	100	9.0 ± 10.0***	73.45
	Neoline	50	15.2 ± 9.7***	55.16
	Neoline	25	29.0 ± 18.5	22.42
	Neoline	12.5	30.4 ± 25.1	10.32
Fuziline	H_2O	–	34.3 ± 8.0	–
	Pethidine hydrochloride	40	0***	100
	Acetyl salicylic acid	200	3.1 ± 3.3***	90.96
	Fuziline	400	13.0 ± 10.2***	62.10
	Fuziline	200	14.7 ± 10.8**	57.14
	Fuziline	100	23.6 ± 7.9**	31.20
	Fuziline	50	29.5 ± 14.6	13.99
Mesaconitine	H_2O	–	39.3 ± 13.8	–
	Pethidine hydrochloride	40	0***	100
	Acetyl salicylic acid	200	3.1 ± 3.2***	92.11
	Mesaconitine	2	19.3 ± 10.0**	50.89
	Mesaconitine	1	21.8 ± 12.6**	44.53
	Mesaconitine	0.5	20.0 ± 9.6**	49.11
	Mesaconitine	0.25	33.6 ± 11.5	14.50

Compared with H_2O, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

▶ **Table 3** Acute toxicity of hypaconitine, neoline, fuziline, and mesaconitine.

Treatment	LD_{50} (mg/kg)	MTD (mg/kg)
Hypaconitine	12.80	–
Neoline	267.95	–
Fuziline	–	1,000.0
Mesaconitine	6.41	–

LD_{50}: median lethal dose, MTD: maximum tolerance dose

Sample preparation

Each air-dried and powdered sample of the *Aconitum* plants (ca. 1 g) was percolated with 0.5% HCl (90 mL) by sonication for 30 min at room temperature. The aqueous acidic solution was basified with 10% sodium hydroxide solution to pH 9–10 and then further extracted with chloroform three times (20 mL each time). Removal of the solvent evaporated to dryness under nitrogen. The resulting extract was stored at −20 °C until analysis. Prior to UPLC-TOF-MS analysis, each extract was dissolved in 100% MeOH (30 mL). In-source MS/MS fragmentation was conducted (with an aperture voltage of 60 V) on these extracts, following which extracts were diluted by 12-fold for standard MS analysis (with an aperture voltage of 0 V). All the prepared samples were filtered through a 0.45-μm nylon membrane filter prior to analysis [22].

Preparation of standard solutions

Each accurately weighted standard was dissolved in methanol to give stock solutions. Working standard solutions containing 11 reference standards were prepared by diluting the stock solutions with methanol-water (containing 0.05 M HCl) (4 : 1, v/v) [5].

Liquid chromatography

Separation was achieved by UPLC using a Waters ACQUITY separations module, (Waters Corporation) coupled with a QTOF-MS (Xevo G2 QTOF, Waters MS Technologies), controlled by MassLynx

v4.1 software. The separations were carried out over a 2.1 × 50 mm i.d., 1.7 µm UPLC BEH C_{18} reversed-phase column at 40 °C. The mobile phase consisted of 0.1% aqueous formic acid (A) and 0.1% formic acid in acetonitrile (B). The linear gradient elution was performed as follows: 0–0.3 min, 19% B; 0.3–3 min, 19–23% B; 3–9 min, 23–38% B; 9–9.5 min, 38–95% B; 9.5–11.5 min, 95–95% B; 11.5–12.0 min, 95–19% B; 12.0–14.0 min, 19–19% B [23]. A flow rate of 0.3 mL/min was employed for elution, and the injected sample (0.1 mg/mL) volume was set at 0.1 µL.

Mass spectrometry

Mass spectra were recorded using a Xevo G2 QTOF (Waters MS Technologies) equipped with an electrospray ionization source and controlled by MassLynx v 4.1 software. MS full scans were acquired in the positive mode over the range *m/z* 100–1000 Da in two channels with a scan time of 1 s. The capillary voltages were set at 3000 V (positive mode) and 2500 V (negative mode), respectively, and the cone voltage was 20 V. Nitrogen gas was used both for the nebulizer and in desolvation. The desolvation and cone gas flow rates were 600 and 20 L/h, respectively. The desolvation temperature was 250 °C, and the source temperature was 100 °C [24].

Chemometric data analysis

The UPLC-QTOF-MS data were processed by MarkerLynx XS v4.1 software (Waters Corp.) and MS full scan data of 29 samples were analyzed by untargeted PCA and OPLS-DA. The parameters of PCA were set as follows: a retention time range of 0.50–950 min, mass range 100–1000 Da, and a mass tolerance of 50 mDa. The intensity threshold (counts) of collection parameters was set at 500, the mass window was set as 0.05, the retention time tolerance was set as 0.20, and the noise elimination level was set as 6.00 [24].

Analgesic activity and acute toxicity in mice

The analgesic activity of fuziline, hypaconitine, mesaconitine, and neoline was performed by the writhing model described by Zhao et al. [25]. The animal protocol, SYXK(Sichuan) 2013–185, was approved on November 12, 2013. Acetic acid (CH_3COOH) was provided by Chengdu Chemical Reagent Factory Co. Ltd., China (purity ≥ 99.5%). Acetyl salicylic acid (Nanjing Baijingyu Pharmaceutical Co. Ltd., purity ≥ 98.5%) and pethidine hydrochloride injection (Qinghai Pharmaceutical Factory, purity ≥ 99.0%) were selected as positive reference standards, and H_2O as the negative control. Adult Kunming mice (18–22 g) were randomized into groups of 10 each, with 50% male and 50% female. Using gavage administration, the negative controls were treated with H_2O, the positive control with pethidine hydrochloride (40 mg/kg), and the test groups with hypaconitine (4, 2, 1, and 0.5 mg/kg), neoline (200, 100, 50, 25, and 12.5 mg/kg), fuziline (400, 200, 100, and 50 mg/kg), or mesaconitine (2, 1, 0.5, and 0.25 mg/kg). All chemicals were dissolved in H_2O and sonicated. Acetic acid was dissolved in normal saline (0.90% w/v) to a final concentration of 0.6%, injected intraperitoneally (0.2 mL/per mice), and the contraction of abdominal muscles (writhes) was observed for 10 min. The number of writhes were counted to assess analgesic activity of various groups, and expressed as the percentage inhibition of abdominal constrictions between the control group and the aconitum alkaloid-treated groups.

Acute toxicity of the four compounds was tested based upon a previously published method, with minor modifications [26]. The test mice and method of dissolution were the same as described for the analgesic activity mentioned above. The compounds were prepared as aqueous suspensions with a concentration of 0.5 g/mL and fed to animals with 0.2 mL/10 g by gavage with various concentrations. The oral LD_{50} and MTD values for test alkaloids were measured using at least four different doses, and a t-test was used to calculate the significance by SPSS 19.0.

Supporting information

Plant collection data for the 29 samples (8 species) used in this research is available as Supporting Information.

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Conflict of Interest

The authors declare no conflict of interest.

References

[1] Li LQ, Kadota Y. *Aconitum*. In: Wu ZY, Raven P, eds. Flora of China 2001. Beijing: Science Press and St. Louis: Missouri Botanical Garden Press; 2001: 149–222

[2] Ma L, Gu R, Tang L, Chen ZE, Di R, Long C. Important poisonous plants in Tibetan ethnomedicine. Toxins (Basel) 2015; 7: 138–155

[3] Nyirimigabo E, Xu Y, Li Y, Wang Y, Agyemang K, Zhang Y. A review on phytochemistry, pharmacology and toxicology studies of *Aconitum*. J Pharm Pharmacol 2015; 67: 1–19

[4] Yu M, Yang YX, Shu XY, Huang J, Hou DB. *Aconitum carmichaelii* Debeaux, cultivated as a medicinal plant in western China. Genet Resource Crop Ev 2016; 63: 919–924

[5] Luo H, Huang ZF, Tang XL, Yi JH, Chen SY, Yang AD, Yang J. Dynamic variation patterns of aconitum alkaloids in daughter root of *Aconitum carmichaelii* (fuzi) in the decoction process based on the content changes of nine aconitum alkaloids by HPLC-MS-MS. Iran J Pharm Res 2016; 15: 263–273

[6] Wang FP. A deliberation on methodology of modernization of traditional Chinese medicines based on the research and development of new drugs from "Cao Wu". Prog Chem 2009; 21: 63–65

[7] Xiao P, Wang F, Gao F. A pharmacophylogenetic study of *Aconitum* L. (Ranunculaceae) from China. Acta Phytotaxon Sin 2006; 44: 1–46

[8] Nyirimigabo E, Xu YY, Li YB, Wang YM, Agyemang K, Zhang YJ. A review on phytochemistry, pharmacology and toxicology studies of *Aconitum*. J Pharm Pharmacol 2014; 67: 1–19

[9] Nordstrom A, Maille GO, Qin C, Siuzdak G. Nonlinear data alignment for UPLC-MS and HPLC-MS based metabolomics: quantitative analysis of endogenous and exogenous metabolites in human serum. Anal Chem 2006; 78: 3289–3295

[10] Konishi Y, Kiyota T, Draghici C, Gao JM, Yeboah F, Acoca S, Jarussophon S, Purisima E. Molecular formula analysis by an MS/MS/MS technique to expedite dereplication of natural products. Anal Chem 2007; 79: 1187–1197

[11] Cui P, Han H, Wang R, Yang L. Identification and determination of aconitum alkaloids in *Aconitum* herbs and Xiaohuoluo pill using UPLC-ESI-MS. Molecules 2012; 17: 10242–10257

[12] Wu SB, Meyer RS, Whitaker BD, Litt A, Kennelly EJ. A new liquid chromatography-mass spectrometry-based strategy to integrate chemistry, morphology, and evolution of eggplant (*Solanum*) species. J Chromatogr A 2013; 1314: 154–172

[13] Wu SB, Wu J, Yin Z, Zhang J, Long CL, Kennelly EJ, Zheng S. Bioactive and marker compounds from two edible dark-colored *Myrciaria* fruits and the synthesis of jaboticabin. J Agric Food Chem 2013; 61: 4035–4043

[14] Sun H, Wang M, Zhang AH, Ni B, Dong H, Wang XJ. UPLC-Q-TOF-HDMS analysis of constituents in the root of two kinds of *Aconitum* using a metabolomics approach. Phytochemical Anal 2012; 24: 263–276

[15] Shen Y. Chemical constituents and their bioactivities of *Aconitum carmichaeli* Debx (Fuzi) [Master dissertation]. Kunming: Yunnan University of TCM; 2004

[16] Shen Y. Studies on chemical constituents of two medicinal plants of *Aconitum* [Doctoral dissertation]. Kunming: Kunming Institute of Botany, Chinese Academy of Sciences; 2010

[17] Kang Y, Łuczaj ŁJ, Ye S. The highly toxic *Aconitum carmichaelii* Debeaux as a root vegetable in the Qinling Mountains (Shaanxi, China). Genet Resour Crop Ev 2012; 59: 1569–1575

[18] Zhou G, Tang L, Zhou X, Wang T, Kou Z, Wang Z. A review on phytochemistry and pharmacological activities of the processed lateral root of *Aconitum carmichaelii* Debeaux. J Ethnopharmacol 2015; 160: 173–193

[19] Ohta H, Seto Y, Tsunoda N, Takahashi Y, Matsuura K, Ogasawara K. Determination of aconitum alkaloids in blood and urine samples. II. capillary liquid chromatographic 2-frit fast atom bombardment mass spectrometric analysis. J Chromatography B 1998; 714: 215–221

[20] Singhuber J, Zhu M, Prinz S, Kopp B. *Aconitum* in traditional Chinese medicine: a valuable drug or an unpredictable risk. J Ethnopharmacol 2009; 126: 18–30

[21] Zhang L, Yi X, Li G, Zi S, Chen Y, Shen Y. Studies on diterpenoid alkaloids in *Aconitum transectum*. China Pharmacist 2016; 19: 222–225

[22] Wang YR, Cai SN, Chen Y, Deng L, Zhou XM, Liu J, Xu X, Xia Q, Lin M, Zhang JL, Huang WL, Wang WJ, Xiang CH, Cui GZ, Du LF, He H, Qi BH. Separation and purification of five alkaloids from *Aconitum duclouxii* by counter-current chromatography. J Sep Sci 2015; 38: 2320–2326

[23] Zhang Q, Wang CH, Ma YM, Zhu EY, Wang ZT. UPLC-ESI/MS determination of 17 active constituents in two categorized formulas of traditional Chinese medicine, Sanhuang Xiexin Tang and Fuzi Xiexin Tang: application in comparing the differences in decoctions and macerations. Biomed Chromatogr 2013; 27: 1079–1088

[24] Li P, AnandhiSenthilkumar H, Wu SB, Liu B, Guo ZY, Fata JE, Kennelly EJ, Long CL. Comparative UPLC-QTOF-MS-based metabolomics and bioactivities analysis of *Garcinia oblongifolia*. J Chromatogr B Analyt Technol Biomed Life Sci 2016; 1011: 179–195

[25] Zhao DK, Ai HL, Zi SH, Zhang LM, Yang SC, Guo HC, Shen Y, Chen YP, Chen JJ. Four new C_{18}-diterpenoid alkaloids with analgesic activity from *Aconitum weixiense*. Fitoterapia 2013; 91: 280–283

[26] Tanii H, Hashimoto K. Studies on the mechanism of acute toxicity of nitriles in mice. Arch Toxicol 1984; 55: 47–54