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Establishment of a bioassay for the toxicity evaluation and quality control of *Aconitum* herbs

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ABSTRACT

Currently, no bioassay is available for evaluating the toxicity of *Aconitum* herbs, which are well known for their lethal cardiotoxicity and neurotoxicity. In this study, we established a bioassay to evaluate the toxicity of *Aconitum* herbs. Test sample and standard solutions were administered to rats by intravenous infusion to determine their minimum lethal doses (MLD). Toxic potency was calculated by comparing the MLD. The experimental conditions of the method were optimized and standardized to ensure the precision and reliability of the bioassay. The application of the standardized bioassay was then tested by analyzing 18 samples of *Aconitum* herbs. Additionally, three major toxic alkaloids (aconitine, mesaconitine, and hypaconitine) in *Aconitum* herbs were analyzed using a liquid chromatographic method, which is the current method of choice for evaluating the toxicity of *Aconitum* herbs. We found that for all *Aconitum* herbs, the total toxicity of the extract was greater than the toxicity of the three alkaloids. Therefore, these three alkaloids failed to account for the total toxicity of *Aconitum* herbs. Compared with individual chemical analysis methods, the chief advantage of the bioassay is that it characterizes the total toxicity of *Aconitum* herbs. An incorrect toxicity evaluation caused by quantitative analysis of the three alkaloids might be effectively avoided by performing this bioassay. This study revealed that the bioassay is a powerful method for the safety assessment of *Aconitum* herbs.

1. Introduction

Nowadays aconite poisoning still frequently occurs in many countries, mostly related to the application of traditional herbal medicines of the genus *Aconitum* [1]. For centuries various species of *Aconitum* have been used by different populations as poisons and medicines, with certain species still being used in Chinese and Japanese herbal medicines [2]. In the Chinese Pharmacopoeia (Ch.P.) 2010 [3], two species of *Aconitum* are recorded. The *Aconitum carmichaelii* Debx. is listed by its two derivatives: the dried mother root named "Chuanwu" in Chinese, and the daughter root called "Fuzi" and known as "Bushi" in Japan. The second species is *Aconitum kusnezoffii* Reichb., whose roots are monographed, namely "Caowu". Compared with Chuanwu and Caowu, Fuzi is more popular and is prescribed more frequently. As widely used in traditional Chinese medicines (TCMs), they have similar pharmacological actions and are commonly applied for various diseases, such as collapse, syncope, rheumatic fever, painful joints, gastroenteritis, diarrhea, oedema, bronchial asthma, various tumors, and some endocrinal disorders like irregular menstruation [4–6]. However, all *Aconitum* herbs are highly toxic and have a narrow margin of safety between therapeutic and toxic doses [7,8]. The cardiotoxicity and neurotoxicity of these herbs are potentially lethal, and the onset of poisoning symptoms occurs rapidly, often within 10–90 min after ingestion [9–13]. The cases of poisoning and even death are usually reported owing to the improper use of *Aconitum* herbs in Asian countries [9,13–17]. In Western countries, aconite poisoning usually occurs after accidentally or deliberately ingesting the wild *Aconitum* plants [18–20]. The high toxicity of *Aconitum* herbs is attributed to *Aconitum* alkaloids, especially diester diterpenoid alkaloids (DDAs), such as aconitine, mesaconitine, and hypaconitine [1,2,6]. It is recognized that these three alkaloids are the major toxic ingredients of *Aconitum* herbs, and as reported, the LD_{50} values of aconitine in mice are 1.8 mg/kg (oral administration), 0.31 mg/kg (intraperitoneal injection), and 0.12 mg/kg (intravenous injection) [21]. For the clinical safety use, the tubers and roots of aconites are applied only after careful processing (usually soaking and steaming) which could greatly reduce their toxicity

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[3,4,22]. Therefore, the safety assessment is a top priority for the quality control of *Aconitum* herbs.

Currently, the method of choice for assessing the safety of *Aconitum* herbs is the quantitative analysis of its three major toxic DDAs (aconitine, mesaconitine, and hypaconitine) by liquid chromatography, as recommended in the Ch.P. 2010 [3,22–26]. In general, upper limits of the three DDAs' content are established in order to ensure the drug safety. For example, the total content of the three DDAs in Fuzi should not be more than 0.020%, as officially prescribed in the Ch.P. 2010 [3]. However, the existing methods and standards of *Aconitum* herbs' safety assessment cannot meet the needs of clinical practice and production of these traditional medicines for the reason that besides the three DDAs, many are the toxic ingredients in *Aconitum* herbs [27,28]. Moreover, despite their highest toxicity when compared with the other detected alkaloids, the contents of the three DDAs are found to be very low [6,22]. The content of the three DDAs may not be able to represent the major or total toxicity of some *Aconitum* herbs. Poisoning incidents in clinic could be caused by the incorrect toxicity evaluations obtained from quantitative analysis methods. In addition, the toxicity of the three DDAs is different (aconitine > mesaconitine > hypaconitine), and thus, samples with the same total DDAs content often have different total toxicity [27,28]. Since the exact toxicity cannot be obtained directly from quantitative analysis, this method is not reliable for the toxicity evaluation of *Aconitum* herbs. Accordingly, limiting the total content of the three DDAs is not a reliable method for the quality control of *Aconitum* herbs.

In order to satisfy the requirements of production and clinical practice, a direct bioassay was established in the present study to evaluate the total toxicity of *Aconitum* herbs. In this bioassay, the minimum lethal doses (MLD) of test sample and standard were respectively determined. The toxic potency was calculated by comparing their MLD. The application of this bioassay was tested by analyzing 18 samples of *Aconitum* herbs. Furthermore, the results of the toxicity analysis were compared with those obtained from an ultra performance liquid chromatography (UPLC) method.

2. Materials and methods

2.1. Chemicals and solvents

Chemical standards of aconitine, mesaconitine, and hypaconitine were supplied by the National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China. The purity of the three standards was each above 99.5%. No impurities were found in these chemical standards by UPLC-DAD analysis. HPLC grade acetonitrile was purchased from Fisher Chemicals (Pittsburg, PA, USA). High purity water was obtained from a Millipore Milli-Q water purification system (Bedford, MA, USA). Analytical grade absolute ethanol, ammonium bicarbonate, and 25% ammonium hydroxide solution were purchased from Beijing Chemical Reagents Company (Beijing, China).

2.2. Sample collection and preparation

Crude Fuzi and Crude Chuanwu, the dried mother roots and daughter roots respectively of *A. carmichaelii* Debx., were harvested in their trueborn cultivating site in Jiangyou, Sichuan, China. Three different batches of Crude Fuzi (1–3) were collected from the same production area but different harvest time. Yanfuzi, Heishunpian, Shufupian, Huangfupian and Baifupian were processed from Crude Fuzi 3 using different procedures. Baifupian 1–7 were of different batches. Crude Caowu, the dried roots of *A. kusnezoffii* Reichb., was collected from the wild in Liaoning, China. The Zhichuanwu and the Zhicaowu were the processed products of the Crude Chuanwu and the Crude Caowu, respectively. All the raw materials were collected from July to September 2009 and processed by Sichuan Jiangyou Zhongba Fuzi Science and Technology Development Co., Ltd. All the procedures were described in detail in the Ch.P. 2010 for each processed product [3]. The processed products were dry except Yanfuzi, which was wet with a moisture content of 102.91% [29].

2.3. Animals

Mice (ICR), rats (SD, Wistar, and F344), guinea pigs (Dunkin Hartley), and domestic pigeons were obtained from the Laboratory Animal Center of Academy of Military Medical Sciences, Beijing, China, and kept under standard laboratory conditions. Animals of both sexes and different weight ranges were used. Before all experiments, the animals were fasting for 24 h with free access to water. The present study conformed to the Regulations for the Administration of Affairs Concerning Experimental Animals, National Committee of Science and Technology of China (31 October 1988) and Instructive Notions with Respect to Caring for Laboratory Animals, Ministry of Science and Technology of China (30 September 2006).

2.4. Establishment of the bioassay

2.4.1. Standards

Standard, one of aconitine, mesaconitine, and hypaconitine, was accurately weighed and dissolved in absolute ethanol to produce a 1 mg/ml standard stock solution. This stock solution was stored at 4 °C and diluted to appropriate concentration with physiological saline as the standard solution on the day of the assay.

2.4.2. Sample extraction

The test sample (*Aconitum* herbs) was extracted with aqueous ethanol. The extraction solution was concentrated to one-tenth of its original volume in vacuo in a rotary evaporator at 40 °C as stock solution, and this stock solution was diluted to appropriate concentration with physiological saline as the test solution. Before infusion, each solution was filtered through a 0.22 μm microporous membrane.

2.4.3. Assay

When measured, the animal was fastened and a fine needle connected with a microburette was inserted into its vein. The standard solution or test solution was administered slowly by intravenous infusion which could ensure the rapid onset of drug action, until the animal was dead. The dilation of pupil and cessation of breath were considered as the critical point of death. The lethal dose of test solution (ml/kg) should be approximately the same as that of standard solution by adjusting the concentration of test solution. Besides, the infusion volume should not exceed the tolerance volume of animals for a single intravenous injection of physiological saline. The number of animals used for test group should be the same as standard group. The body weight and infusion volume were recorded and the MLD was calculated as mg or g per kg of body weight. The toxic potency of test sample (TPT) and its percentage of fiducial limits (*FL%*) were calculated by using the statistical method for direct bioassays described detailedly in the Ch.P. 2010 [30].

In this bioassay, the response (death), produced by standard or test sample which took effect rapidly, was clear-cut and easily recognized. Exact dose could be measured without time lag.

2.5. Standardization of the bioassay

In order to improve the precision and reliability of the bioassay, the effects of experimental conditions relating to experimental animal, standard, infusion speed, and extraction of test sample were

investigated with the MLD as a target. The standardized bioassay was established based on the optimized experimental conditions.

Experimental animal. After a preliminary screen and global consideration of the operation, costs, and drug requirements, we chose mice, rats, guinea pigs, and pigeons as the test animals for this bioassay. The MLD of aconitine was determined to investigate the relative sensitivity of these animals to toxicity. Mice and rats were infused via the tail vein, guinea pigs via the neck vein, and pigeons via the wing vein. In addition, the poisoning symptoms in these animals were observed and recorded. Then, the effect of strain, sex and body weight of the chosen animal was respectively investigated.

Standard. The MLD of aconitine, mesaconitine, and hypaconitine were compared to choose the most toxic one as standard. Each substance was made into 1 mg/ml solution with absolute ethanol as stock solution, which was subsequently diluted to 0.01 mg/ml with physiological saline as infusion solution. Then, the stability of the chosen standard's stock solution and the concentration of the chosen standard's infusion solution were also investigated.

Infusion speed. The MLD of the standard's infusion solution was determined with different infusion speeds to investigate the effect of infusion speed.

Extraction of test sample. In order to accurately characterize the total toxicity of *Aconitum* herbs, the extract should contain as many toxic substances as possible. We selected ethanol, miscible with water and less toxic, as the extraction solvent for the extract. To study the impact of the extraction method, Crude Fuzi 1 was extracted separately by three extraction methods which are often used in the extraction of TCM. The specific methods were performed as follows: three equal portions of Crude Fuzi 1 powder (through a 100 mesh sieve) were soaked in 10 times amount of 85% ethanol (v/w) for 30 min; one portion was then soaked continuously for 30 min (soak method); another portion was subjected to an ultrasonic treatment for 30 min (ultrasonic method); the last portion was boiled and refluxed for 30 min (reflux method); and finally, the MLD of the three extracts were measured, respectively. After the extraction method was selected, its experimental conditions were optimized. In addition, the stability of the test sample's stock solution was also investigated.

2.6. Sample analysis

2.6.1. Bioassay

The standardized bioassay was applied to measure the toxic potency of 18 samples of *Aconitum* herbs. The results were compared with those obtained from the chemical analysis.

2.6.2. Chemical analysis

The content of aconitine, mesaconitine, and hypaconitine in the same sample extracts used in the bioassay were analyzed by UPLC using a Waters Acquity system equipped with a binary solvent delivery pump, an auto sampler and a photo diode array detector. The mobile phase consisted of (A) 10 mM ammonium bicarbonate in water with pH 10 adjusted by 25% ammonium hydroxide solution and (B) acetonitrile using a gradient program of 5–30% B in 0–10 min, 30–60% in 10–25 min, and 60% in 25–30 min. A Waters Acquity UPLC™ HSST3 column (100 mm × 2.1 mm, 1.8 μm) was used and the column temperature was maintained at 25 °C. The flow rate was 0.3 ml/min. The detector wavelength was set at 235 nm.

2.7. Statistical analysis

The MLD was expressed as mean ± standard deviation (S.D.). The differences between groups were analyzed by ANOVA. $P < 0.05$ was considered statistically significant.

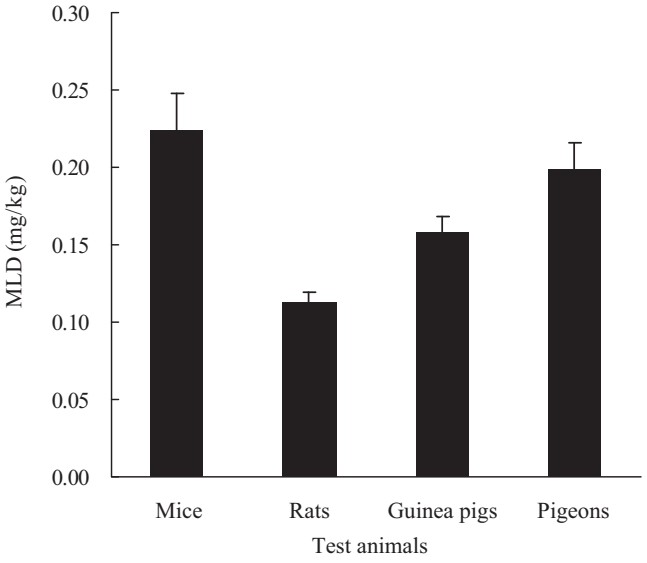

Fig. 1. The MLD of aconitine in different test animals. Mice (ICR, 20 ± 2 g), rats (SD, 180 ± 10 g), guinea pigs (Dunkin Hartley, 300 ± 30 g), and domestic pigeons (350 ± 50 g); male. The aconitine concentration was 0.01 mg/ml. The minimum lethal doses (MLD) were expressed as mean ± S.D. (n = 10).

3. Results and discussion

3.1. Effect of animal species, strain, sex, and body weight

The MLD of the different animal groups were shown in Fig. 1. Most of the animals were dead within several minutes, with tremor, emesis, and/or stool evacuation observed before dying. The endpoint of the infusion was easy to be predicted. According to the MLD, the order of sensitivity was as follows: rat > guinea pig > pigeon > mouse. Because intravenous administration in mice and rats required only one person to complete, it was simpler than intravenous administration in guinea pigs or pigeons. Additionally, the relative standard deviation (RSD) of the mice group was much greater than that of the other groups, and the small lethal volume of each mouse (0.4–0.5 ml) probably increased the experimental error. Thus, rats were ultimately chosen as the experimental animals for this bioassay.

Three strains of male rats, Wistar, SD, and F344, were selected to study the impact of toxicity on different animal strains. As shown in Table 1, the sensitivity order of the three strains was as follows: F344 > SD > Wistar. There were no significant differences ($P > 0.05$) in MLD between the groups, and the RSDs were similar. These results indicated that rat strain had little effect on the MLD of aconitine. Taking economic costs into account, the SD rats were specified as the experimental animals. Then the MLD in female SD rats was determined using the same aconitine solution. Although there was no significant difference in MLD between the male and female groups, the RSD of the male group was smaller than that of the female group.

Table 1
The MLD of aconitine in the animals with different strain and sex.

Strain	Sex	MLD (mg/kg)	RSD (%)
Wistar	Male	0.1074 ± 0.0068	6.32
F344	Male	0.0980 ± 0.0044	4.53
SD	Male	0.1036 ± 0.0045	4.38
	Female	0.1066 ± 0.0077	7.20

The weight of each animal was 180 ± 10 g. The aconitine concentration was 0.01 mg/ml. The minimum lethal doses (MLD) were expressed as mean ± S.D. (n = 6).

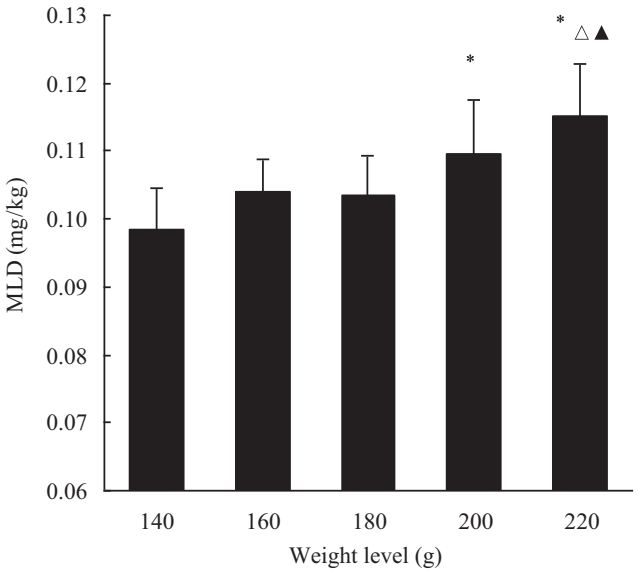

Fig. 2. The MLD of aconitine in rats with different weight levels. Comparing with its weight level, the weight difference of the rat was less than 2 g for each group. The aconitine concentration was 0.01 mg/ml. The minimum lethal doses (MLD) were expressed as mean ± S.D. (n = 6). *P < 0.05 vs. 140 g group; △P < 0.05 vs. 160 g group; ▲P < 0.05 vs. 180 g group.

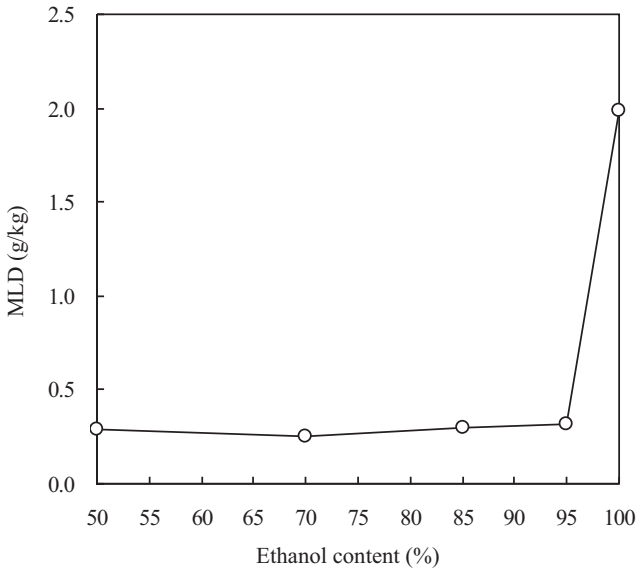

Fig. 3. The MLD of Crude Fuzi 1 extracted by the aqueous ethanol with different ethanol content. The minimum lethal doses (MLD) were expressed as mean ± S.D. (n = 6).

This finding implied that males were more uniform in response than females. Therefore, the male SD rats were employed exclusively.

The MLD of aconitine in rats with different weight were determined to evaluate the effect of animal weight. The results were presented in Fig. 2. The MLD increased with an increase in weight level, suggesting that the sensitivity of SD rats decreased with an increase in animal weight. There were no significant differences between groups with weight level differences of 20 g. When the difference in weight level was 40 g, only the MLD between the 180 g and 220 g groups were significantly different (P < 0.05). When the difference was 60 g or 80 g, all the MLD were significantly different (P < 0.05). These results indicated if the animal weight was varied too much, it would produce significant effects on the MLD. Based on the above analysis, we recommended that the rats weighed between 160 g and 200 g and the weight difference in the same group should be less than 40 g. That is, the rats of 180 ± 20 g were used in this bioassay.

3.2. Optimization of standard

The MLD of aconitine, mesaconitine, and hypaconitine were 0.1121 ± 0.0068, 0.1580 ± 0.0106, and 0.2919 ± 0.0140 mg/kg (n = 6), respectively. The toxicity order was as follows: aconitine > mesaconitine > hypaconitine. We selected aconitine as the standard for the bioassay and defined 1 mg of aconitine as 1000 units (u).

According to our experiment, the volume of physiological saline tolerated by a 180 g rat in a single intravenous injection was about 5–8 ml. The infusion volume should not exceed this tolerance volume, and in order to reduce errors, it should also not be too small. As presented in Table 2, three concentration levels were designed based on this tolerance volume to investigate the effect of aconitine concentration. When the concentration was reduced, the MLD and its RSD decreased too. Compared with the 0.0100 mg/ml group, the MLD of the 0.0075 mg/ml group and 0.0050 mg/ml group were significantly different (P < 0.05). It indicated that the MLD could be significantly reduced and the precision of the results could be improved by optimizing the standard concentration. Considering the tolerance volume, the concentration of standard solution was restricted to 0.0050 mg/ml (5 u/ml).

3.3. Effect of infusion speed

The infusion speed was altered to assess its effects on the MLD using the aconitine solution (0.0050 mg/ml). At lower injection speeds, the MLD and its RSD were lower (Table 2). Compared with the 1.0 ml/min group, the MLD of the 0.2 ml/min group and the 0.5 ml/min group were significantly different (P < 0.05), but the latter two groups were not significantly different. The injection speed was set to 0.5 ml/min for the bioassay, and at this speed, the injection time was about 6–8 min.

3.4. Optimization of test sample's extraction method

The results demonstrated that the MLD of Crude Fuzi 1 varied significantly due to the different extraction methods. The MLD obtained from the ultrasonic method (0.2950 ± 0.0169 g/kg, n = 6) and the reflux method (0.3212 ± 0.0176 g/kg, n = 6) were far less than the MLD from the soak method (0.4180 ± 0.0171 g/kg, n = 6), indicating that ultrasonic and heating might be beneficial to the dissolution of toxic ingredients. Also, heating could cause hydrolysis of DDAs to reduce the toxicity of test sample, which probably made the MLD of the reflux method group greater than that of the ultrasonic method group. Therefore, the ultrasonic method was more appropriate for the extraction of toxic ingredients.

The influence of the extraction solvent was assessed by varying the ethanol content (100, 95, 85, 70, and 50%). Fig. 3 illustrates that ethanol content made a large impact on extraction efficiency. When absolute ethanol was used, the MLD was the biggest. The MLD began to decrease with a reduction of the ethanol content and reached the minimum at 70%. With the ethanol content less than 70%, the MLD began to increase again, possibly caused by the hydrolysis reaction of DDAs due to the increase of water in the solvent. The MLD was significantly different between each group (P < 0.05). Thus, 70% aqueous ethanol was selected as the extraction solvent.

The effect of solvent-to-solid ratio, repeat times, and extraction time on the extraction efficiency was investigated with three different settings for each factor. The results were shown in Fig. 4.

Table 2
The MLD of the standard solution with different concentration and infusion speed.

Concentration (mg/ml)	Infusion speed (ml/min)	MLD (mg/kg)	RSD (%)
0.0100	0.5	0.1098 ± 0.0056	5.15
0.0075	0.5	0.0990 ± 0.0061*	6.15
0.0050	0.5	0.0942 ± 0.0038*	4.06
	0.2	0.0919 ± 0.0036	3.90
	1.0	0.1072 ± 0.0067△,▲	6.26

The minimum lethal doses (MLD) were expressed as mean ± S.D. (n = 6).
* P < 0.05 vs. 0.0100 mg/ml group.
△ P < 0.05 vs. 0.5 ml/min (0.0500 mg/ml) group.
▲ P < 0.05 vs. 0.2 ml/min group.

The MLD decreased with an increase in solvent-to-solid ratio from 5:1 to 15:1. There were significant differences (P < 0.05) between 5:1 group and 10:1 group or 15:1 group, but the latter two groups were not significantly different. No significant changes in the MLD were observed at the three different repeat times settings. The MLD decreased with an increase in extraction time from 15 min to 60 min. Compared with the 15 min group, the MLD of the 30 min group and 60 min group were significantly different (P < 0.05), but there was no significant difference between the latter two groups. Based on the above analysis, the extraction conditions were defined as follows: solvent-to-solid ratio of 10:1, extraction time of 30 min, and repeat of once.

3.5. Effect of ethanol in infusion solution

The MLD of three aconitine solutions with the same aconitine concentration (0.005 mg/ml) but different ethanol contents (0.5, 1, and 3%) were determined respectively to estimate the influence of ethanol content. The MLD were 0.1058 ± 0.0055, 0.1109 ± 0.0088, and 0.0997 ± 0.0048 mg/kg (n = 6) corresponding to the ethanol content from small to large, and there were no significant differences between the groups. These results demonstrated that an ethanol content of the infusion solution less than 3% had no significant impact on the MLD. In the extraction method for the test samples, the extraction solution was concentrated to remove ethanol, resulting in a final ethanol content of about 1–1.5%. The ethanol content would be much less after dilution with physiological saline. Moreover, the ethanol content of the standard solution was 0.5%. Therefore, the ethanol in the infusion solution had no significant influence on the MLD in this bioassay.

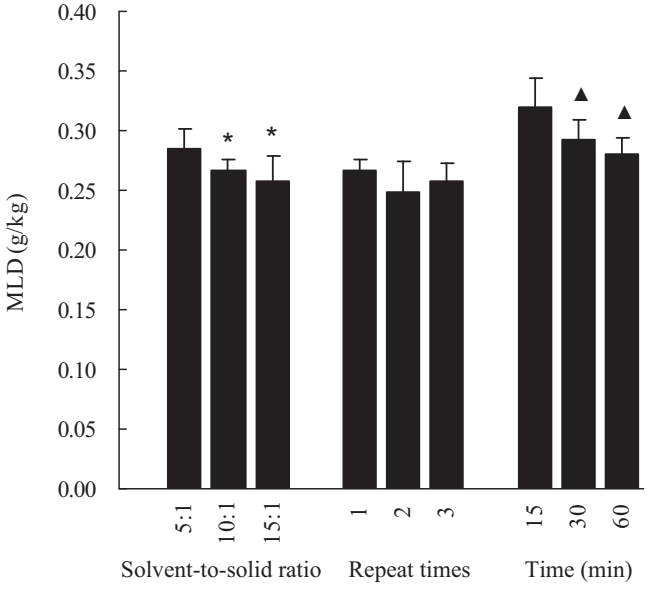

Fig. 4. The effect of solvent-to-solid ratio, repeat times and extraction time on the MLD. The minimum lethal doses (MLD) were expressed as mean ± S.D. (n = 6). *P < 0.05 vs. 5:1 group; ▲P < 0.05 vs. 15 min group.

3.6. Stability of stock solution

The stock solution of aconitine was tested after being stored for 0, 3, 7, and 14 d. As the storage time increased, the MLD (0.1058 ± 0.0055, 0.1105 ± 0.0058, 0.1157 ± 0.0094, and 0.1318 ± 0.0082 mg/kg; n = 6) increased accordingly. The data began to show significant difference compared with the first day at 14 d of storage (P < 0.05). Therefore, the stock solution of aconitine was stable within a week. The stock solution of Crude Fuzi 1 was prepared under the optimized experimental conditions and stored at 4 °C. After the stock solution of Crude Fuzi 1 was stored for 0, 12, and 24 h, the MLD were 0.2505 ± 0.0130, 0.2611 ± 0.0133, and 0.2631 ± 0.0247 g/kg (n = 6), respectively. There were no significant differences between the groups, which indicated that the stock solution of Crude Fuzi 1 was stable within at least 24 h.

3.7. Number of animals and FL%

Bioassays for the same sample were performed under the optimized experimental conditions with 3–36 animals per group. The *FL%* of the bioassays varied between 20.32% and 5.21%. The upper limit of *FL%* for this bioassay was artificially set to 10%. At least six animals per group were required to obtain a toxic potency whose *FL%* was less than 10%.

In this bioassay, the occurrence of false positives and false negatives is mostly related to the tolerance degree of animals to the toxins. Strong tolerance may lead to false negatives and the opposite may lead to false positives. If the *FL%* was more than 10% in an assay, we would increase the number of animals until the *FL%* was less than 10%. This statistical method could availably reduce the effects of false positives and false negatives on the results. However, such cases (n > 6) rarely occurred in our measurements, which might be due to the investigation on the species, strain, sex, and weight of animals when the bioassay was established. It suggested that the toxicity sensitivity of the selected animals to *Aconitum* herbs was consistent.

3.8. The standardized bioassay

Preparation of standard solution. Aconitine is dissolved in absolute ethanol to produce a 1000 u/ml standard stock solution. The stock solution is diluted to 5 u/ml with physiological saline as the standard solution on the day of the assay. The standard solution must be filtered through a 0.22 μm microporous membrane before infusion. The stock solution is stored at 4 °C and may be used within one week if it remains clear.

Preparation of test solution. The test sample powder (through a 100 mesh sieve) is soaked for 30 min with 10 times amount of 70%

aqueous ethanol (v/w), and then soaked continuously under ultrasonic irradiation for 30 min. The extraction solution is concentrated to one-tenth of its original volume in vacuo in a rotary evaporator at 40 °C as the stock solution. The stock solution is diluted to the appropriate concentration with physiological saline as the test solution, which must be filtered through a 0.22 μm microporous membrane before infusion. The concentration of the test solution (u/ml), calculated on the basis of assumed toxic potency of sample (u/g), is adjusted to make the lethal dose (ml/kg) approximately the same as that of the standard solution. The stock solution of the test sample is stored at 4 °C and may be stable within 24 h.

Assay. Male SD rats of 180 ± 20 g are fasting for 24 h with free access to water before the assay. On the day of the assay, the rats are randomly distributed at random into two equal groups with at least six animals in each group. One is a standard group, and the other is a test group. Each rat is fastened and a fine needle connected with a microburette is inserted into its tail vein. The standard solution or test solution is infused continuously at the speed of 0.5 ml/min, until the rat is dead. The rats may have tremor, emesis, or evacuation of stool before dying, but only the dilation of pupil and cessation of breath are considered as the critical point of death. The body weight (kg), concentration of the infusion solution (u/ml), and infusion volume (ml) are recorded, and the MLD is calculated as u per kg of body weight using the following formula:

$$\text{MLD} = \frac{\text{concentration of infusion solution} \times \text{infusion volume}}{\text{body weight}}$$

The TPT is calculated by comparing the MLD of test sample with that of the standard, according to the statistical method for direct assays which are described detailedly in the Ch.P. 2010 [30]. The *FL%* should not be greater than 10%.

3.9. Repeatability of the standardized bioassay

Crude Fuzi 1 was repeatedly analyzed for six times using the standardized bioassay. The main toxic potency was 472.01 ± 23.47 u/g and the RSD was 4.97% (n = 6). The results revealed that the bioassay had good repeatability.

3.10. Chemical contents of the three DDAs in Aconitum herbs

The linear regression equations and correlation coefficients (R^2) were $y = 57.19x + 63.840$ ($R^2 = 0.9996$, $n = 8$) for aconitine, $y = 68.19x + 26.846$ ($R^2 = 0.9997$, $n = 8$) for mesaconitine, and $y = 56.21x + 45.405$ ($R^2 = 0.9996$, $n = 8$) for hypaconitine. The linear range was 0.5–100 ng for all three equations. These equations were employed to calculate the amount of the three alkaloids in sample extracts. The repeatability was assessed by six replicated analyses of Crude Fuzi 1, and the RSD on the amount of each analyte were all less than 3%. Typical UPLC chromatogram of the three alkaloids in *Aconitum* herbs was shown in Fig. 5. The content of the three alkaloids in samples were shown in Table 3.

3.11. Toxic potency of Aconitum herbs

The toxic potency of mesaconitine and hypaconitine, assessed by the bioassay with aconitine as standard, were 709.90 u/mg and 383.86 u/mg, respectively. The total toxicity of aconitine, mesaconitine, and hypaconitine in each sample was defined as the sum of the toxic potencies of the three alkaloids (TPA). TPA = 1000 × aconitine content + 709.90 × mesaconitine content + 383.86 × hypaconitine content. In the Ch.P. 2010, the total content of the three DDAs in Fuzi (processed products) is restricted to less than 0.020%. However, this official limit varies between 76.77 u/g and 200 u/g according to the bioassay, which is actually a wide range not a point. The minimum of the range is calculated as hypaconitine whose toxicity is the lowest in the three DDAs, while the maximum is calculated as aconitine whose toxicity is the highest. Obviously, it is unreliable and unfavorable for the quality control of such medicines which have a narrow margin of safety between therapeutic and toxic doses. For the clinical safety, we propose 76 u/g as the toxicity limit for Fuzi.

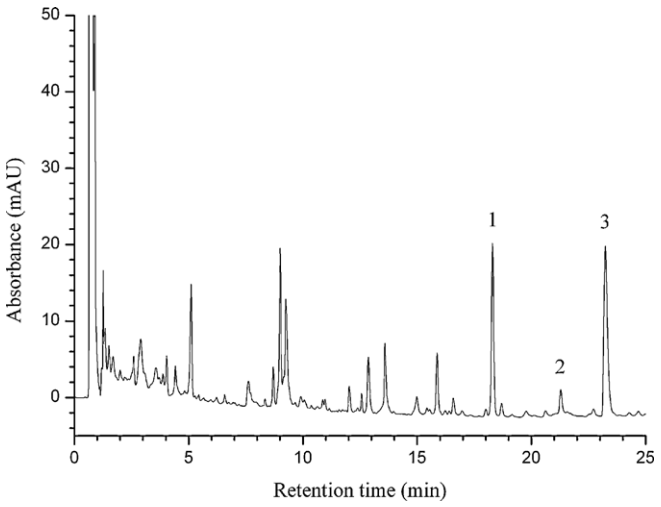

Fig. 5. Typical UPLC chromatogram of the three alkaloids in a *Aconitum* herb (Crude Fuzi 1): mesaconitine (1), aconitine (2), and hypaconitine (3). For conditions see Section 2.5.

As listed in Table 3, the TPT of Crude Fuzi 1–3 varied with different harvest time. For every type of *Aconitum* herbs, the TPT of the processed products were less than that of their crude drug, and especially, the TPT of Crude Fuzi 3 was about 10–50 times higher than that of Baifupian 1–7. These data indicated that the toxicity of processed products decreased greatly after processing. Decreasing toxicity by processing is a common way to ensure the safety of *Aconitum* herbs in TCM clinical application. The mechanism of such way is as follows: after processing by methods such as soaking and steaming, the DDAs in *Aconitum* herbs are hydrolyzed to monoester alkaloids or unesterified alkaloids, resulting in a reduction of the toxicity [1,6]. However, the TPT of Baifupian 1–7, processed by the same method from Crude Fuzi 3, presented great difference. The highest TPT was 5 times greater than the lowest TPT, suggesting that the processing method was very unsteady. This unsteady processing method might lead to risks for the clinical use of *Aconitum* herbs.

The differences between the TPA and TPT for each type of the crude drugs were small, and their TPT/TPA was about 1–2. These data indicated that the three DDAs were the major toxic ingredients in the crude drugs. After processing, the TPA declined similarly to the TPT, but the amplitude of the decrease was much greater than that of the TPT. The TPT/TPA was about 2–10 for the processed products. This finding implied that the three DDAs were not the major toxic ingredients in the processed products, unlike in the crude drugs. In addition, the TPA of Baifupian 2 was found to be smaller than that of Baifupian 1 and Baifupian 3, but its TPT was greater than the latter two samples. This phenomenon was not unique and also occurred between other processed products. According to the limit published in the Ch.P. 2010, the toxic alkaloid content of Yanfuzi calculated as toxic potency in this study might be unacceptable but was qualified by UPLC analysis. Incorrect toxicity evaluations might be one of the reasons that aconite poisoning frequently occurs in clinic. Based on the mechanism of decreasing toxicity by processing, we speculated that the amount of the three DDAs in the crude drugs was reduced by processing due to hydrolysis reaction, but other toxic ingredients did not

Table 3
The results of sample analysis.

Samples	UPLC				Bioassay		TPT/TPA
	Aconitine (mg/g)	Mesaconitine (mg/g)	Hypaconitine (mg/g)	TPA (u/g)	TPT (u/g)	*FL*% (%)	
Crude Fuzi 1	0.0264	0.1840	0.3027	273.25	466.21	6.33	1.71
Crude Fuzi 2	0.0549	0.2116	0.2414	297.83	518.30	9.32	1.74
Crude Fuzi 3	0.0511	0.2448	0.3199	347.70	541.82	6.00	1.56
Baifupian 1	0.0006	0.0196	0.0202	22.23	30.49	8.68	1.37
Baifupian 2	0.0007	0.0070	0.0264	15.75	46.50	7.82	2.95
Baifupian 3	0.0021	0.0067	0.0241	16.13	26.74	5.91	1.66
Baifupian 4	–	0.0017	0.0004	1.38	10.69	7.22	7.75
Baifupian 5	0.0026	0.0208	0.0093	20.97	50.66	7.80	2.42
Baifupian 6	0.0111	0.0605	0.0187	61.26	56.10	7.06	0.92
Baifupian 7	–	0.0001	0.0112	4.38	23.80	9.81	5.43
Yanfuzi	0.0082	0.0259	0.0390	41.56	106.15	9.58	2.55
Heishunpian	0.0001	0.0017	0.0050	3.29	33.33	6.91	10.14
Shufupian	0.0011	0.0009	0.0072	4.54	16.01	8.12	3.53
Huangfupian	0.0274	0.0938	0.0823	125.52	149.48	7.49	1.19
Crude Caowu	0.0161	0.0749	0.0691	95.76	164.20	6.78	1.71
Zhicaowu	0.0458	0.0004	0.0054	48.24	108.52	8.26	2.25
Crude Chuanwu	0.0588	0.2193	0.2117	295.70	356.82	8.69	1.21
Zhichuanwu	0.0006	0.0055	0.0333	17.24	55.46	7.44	3.22

TPT, the toxic potency of test sample; *FL*%, the percentage of fiducial limits; TPA, the sum of toxic potency of the three alkaloids (aconitine, mesaconitine, and hypaconitine). TPA = 1000 × aconitine content + 709.90 × mesaconitine content + 383.86 × hypaconitine content. TPT/TPA is a ratio.

have such reaction and their toxicity did not change a lot, resulting in a decrease in the proportion of TPA in TPT. Once again it proved that the three DDAs were not the major toxic ingredients in the processed products, in contrast to the previous reports on *Aconitum* herbs [1,6,22]. Consequently, the results obtained from the sample analysis confirmed that the content of the three DDAs could not characterize the total toxicity of *Aconitum* herbs, particularly the processed products. The total toxicity of the processed products was generally much greater than the toxicity of the three DDAs.

4. Conclusions

For the first time, we established a bioassay for evaluating the toxicity of *Aconitum* herbs. We found that for all *Aconitum* herbs the total toxicity was greater than the toxicity of the three DDAs. Some of the processed products contained a smaller amount of the three DDAs but exhibited greater total toxicity. In other words, the content of three major toxic alkaloids could not represent the total toxicity of *Aconitum* herbs, and the bioassay could characterize their total toxicity by determining their toxic potencies. Obviously, this bioassay as a new method offered unique advantages over the current commonly used method for the safety assessment of *Aconitum* herbs. An incorrect toxicity evaluation caused by quantitative analysis of the three DDAs might be effectively avoided by this bioassay.

Traditional herbal medicines are a complex system with multi-component and multi-target characteristics. Synergistic or antagonistic interactions may exist between the different components [31,32]. The simple analysis is not often sufficient for characterizing the overall efficacy or toxicity, which was also proved through the present study. In addition, the toxic potency obtained from the correlated animal experiments make the toxicity evaluation of *Aconitum* herbs more intuitive and reliable.

In this bioassay, the total toxicity was expressed as toxic potency, namely aconitine equivalent. This metric is convenient for the uniform toxicity reporting of *Aconitum* herbs in manufacturing practice and the toxicity comparison between different medicines. Based on the toxic potency, doctors can adjust the dose to ensure the safe medication in clinic. Besides, more steady and reliable processing method could be established and applied to control the quality of the processed products with the toxic potency as the evaluation target.

A bioassay for the safety assessment of *Aconitum* herbs is crucial to provide a proper guidance for the clinic use of these toxic herbal medicines. However, the laboratory animals' sacrifice is still required by this method. Although the direct bioassay generally needs fewer animals than the indirect bioassay which determines the median lethal dose, continuous efforts aiming at reducing or replacing the use of animals are still needed. Finally, this bioassay was found to be reliable for the safety assessment of *Aconitum* herbs, and its application might significantly reduce the occurrence of aconite poisoning in clinic. Moreover, the establishment of this bioassay could provide a paradigm for the quality control of other toxic TCM.

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