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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