

Analgesic Activity, Toxicity and Alkaloid Composition Variations of Fermented *Aconitum Carmichaeli* Debx

Yi Le, Zaichang Yang*, Chong Qiao

School of Pharmaceutical Sciences, Guizhou University, Guiyang 550025, China
 349062458@qq.com

Aqueous extract of *Aconitum carmichaeli* Debx is fermented by *saccharomyces cerevisiae* where the transformations in alkaloids are analyzed with HPLC. In the yeast-fermented *Aconitum* chloroform extract, the content of hyaconitine is lessened from 63.6% to 14.8%; the content of songorine increases from 7.5% to 34.5% in relation to original *Aconitum*. In tail clip test on mice, both original (ED₅₀, 0.25 ± 0.04 mg/kg) and yeast-fermented *Aconitum* (ED₅₀, 0.44 ± 0.12 mg/kg) showed a potent analgesic action. However, the fermentation process may certainly relieve the toxicity of *Aconitum*. The LD₅₀ values of original and yeast-fermented *Aconitum* are about 1.44 mg/kg and 13.80 mg/kg, respectively, but the latter has a greater therapeutic scope than the former. This discovery may provide a way to develop new analgesics.

1. Introduction

Opioids and Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) are the main pharmaceuticals for pain management, however both of these classes suffer from setbacks in clinical use. Some NSAIDs have a certain efficacy against gastric damage as well as kidney and liver toxicity, while the opioids can be incident to produce addiction, tolerance and dependence on human body accompanied by symptoms such as constipation, nausea, respiratory depression and sedation. For some indications such as neuropathic pain, these classical medications appear to be inefficacy in a substantial number of patients. Now we desperate for a class of novel and safe analgesics with high efficacy against algogenesis (Buschmann et al., 2004)

Aconitum carmichaeli Debx, also known as Wu-tou in Chinese, is a flowering plant species of the genus *Aconitum* in the family Ranunculaceae. The root of Wu-tou has long been used in the traditional Chinese medicine for anti-inflammation and pain relief (Rathbone and Bruce, 2002). The alkaloids of *Aconitum*, which include aconitine, mesaconitine, hyaconitine and so on, are believed to possess analgesic activity, however, these C19 diterpenoid alkaloids are highly toxic. Patients who take *Aconitum* may die from ventricular arrhythmias, most likely incident within the first 24 h. The potential toxicity of *Aconitum* limits its clinical use (Chan et al., 1994).

Isolated early, many of the pure compounds with biological activity are alkaloids. As many valuable drugs are derived from such natural compounds, it is a hotspot that new or improved drugs may be synthesized by the compounds or intermediates transformed from alkaloids. Studies have been made on the structure modification of microorganisms with enormously diverse alkaloids. Fungi, bacteria, mammal cell, and yeast are widely used in biotransformation. An attempt is to ferment the *Aconitum* root using yeast for several reasons: first, yeast has best used as a catalyst for biotransformation processes; secondly, yeast has a long history of application in food and beverage production with a proven safety record for human consumption. Thirdly, yeast is inexpensive and easy to extract (Rathbone and Bruce, 2002; Nikolova and Ward, 1992; Venisetty and Ciddi, 2003; Walker, 1998). It is therefore an optimal microorganism to be used for biotransformation of *Aconitum*.

In the current study, we are interested in what happened in *Aconitum* during the fermentation process with yeast. *Saccharomyces cerevisiae* is used to ferment the *Aconitum* root in laboratory. The contents of *Aconitum* before and after fermentation are analyzed with HPLC. The analgesic activity and the acute toxicity of *Aconitum* before and after fermentation are also evaluated in the animal test models.

2. Materials and methodologies

2.1 Plant material

The fresh roots of *Aconitum carmichaeli* Debx are collected in Guanling County, Guizhou province, China, and identified by Prof. Deyuan Chen, Department of Herbal Sciences, Guiyang College of Traditional Chinese Medicine, Guiyang, China. It is chopped into small pieces and air-dried in a darkened, ventilated cabinet until the moisture content is less than 5%. A specimen is stored at the laboratory in Guizhou University.

2.2 Yeast culture

The *Saccharomyces cerevisiae* strain (CICC, 1942) used in this study is purchased from CICC (Chinese Industry Culture Collection, Beijing, China). Cells are cultured aerobically in YEP (yeast extract peptone) medium (50 ml, pH 5.0) containing (g/L): 20 g peptone, 10 g yeast extract, and 20 g of glucose at 28°C for 48h.

2.3 Fermentation

The dry pieces of Aconitum root (200g) is marinated in 500 ml distilled water for 30 min, then boiled it for 1 h to obtain 200 ml liquid extracts (pH 7.4) which is poured into two flasks (each 100 ml), where 2 g glucose is added to the liquid extract, respectively. After pressure sterilization (0.15 Mpa for 20 minutes), one flask is inoculated with 5 ml yeast culture (1.2×10^8 cfu/mL), and another is used as a negative control before it is fermented by yeast. Two flasks are placed on a rotary platform incubator shaker at 200 rpm at 28°C for 4 days. During the fermentation, specimen (1 mL) is taken out of the flask for alkaloid and cfu detection of yeast within 4 days after inoculation with yeast.

2.4 Preparation of chloroform extract

The above yeast-fermented and unfermented (control) liquors are separated by filter paper to collect the liquid fraction, extracted with chloroform (100 mL) in tap funnel for three times. The extracts are evaporated to dryness under vacuum and dried in vacuum desiccators (160 mg for yeast-fermented specimen, 172 mg for control specimen).

2.5 HPLC analysis

Standard specimens of mesaconitine, hyaconitine and songorine (Figure 1) were kindly provided by Dr. Pan Weidong (Key Laboratory of Chemistry for Natural Products of Guizhou Province and Chinese Academy of Sciences, Guiyang, China). Mesaconitine, hyaconitine and songorine are accurately weighed and dissolved in methanol to produce a stock standard solution at a final concentration of 0.115 mg/mL, the test solution specimen at a final concentration of 0.785 mg/mL. A HP1100 HPLC system used in the study consisted of a G1315ADAD detector, a G1311A quaternary pump, a G1322A vacuum degasser and a G1367A well plate autosampler. Zorbax C8 reverse phase column (ID 4.6×250mm, particle size 5µm) is used as the stationary phase. The mobile phase consisted of glacial acetic acid (0.2%, adjusted to pH 5.43 with triethylamine): methanol (55:45) at a flow rate of 0.8 mL/min. Detection is carried out at 231 nm at 25°C with injection volume of 3 µL for all HPLC flows.

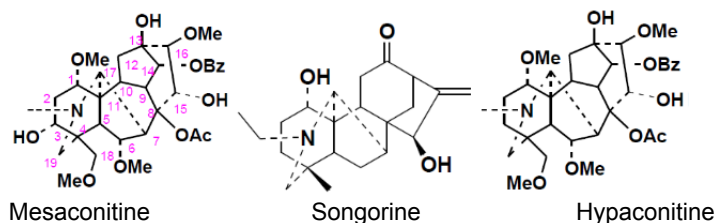


Figure 1: Structures of mesaconitine, songorine and hyaconitine

2.6 Animals for tail clip test and determination of median lethal dose (LD50)

The in vivo experiments are conducted using male Kunming mice (20-22 g) purchased from Animal Center of Guiyang Medical College, Guiyang, China, in an animal room at 20±2°C with a 12h light/dark cycle, where food and tap water are supplied. All experiments are done in compliance with the internationally accepted standards for animals and have been approved by the Guizhou University.

2.7 Tail clip test in mice

This analgesic test bases on a modified method as proposed by Bianchi and Franceschini (Bianchi and Franceschini, 1954). The chloroform extract of Aconitum (CEA) and chloroform extract of yeast-fermented

Aconitum (CEYFA) are dissolved in physiological saline to obtain the stock solution of 1 mg/mL, sterilized by filtration through a 0.45 μ m filter and further diluted in the experiments. Mice (n =10) is injected intraperitoneally with saline (negative control), and graded doses (0.10, 0.17, 0.30, 0.50 or 0.80 mg/kg) of CEA and CEYFA or morphine sulfate (10mg/kg, reference drug). An artery clip is applied to the root of the tail (approximately 1 cm from the body) to induce pain. The animal quickly responds to the noxious stimuli by biting the clip or the tail near the location of the clip. The time between stimulation onset and response is measured by a stopwatch. The presence or absence of analgesia is determined at 15, 30, 45 and 60 min after i.p. administration of the tested extract, respectively, with artery clip applied for 30 sec. The results are expressed as the percentage of mice showing analgesia-insensitivity to the noxious stimulus-after treatment.

2.8 Determination of median lethal dose (LD50)

The preliminary tests indicate that the CEA shows far more toxic than the CEYFA. In formal test the doses and the intervals between the CEA and CEYFA are different. For each extract, mice are divided into 5 groups of 10 each. The CEA solution is intraperitoneally administered at dose of 0.8, 1.2, 1.8, 2.7 and 4.1 mg/kg body weight. The CEYFA solution is also intraperitoneally administered at dose of 4.5, 7.0, 12.0, 20.0 and 34.0 mg/kg body weight. Death is monitored over a period of 24h. LD50 is then calculated with Trimmed Spearman-Kärber Method (Hamilton et al., 1977).

3. Results and discussion

During the periods of fermentation, TLC is used to detect the alkaloids in the liquid extract of Aconitum root for variants. The results show that the content of alkaloids in the liquid extract of Aconitum root has changed significantly on the 4th day after inoculation with yeast. The cfus of yeast in the liquid extract of Aconitum root are 1.8×10^{10} , 1.4×10^{12} , 1.82×10^{11} and 1.2×10^9 cfu/mL on the 1st, 2nd, 3rd and 4th days, respectively, which indicates that the liquid extract of Aconitum root does not inhibit the growth of the yeast.

There are more than 10 kinds of alkaloids in Aconitum root. After the fermentation by *Saccharomyces cerevisiae*, the contents of three kinds of alkaloids, i.e. mesaconitine, hyaconitine and songorine, change in the cultured liquids of Aconitum. HPLC is sufficient for good separation of the studied alkaloids. Under the given HPLC conditions, mesaconitine (Rt 10.565), hyaconitine (Rt 12.006) and songorine (Rt 4.359) show a significant single peak (Figure 2, 3, 4). After fermentation, the songorine increases while the mesaconitine and the hyaconitine all decrease in the cultured liquids of Aconitum, in relation to the aqueous extract of Aconitum (Figure 5, 6).

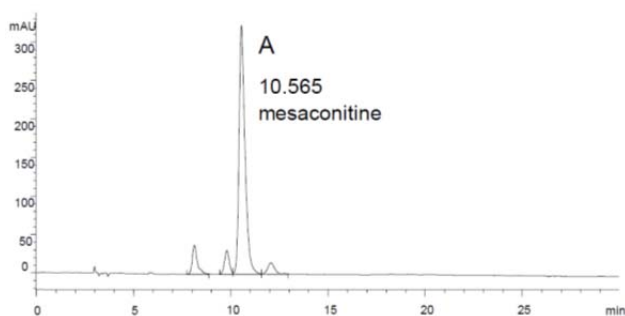


Figure 2: HPLC chromatogram of mesaconitine

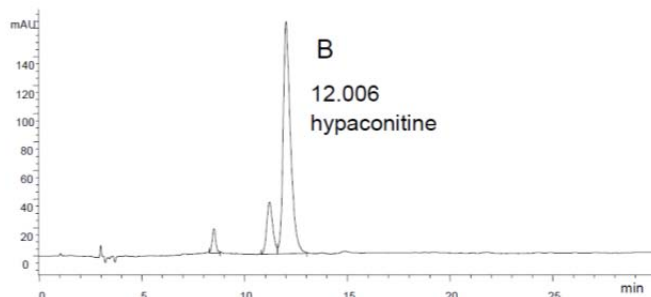


Figure 3: The HPLC chromatogram of hyaconitine

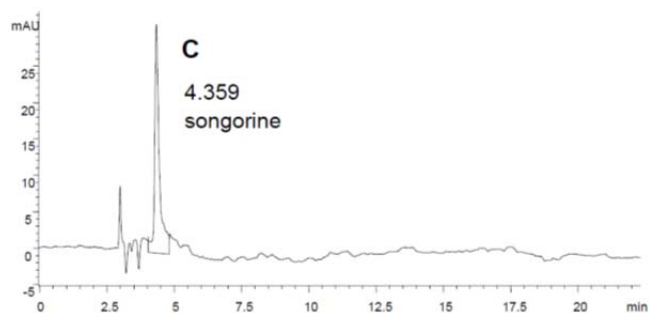


Figure 4: HPLC chromatogram of songorine

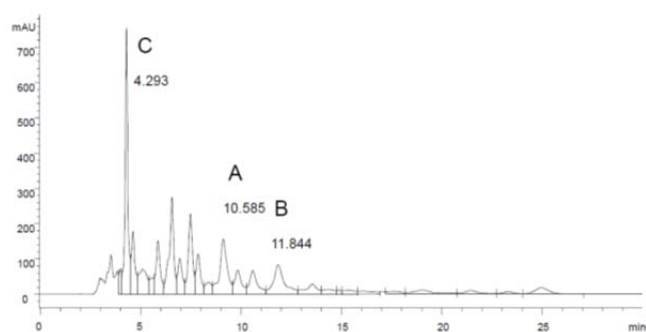


Figure 5: HPLC chromatogram of the chloroform extract of yeast-fermented *Aconitum* (CEYFA)

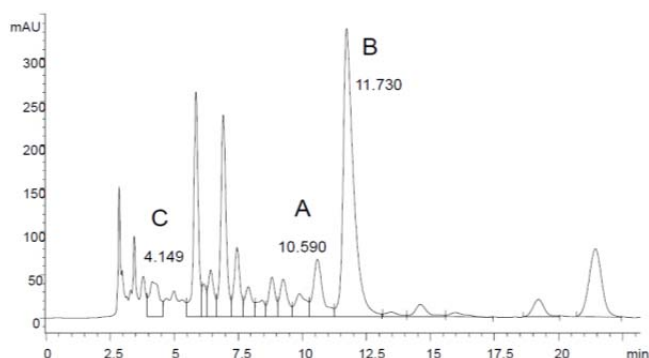


Figure 6: HPLC chromatogram of the chloroform extract of *Aconitum* (CEA)

Table 1: Peak height (mAU) and area (%) of M (mesaconitine), H (hypoconitine) and S (songorine) in the chloroform extract of *Aconitum* and yeast-fermented *Aconitum*

Sample	Height (mAU)			Area (%)		
	M	H	S	M	H	S
Yeast-fermented <i>Aconitum</i>	66	82	753	8.9	14.8	34.5
<i>Aconitum</i>	66	332	40	11.7	63.6	7.5

The above results demonstrate that the yeast produces a less effects on the biotransformation of mesaconitine. There is a hydroxide radical linking to C3 of mesaconitine as the only difference between mesaconitine and hypoconitine structures (Figure 1). It is speculated that the hydroxide radical may be a sterichindrance for yeast enzymes to convert mesaconitine into other molecules. The conversion of hypoconitine into songorine easily takes place in this experiment involving a wide variety of reactions. Dehydrogenases are widely existed in yeast (Pereira, 1998). Some chemical functional groups of hypoconitine, for example, the hydroxide radical that binds C14, should at least be oxidized by yeast dehydrogenases. However, the exact metabolic pathways need to be further studied in the future works.

Tail clip test, as a simple means of testing analgesic drugs, is based on a reflex mechanism which involves the higher center (Bianchi and Franceschini, 1954). The earlier studies reveal that the tail clip test is applicable to detect the analgesic activity of Aconitum (Hikino et al., 1979). The current results (Table 2) show that the two kinds of chloroform extracts of Aconitum exhibit dose-dependent analgesic effects. The ED₅₀ of the CEYFA is 0.44 ± 0.12 mg/kg and the CEA 0.25 ± 0.04 mg/kg. As judged from the ED₅₀ values, the CEA is about 2 times than the CEYFA in suppressing clip-induced stimuli. Hypaconitine and mesaconitine are known as the active compounds of Aconitum for pain management (Ameri, 1998). Interestingly the hypaconitine in CEA is about 6 times than that in CEYFA and the analgesic effect of CEA should be much more potent than CEYFA, but there is no such a big difference between CEA and CEYFA in analgesic activity. This can be explained by the fact that songorine increases to about 5 times in CEYFA than in CEA. The results suggest that songorine may be also a compound with analgesic activity. This assumption deserves to be further verified in the future works.

In order to investigate whether fermentation process can reduce the toxicity of Aconitum, mice are used to determine the median lethal dose (LD₅₀) of the chloroform extract of Aconitum (CEA) and the chloroform extract of yeast-fermented Aconitum (CEYFA). The results show that the fermentation may greatly relieve the toxicity of Aconitum (Table 3) with LD₅₀ value of CEA about 1.44 ± 0.07 mg/kg. This result coincides with that in earlier study (Hikino et al., 1979) at the hypaconitine LD₅₀(mice, s.c.) of 1.19 mg/kg. The CEA contains many compounds but the percentage of hypaconitine in CEA is only 63.6%. The somewhat lower acute toxicity in this experiment may be caused due to the method of drug application and the impurity of CEA.

The LD₅₀ of CEYFA is 13.80 ± 1.92 mg/kg, which indicates obviously that the toxicity of CEYFA is lower than that of CEA. Two factors might lead to the reduction of toxicity in CEYFA. First, the percentage of hypaconitine in the CEYFA is reduced from 63.6% to 14.8% in relation to CEA. It is known that activation of Na⁺ channels by aconitine and hypaconitine is responsible for their cardiotoxic. The reduction of hypaconitine in CEYFA should cause the mitigation of toxicity of CEYFA theoretically. Secondly, songorine increases relatively in CEYFA. Songorine was reported once to have antiarrhythmic actions (Ameri, 1998), therefore, it can resist the cardiotoxicity produced by aconitine, hypaconitine and so on. It is certain that the toxicity of Aconitum can be reduced to a relatively low level after it is fermented by yeast.

In summary, the apparent changes take place in the contents of alkaloids of Aconitum after fermentation by yeast. In the extract of yeast-fermented Aconitum, hypaconitine decreases and songorine increases relatively. The variants cause a slight weakness in analgesic effect of yeast-fermented Aconitum, but these lead to a significant reduction of toxicity of yeast-fermented Aconitum. Yeast-fermented Aconitum has a greater therapeutic efficacy than original Aconitum. This discovery may provide a basis for developing new analgesic agents.

Table 2: Effects of the chloroform extract of Aconitum and yeast-fermented Aconitum on the tail clip test in mice

Group (n=10)	Dose (mg/Kg)	Analgesia (%)			
		15min	30min	45min	60min
	-	0	0	0	0
Saline control	0.10	10/20	10/20	10/20	10/20
	0.17	20/40	20/40	20/40	20/40
	0.30	30/50	30/50	30/50	30/50
Yeast-fermented Aconitum	<i>Aconitum</i> / 0.50	40/90	40/90	40/90	40/90
	0.80	80/100-	80/100	80/100	80/100
Morphine	10	100	100	100	100

*At the dose of 0.80 mg/kg, one mouse in group of Aconitum died within 15 min.

Table 3: Death monitored over a period of 24 h after the extracts of *Aconitum* are intraperitoneally administered to mice

Sample	Number of animal (n)	Dose range tested i.p. (mg/kg)	Highest dose without lethality (mg/kg)	LD50 value (mg/kg)
Yeast-fermented <i>Aconitum</i>	10	2.60-34.0	2.60	13.80±1.92
<i>Aconitum</i>	10	0.54-4.1	0.54	1.44±0.07

*Values represent means ± SEM

Acknowledgement

This work was financially supported by Natural science and technology foundation of Guizhou Province, China ([2010]2225). Project supported by Guizhou Province Natural Science and Technology Fund Project ([2010]2225).

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