

Radix Aconiti kusnezoffii Exhibits an Antinociceptive Activity Involvement at Central and Peripheral Nervous System

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ABSTRACT

The dry root of *Aconiti kusnezoffii*, also named Cao Wu, is widely used as folk medicine in China to treat rheumatic pain, paralysis due to stroke, carbuncle, and furuncle. In the present study we investigated the molecular mechanisms of its analgesic and anti-inflammatory activities. Using the acetic acid-induced writhing and formalin test models in mice, our results clearly showed that the constituents of processed Radix Aconiti Kusnezoffii can reduce both peripherally and centrally induced pain in a dose-dependent manner. Mice treated with processed Radix Aconiti Kusnezoffii has significantly increased levels of glutathione peroxidase, glutathione reductase, and superoxide dismutase activities in the liver compared to untreated controls. Furthermore, treated mice exhibited lower levels of malondialdehyde after carrageenan injection compared to untreated controls. In conclusion, our results demonstrate that the ingredients in the processed Radix Aconiti Kusnezoffii can effectively reduce pain involving in the peripheral and central nervous systems and exhibit anti-inflammatory activity. The pharmacological analgesic and anti-inflammatory properties of Radix Aconiti Kusnezoffii may be attributed to the elevation of hepatic anti-oxidative enzymes to eradicate free radicals.

Key words: Antinociceptive activity, Anti-inflammatory activity, Radix Aconiti Kusnezoffii (Cao Wu).

INTRODUCTION

The Ranunculaceae family is relatively primitive in evolution, and contains a great number of medicinal plants. Members of the genus *Aconitum* are widely distributed across North America and the northern part of Asia. This perennial plant can grow up to 3 ft (1 m) with tuberous roots, palmately lobed leaves, blue or white flowers with large hoodlike upper sepals, and an aggregate of follicles⁽¹⁾. There are 167 species of *Aconitum* found in mainland China, and 44 of them have been used to treat rheumatic pain, paralysis due to stroke, carbuncle, and furuncle⁽²⁾. However, the narrow therapeutic window of *Aconitum* limits its medical applications⁽³⁾. Several diterpenoid alkaloids have been isolated and identified from the roots of *Aconitum*, and the major constituents have been reported to have analgesic, antipyretic, and local anaesthetic properties⁽⁴⁾.

Radix Aconiti Kusnezoffii, named Cao Wu in Chinese, is the dry root of *Aconiti kusnezoffii* Reichb, which belongs to the plant family of Ranunculaceae⁽⁵⁾. The major components of Radix Aconiti Kusnezoffii are alkaloids, such

as aconitine, mesaconitine, jesaconitine, and hyaconitine, as well as non-alkaloid constituents such as sucrose, daucosterin, meso-inositol, and higher fatty acids⁽⁶⁾. The crude Radix Aconiti Kusnezoffii was reported as highly toxic in early Chinese medicinal literatures⁽⁷⁻⁸⁾, and its misuse can be fatal. However, with the traditional preparation method, processed Radix Aconiti Kusnezoffii can be used clinically with the significant reduction in toxicity.

Inflammation is a response to tissue damage that involves enzyme activation, cytokine release, organ swelling, cell migration, and tissue repair⁽⁹⁾. The release of free radicals from damaged tissues has been demonstrated to play a very important role during inflammation, and can be identified within the first six hours after λ -carrageenan administration, along with elevated levels of prostaglandins and nitric-oxide. Several studies also indicated that inflammation can stimulate malondialdehyde (MDA) production in response to cell membrane damage arising from free radicals⁽¹⁰⁾. Therefore, in this study, we analyzed the active components and evaluated the antipyretic activity and anti-inflammatory effects of concocted *Actonitum* using the acetic acid-induced body twist test and formalin-induced pain assay. To further investigate the anti-inflammatory mechanism, we determined

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MDA levels in paw edema, as well as the concentrations of lipid peroxide, superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione reductase (GRx) in the liver. We also evaluated the anti-inflammatory effects of the *Radix Aconiti kusnezoffii* in the carrageenan induced acute and chronic inflammation models in rats.

MATERIALS AND METHODS

I. Collection of Plants

Radix Aconiti Kusnezoffii were collected from a pharmacy in Chengdu, the capital of Sichuan Province in China, and identified by Prof. Y. C. Wu (China Medical University, Taiwan). Voucher specimens of *Radix Aconiti Kusnezoffii* have been deposited at the Herbarium of Graduate Institute of Chinese Pharmaceutical Sciences, College of Pharmacy, China Medical University, Taichung, Taiwan, under the reference numbers of CMC7021 and CMC7022. Aconitine, acetic acid, formalin, λ -carrageenan and indomethacin were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). The SOD, GPx, GR and MDA activity kits were purchased from Randox Laboratory Ltd. Mesaconitine and hypaconitine were purchased from Boppard (H. K. Co., Ltd., Hong Kong). All other reagents were obtained from standard sources.

II. Preparation of the Plant Extract

One kilogram of *Radix Aconiti Kusnezoffii* powdered samples (100 mesh) were immersed in 5000 mL distilled water for 30 min, and then boiled for 1 h. Filtration and collection of the extracts were repeated three times. The combined decoctions were evaporated to dryness. The percentage of the lyophilized extract yield in processed *Radix Aconiti Kusnezoffii* was 18.34% (w/w). The lyophilized extract was dissolved in saline solution prior to its use in pharmacological tests.

III. Animals

Male BALB/c albino mice weighing 18 to 22 g were obtained from the National Laboratory Animal Center (Taipei, Taiwan). The animals were fed standard chow (Purina Mills, Inc.), given water *ad libitum*, and maintained under well-ventilated conditions with a 12h:12h light-dark cycle. All animal procedures were performed according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, as well as the guidelines of the Animal Welfare Act. All tests were conducted under the guidelines of the International Association for the Study of Pain⁽¹¹⁾.

IV. Acute Oral Toxicity Studies

Acute oral toxicity studies were performed according to OECD-423 guidelines (acute toxic class method)⁽¹²⁾. Mice

($n = 10$) of either sex, selected by random sampling, were employed in this study. The animals were fasted for 4 h with free access to water only. Aqueous extracts of the tested *Aconitum* plants and alkaloids were administered orally at the desired doses and survival was observed over 3 days. If mortality was observed in 2/3 or 3/3 animals, the dose administered was considered toxic. However, if mortality was observed in only 1/3 of animals, the same dose was repeated to confirm the toxic effect again.

V. Carrageenan-induced Paw Edema

The carrageenan-induced paw edema test, an acute inflammatory model first proposed by Winter *et al.*⁽¹³⁾, was used with modifications. Animals were fasted for 24 h before the experiment, with free access to water. A 0.05 mL of 1% (w/v) carrageenan solution in normal saline was injected intradermally into the plantar side of the right hindpaw of each mouse. Animals received an oral (p. o.) dose of *Aconitum* extract or an intraperitoneal (i. p.) injection of indomethacin 1 h prior to carrageenan injection. Paw volumes were measured immediately after carrageenan injection using a plethysmometer (Model PP0, Diagnostic & Research Instruments Co., Ltd., Taiwan) at 1 h intervals until 4 h after the injection of the carrageenan.

To induce inflammation, a suspension (50 μ L) of λ -carrageenan was injected subcutaneously into the right hind limbs of one mice. After 2 h of induction, mice in the test group were treated orally with different doses of the crude extract, in a volume of 0.1 mL/10 g body weight. Mice in the control group were given i. p. injections of indomethacin (10 mg/kg) after 1.5 h of induction. After 3 h of induction, both groups were sacrificed using pentobarbital, and the blood, hepatic lobar tissue, and foot tissue were collected. One gram of liver tissue was homogenized with 1 mL normal saline using a homogenizer in an ice bath. The homogenate was transferred to Eppendorf tubes and centrifuged at 12,000 rpm for 10 min at 4°C. The clear upper layer, which was the liver extract, was then aliquotted into new Eppendorf tubes and stored at -80°C for subsequent use.

VI. Antioxidant Enzymes Activity Measurements

(I) The Superoxide Dismutase (SOD) Assay

The liver extract (15 μ L) was placed in an Eppendorf tube and mixed for 15 min with 90 μ L ice-cold double distilled water at 4°C. Then, 20 μ L of the mixture was added to 260 μ L 0.1 M phosphate buffer containing 3-cyclohexylamino-1-propanesulfonic acid (CAPS 40 mM) and EDTA (0.96 mM, pH 7.0). A 5 μ L volume of the mixture was added to 340 μ L of mixed substrate (xanthine, 0.05 mM; I.N.T., 0.025 mM) and xanthine oxidase to determine the colorimetric reaction. Light absorbance was recorded at 37°C and 505 nm on a Hitachi U 2000 Spectrophotometer. One unit of SOD activity was defined as the rate of inhibiting the spontaneous oxidation of I.N.T. within a certain time unit

(U), and the SOD activity in the liver tissue is represented as U/mg protein⁽¹⁴⁾.

(II) *Glutathione Reductase (GRx) Assay*

This assay was based on the method by Guntherber and Rost⁽¹⁵⁾. Liver extract (100 μ L) was added to 100 μ L cold ddH₂O and kept at 2 to 8°C for 10 min, followed by centrifugation at 12,000 rpm for 5 min. The supernatant (25 μ L) was collected and diluted with 475 μ L normal saline. Finally, 50 μ L of the mixture was added to a cuvette, followed by the sequential addition of equal amounts of GRx buffer (250 mM, kupferrphosphate, 0.5 mM EDTA, pH 7.3), substrate oxidized glutathione (GSSG) 2.2 mM and NADPH (0.17 mM). Light absorbance was read at 340 nm, and GRx activity was expressed as U/mg protein.

(III) *Glutathione Peroxidase (GPx) Assay*

This assay was based on the method by Paglia and Valentine⁽¹⁶⁾, who utilized the reaction of glutathione and cumene hydroperoxide with GPx, and the following rapid reduction of GSSG by NADPH and GRx. The coupled reaction was accompanied by the oxidation of NADPH to NADP⁺. GPx activity was defined by the decreased rate in light absorbance. Twenty-five microliter of the extract was diluted with 500 μ L of diluting agent. Five hundred microliter of the clear supernatant was immediately mixed with Reagent 1 (glutathione, 4 mM; GRx, 0.5 U/L; NADPH, 0.28 mM) and Reagent 2 (cumene hydroperoxide, 0.18 mM). Light absorbance was read at 340 nm at 37°C and the rate of absorbance change was calculated. GPx activity was shown in U/mg protein.

(IV) *MDA Assay*

The quantitative assay for the lipid peroxide malondialdehyde (MDA) was performed by the method described by Tatum, Changchit and Chow in 1990⁽¹⁷⁾, based on the fact that MDA and thiobarbituric acid (TBA) can form a red adduct, TBARS, under acidic condition at high temperature. One gram of foot tissue was homogenized with 2 mL normal saline and centrifuged at 12,000 rpm for 5 min at 4°C. The supernatant was combined with 45 μ L BHT reagent and 300 μ L TBA reagent in a mixer and heated in water bath at 90°C for 45 min. After cooling to room temperature for 10 min, 495 μ L of *n*-butanol was added and mixed for 1 min. After centrifugation at 12,000 rpm for 15 min at 4°C, 100 μ L of the supernatant (pink color) was used to measure light absorbance at 532 nm for four repeats on an ELISA reader.

VII. *Detection of Aconitum Alkaloids*

Aconitine, hypaconitine, and mesaconitine were weighed accurately (1 mg) and dissolved in 10 mL absolute methanol to make stock solutions. The dried powder of processed Radix Aconiti Kusnezoffii was weighed

accurately (1 g) and mixed with 30 mL abs. MeOH and then extracted ultrasonically for 35 min. The extraction process was repeated twice, and the extracts were combined and evaporated to dryness under a stream of nitrogen gas. The dried residues were dissolved in 10 mL abs. MeOH and filtered through a membrane filter (0.45 μ m) prior to injection.

Analysis were carried out on an HPLC system consisting of a Waters 2695 separation module and a Waters 2996 photodiode array detector (Waters, Milford, MA, USA) equipped with an XBridge Shield RP-18 column (4.6 ID \times 250 mm, particle size 5 μ m). The eluents were A (aqueous 0.03 M ammonium hydrogen carbonate, adjusted to pH 9.5 with concentrated ammonia) and B (100% acetonitrile). The flow rate was maintained at 0.8 mL/min with an isocratic system consisting of 85% solvent A and 15% solvent B. The detection wavelength was set at 235 nm. The column temperature was 35°C, and the sample injection volume was 10 μ L⁽¹⁸⁾.

VIII. *Antinociceptive Activity*

(I) *Acetic Acid-induced Writhing Test*

The acetic acid-induced writhing test was performed using a modification of the method described by Koster *et al.*⁽¹⁹⁾. Mice were first pretreated with an oral (p. o.) doses of either aqueous extracts or alkaloids, or with the standard antinociceptive compound indomethacin (10 mg/kg i. p.). Test compounds were administered 1 h before the i.p. injection of acetic acid (0.75%, v/v, 0.1 mL/10 g body weight) to induce the typical stretching response. After induction, pairs of mice were placed in separate boxes and the number of writhes or stretches for each animal were counted for a period of 5 min within a 15 min period of double-blind observation. The antinociceptive effect was measured by calculating the mean reduction in the number of abdominal constrictions for mice treated with Radix Aconiti kusnezoffii extracts or indomethacin, as compared with the vehicle-treated group. The evaluation of antinociceptive activity was expressed as inhibition or percent reduction in the number of total abdominal writhes

(II) *Formalin Test*

The method used was similar to that described by Hunskaar and Hole⁽²⁰⁾. To evoke a nociceptive response, mice were injected under the dorsal surface skin of the right hind paw with 20 μ L of dilute formalin (1%) using a 30 gauge needle. Each mouse was placed immediately into a glass cylinder fitted with mirrors to enable a total panoramic view of the animal's behavior. The number of paw shakes and the accumulated time spent in licking the injected paw were taken as indicators of the nociceptive response. Two periods of high licking and shaking activity were considered: the first one was obtained immediately after injection and lasted for 5 min; this is known as early (neurogenic) phase. A second period was observed 20-30

min after formalin injection and dominated the late (inflammatory) phase. Animals were administered with processed *Radix Aconiti Kusnezoffii* extracts (p. o.) or indomethacin (10 mg/kg, i. p.) 1 h before the injection of formalin. The time spent in paw-licking behavior during each phase was used to quantify the pain response.

IX. Statistical Analysis

Statistical differences among groups were determined using two-way repeated-measures ANOVA, followed by

Dunnett range post-hoc comparisons to determine the source of significant differences where appropriate. Values of $p < 0.05$ were considered statistically significant.

RESULTS

I. Quantitative Analysis

HPLC chromatograms of standards and the sample solution are shown in Figure 1. Linear regression analysis

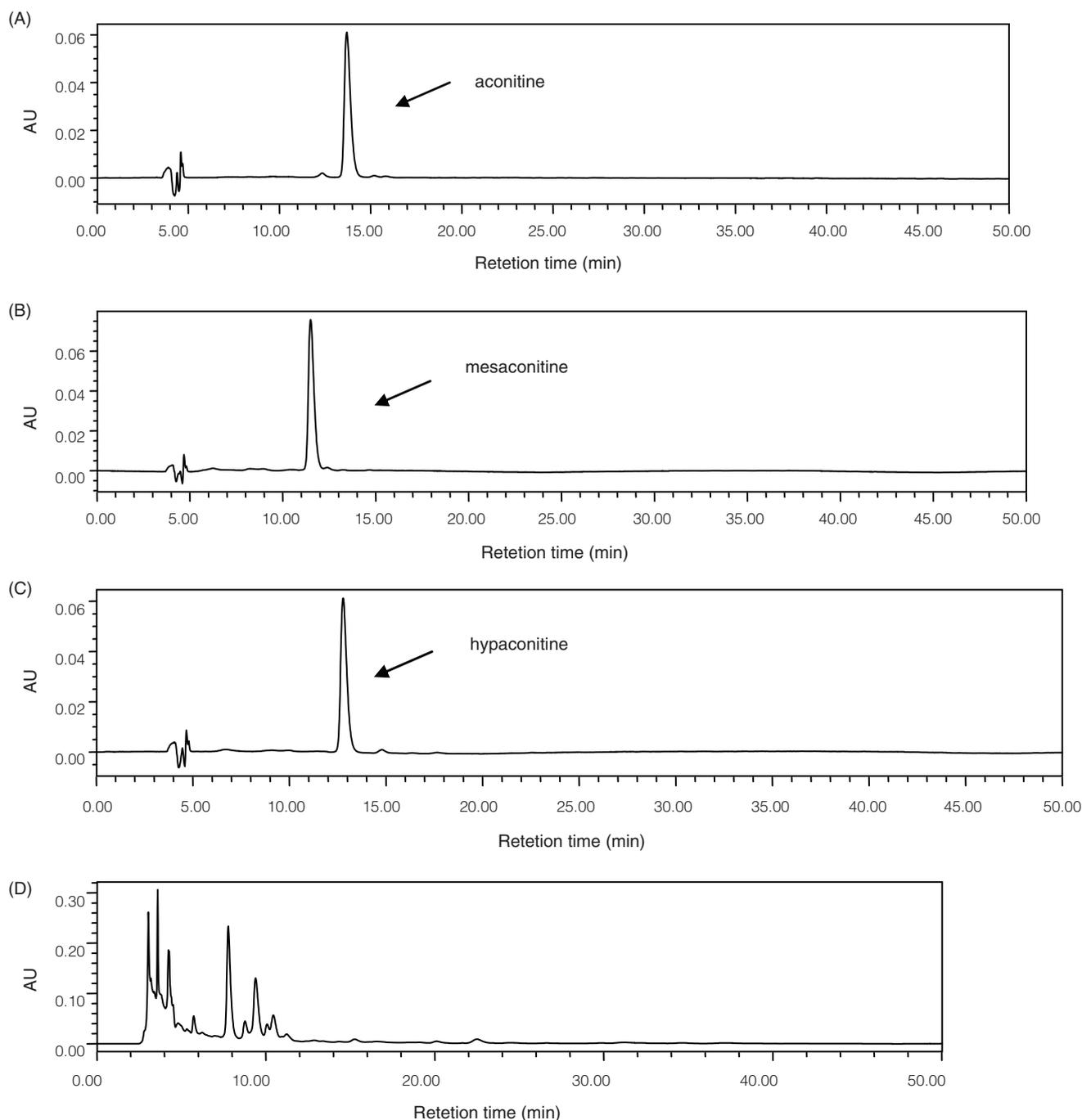


Figure 1. HPLC chromatograms of (A) aconitine, (B) mesaconitine, (C) hyaconitine and (D) *Radix Aconiti Kusnezoffii* extract.

for each alkaloid was performed by the external standard method. The calculated results are given in Table 1, where a and b were the coefficients of the regression equation $y = ax + b$ (x referred to the concentration of the alkaloid ($\mu\text{g/mL}$); y denoted the peak area) and r represented the correlation coefficient of equation. All the alkaloids showed good linearity ($r = 0.999$) in the concentration range. (Table 1) The average recovery for the alkaloids was higher than 98% (Table 2). The precision of the intra-day and inter-day data was indicated by RSD which were less than 3.2% for each alkaloid at three concentrations (Table 3). The order of concentration of three aconitine-type alkaloids in Radix Aconiti Kusnezoffii was mesaconitine ($431.6 \pm 3.2 \mu\text{g/g}$) > aconitine ($105.3 \pm 1.3 \mu\text{g/g}$) > hypaconitine ($67.2 \pm 0.9 \mu\text{g/g}$).

II. Acute Oral Toxicity Test

We found no instance of mortality in mice receiving Radix Aconiti Kusnezoffii, even at the highest oral dose (60 mg/kg) employed, and oral doses of 20, 40, and 60 mg/kg were selected for further pharmacological studies.

Table 1. Linear regression equation of the alkaloids

Compound	Concentration rang ($\mu\text{g/mL}$)	Regression equation	r
Aconitine	0.310 - 40	$y = 10067x - 1084.71$	0.9999
Mesaconitine	0.625 - 40	$y = 15998x - 126.15$	0.9998
Hypaconitine	3.125 - 50	$y = 13642x - 1092.34$	0.9999

Values were obtained from 4 concentrations of each compound.

Table 2. Contents of *Aconitum* alkaloids in Radix Aconiti Kusnezoffii

<i>Aconitum</i> alkaloid	Content ($\mu\text{g/g}$)	Recovery (%)
Aconitine	105.3 ± 1.3	98.47
Mesaconitine	431.6 ± 3.2	98.72
Hypaconitine	67.2 ± 0.9	98.15

Values (mean \pm SEM) were obtained from 4 replicate.

Table 3. Precision of the intra-day and inter-day measurements

Compound	Concentration ($\mu\text{g/mL}$)	RSD (%)	
		Intra-day	Inter-day
Aconitine	20.00	1.13	2.67
	2.50	1.03	1.89
	0.31	3.12	2.51
Mesaconitine	20.00	2.83	2.44
	2.50	1.19	1.59
	0.31	3.03	2.84
Hypaconitine	20.00	1.24	1.92
	2.50	1.39	1.49
	0.31	3.06	2.28

Values were obtained from 4 replicate of each group.

Additionally, mortality was not observed in mice receiving the alkaloid constituents aconitine, mesaconitine, or hypaconitine at an oral dose of 0.5 mg/kg for 3 days.

III. Carrageenan-Induced Paw Edema

Subplantar injection of carrageenan in mice produced a time-dependent increase in paw volume (Figure 2). The increase was observed at 1 h and was maximal 4 h after administration of carrageenan injection in the vehicle-treated groups. Indomethacin markedly reduced the carrageenan-induced paw edema at all assessment time. Radix Aconiti Kusnezoffii also reduced carrageenan-induced paw edema in all phases of the experiments.

IV. Acetic Acid-Induced Writhing Response

The effects of treatments on the writhing response in mice are shown in Table 4. Indomethacin resulted in a marked (83.2%) reduction in the number of writhes. Radix Aconiti Kusnezoffii reduced the number of acetic acid-induced abdominal writhes in a dose-dependent manner ($r = 0.997$). Radix Aconiti Kusnezoffii at the highest dose tested (60 mg/kg) produced a nearly 70% reduction in the induced nociceptive response.

Table 4 also shows that aconitine and mesaconitine (0.5 mg/kg, p.o.) effectively reduced the number of writhes in mice by 75.3 ± 2.5 and $80.4 \pm 3.2\%$, respectively. Hypoconitine (0.5 mg/kg, p.o.) produced only a modest (15.1 \pm 2.1%) reduction in the number of writhes.

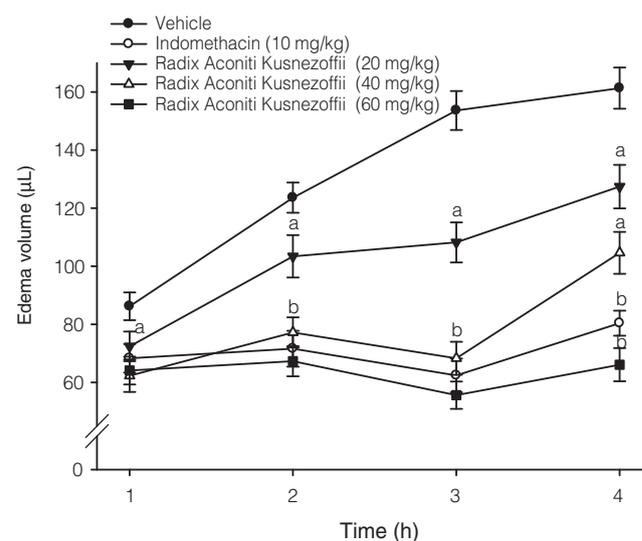


Figure 2. Effects of the oral administration of aqueous extract of Radix Aconiti Kusnezoffii on carrageenin-induced paw edema in mice. Indomethacin (10 mg/kg) was administered intraperitoneally to the separate group of mice. The vehicle (normal saline) used to dissolve the tested medications was given at the same volume. Values (mean \pm SEM) were obtained from each group of 8 animals. ^a $p < 0.05$ and ^b $p < 0.01$ compared to the values of vehicle-treated group at the indicated times, respectively.

V. Formalin-Induced Paw-Licking in Mice

Radix Aconiti Kusnezoffii produced a dose-dependent reduction in paw-licking time in both phases of the formalin-induced paw-licking test (Table 5). In contrast, indomethacin had no significant effect on paw-licking behavior in the early phase of the nociceptive response, but markedly reduced paw-licking time in the late phase of the response (Table 5).

VI. Antioxidant Enzymes Activity and MDA Levels

Oral administration of the extract of Radix Aconiti Kusnezoffii (20, 40, and 60 mg/kg) boosted the activity of the anti-oxidative enzymes SOD, GR, and GRx in the liver

Table 4. Effects of Radix Aconiti Kusnezoffii and *Aconitum* alkaloids on acetic acid-induced writhing response in mice

Treatment	Number of writhes (15 min)	Inhibition (%)
Vehicle	30.4 ± 1.8	—
Indomethacin (10 mg/kg, i.p.)	5.1 ± 1.0 ^b	83.2 ± 2.4
Radix Aconiti Kusnezoffii (20 mg/kg, p.o.)	21.2 ± 2.0 ^a	30.3 ± 2.7
Radix Aconiti Kusnezoffii (40 mg/kg, p.o.)	14.3 ± 1.2 ^a	52.9 ± 1.8
Radix Aconiti Kusnezoffii	9.6 ± 0.6 ^b	69.4 ± 2.9
Aconitine (0.5 mg/kg, p.o.)	7.6 ± 0.9 ^b	75.3 ± 2.5
Mesaconitine (0.5 mg/kg, p.o.)	5.9 ± 0.8 ^b	80.4 ± 3.2
Hypaconitine (0.5 mg/kg, p.o.)	25.8 ± 1.2	15.1 ± 2.1

Values (mean ± SEM) were obtained from each group of 8 animals. ^a*p* < 0.05 and ^b*p* < 0.01 compared to the values of vehicle-treated group, respectively.

Table 5. Effects of Radix Aconiti Kusnezoffii on the early phase and late phase of formalin test in mice

	First phase		Second phase	
	Licking time (sec)	Inhibition (%)	Licking time (sec)	Inhibition (%)
Vehicle	96.4 ± 7.9	—	164.6 ± 9.2	—
Indomethacin (10 mg/kg, i. p.)	90.2 ± 8.4	6.4 ± 1.2	49.2 ± 5.6 ^b	70.2 ± 3.2
Radix Aconiti Kusnezoffii (20 mg/kg, p.o.)	60.8 ± 3.5 ^a	36.9 ± 2.6	53.6 ± 4.3 ^b	67.7 ± 4.2
Radix Aconiti Kusnezoffii (40 mg/kg, p.o.)	45.2 ± 5.4 ^b	53.1 ± 3.2	47.7 ± 3.9 ^b	70.9 ± 2.9
Radix Aconiti Kusnezoffii (60 mg/kg, p.o.)	30.9 ± 2.8 ^b	67.9 ± 4.1	35.4 ± 2.7 ^b	78.4 ± 3.3

Values (mean ± S.E.M.) were obtained from each group of 8 animals. ^a*P* < 0.05 and ^b*P* < 0.01 compared to the values of vehicle-treated group, respectively.

Table 6. Effects of the Radix Aconiti Kusnezoffii on the liver SOD, GPx, and GRx activities in mice

Groups	SOD (U/mg protein)	GPx (U/mg protein)	GRx (U/mg protein)
Carr	39.0 ± 0.5	0.1866 ± 0.0006	0.0648 ± 0.0002
Carr + 20 mg/kg Radix Aconiti Kusnezoffii, p. o.	58.4 ± 0.2 ^a	0.3147 ± 0.0002 ^a	0.1087 ± 0.0001 ^a
Carr + 40 mg/kg Radix Aconiti Kusnezoffii, p. o.	60.5 ± 0.5 ^a	0.3542 ± 0.0003 ^a	0.1178 ± 0.0002 ^a
Carr + 60 mg/kg Radix Aconiti Kusnezoffii, p. o.	82.0 ± 0.5 ^b	0.3753 ± 0.0004 ^b	0.1191 ± 0.0001 ^b

All values represents as mean ± SEM (n = 10). ^a*p* < 0.05 and ^b*p* < 0.01 compared to the carrageenan (Carr) group.

(Table 6). Peritoneal injection of indomethacin and oral administration of Radix Aconiti Kusnezoffii (40 and 60 mg/kg) reduced MDA levels in the hind paws of mice (Figure 3).

DISCUSSION

The genus *Aconitum* contains a toxic diester diterpenoid

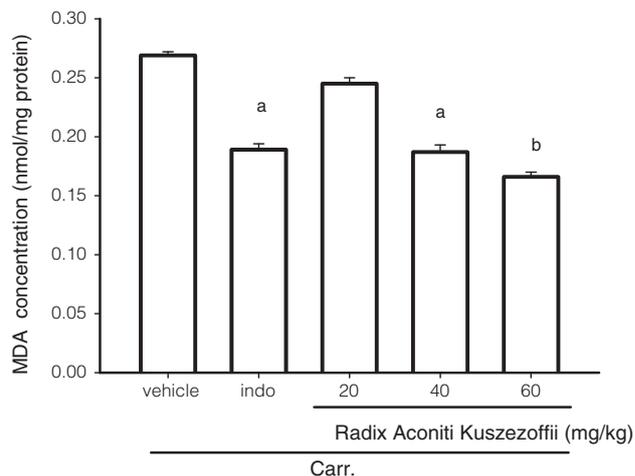


Figure 3. Effects of Radix Aconiti Kusnezoffii and indomethacin (Indo) On carrageenan induced MDA concentration of the edema paw at 3rd hour in mice. Indomethacin (10 mg/kg) was administered intraperitoneally to the separate group of mice. The vehicle (normal saline) used to dissolve the tested medications was given at the same volume. Values (mean ± SEM) were obtained from each group of 8 animals. ^a*p* < 0.05 and ^b*p* < 0.01 compared to the values of vehicle-treated group at the indicated times, respectively.

alkaloids, because it has beneficial pharmacological properties if used at proper dosage and, therefore, is an important ingredient in many traditional herbal medicines. Radix Aconiti Kusnezoffii also contains diester diterpenoid alkaloids, which can be destroyed by heat during concoction. Processed Radix Aconiti Kusnezoffii thus has reduced toxicity and can be used clinically. Therefore, processed Radix Aconiti Kusnezoffii was used in this study for evaluation of its antinociceptive and anti-inflammatory activities.

Experimental animal models for the study of pain rely on four damaging stimuli: mechanical, thermal, electrical, and chemical stimulation⁽²¹⁾. We evaluated the analgesic properties of agents using the acetic acid-induced writhing test and the formalin test in mice. Both involve chemically-induced pain. The writhing response can be suppressed by neural analgesic substances, such as opium and endopeptides, and peripheral anti-pyretic analgesic substances such as steroidal and non-steroidal anti-inflammatory drugs (NSAIDs). Acetic acid-induced pain is sensitive to NSAIDs, anesthetic agents, and other central nervous system-active medicines so that the acetic acid-induced writhing model is widely used to evaluate analgesic effects of compounds⁽²²⁾. The model is based on pain emanating from internal organs, which is mediated through prostaglandins generated from arachidonic by the enzyme cyclooxygenase. Increased prostaglandin levels, particularly PGE_{2 α} , can be detected quantitatively in peritoneal fluid after acetic acid injection using radioimmunoassay. Thus, prostaglandin production plays an important role in the pain response. This study showed that Radix Aconiti Kusnezoffii is able to produce a dose-dependent reduction in acetic acid-induced writhing, to a degree comparable to indomethacin. In addition, the analgesic effect might be associated with the inhibition of arachidonic acid metabolism.

The aconitine-alkaloids aconitine, mesaconitine and hypaconitine were used as standards in analyzing the components of Radix Aconiti Kusnezoffii. Among these alkaloids, mesaconitine was found to be the most abundant constituent of Radix Aconiti Kusnezoffii, followed by aconitine and then hypaconitine. In the acetic acid-induced writhing test, mesaconitine and aconitine exhibited marked analgesic effects, producing significant reductions in body writhing.

In the formalin test, dilute formalin is injected subcutaneously into the hind paw of rats, evoking a sustained damaging stimulation that leads to an obvious, long-lasting, and spontaneous pain response characterized by leg flinches, leg licking, and trembling⁽²³⁾. This model can mimic some elements of human pain syndromes in response to injury and is quite reproducible⁽²³⁾. The paw-licking response to formalin is biphasic. The first or early phase, representing acute neurogenic pain, occurs from 0 to 5 min after formalin injection. Pain in this phase is transmitted mainly through the release of neurotransmitters such as substance P and bradykinin, which directly stimulate nociceptors. The transmission of pain signals in this phase is via A δ and C nerve fibers. This phase represents a central nervous system-induced pain response. After a latent period, a second, late

phase representing inflammatory pain becomes evident over the period of 15 to 30 min after formalin injection. Late phase pain is associated with chemical mediators such as histamine, serotonin, PGE₂, and kinins, which are secreted from cells damaged as a result of the formalin-induced inflammatory reaction. This phase represents a peripherally induced pain response⁽²⁴⁾. Our results showed that Radix Aconiti kusnezoffii had an analgesic effect to reduce both central and peripheral nervous system-induced pain.

A common animal model for the investigation of anti-inflammatory drugs is λ -carrageenan injection into the foot of a mouse⁽²⁵⁾. This triggers increased permeability of local blood vessels, resulting in foot swelling. This method is often employed to evaluate the efficacy of anti-inflammatory agents and the anti-edema effects of natural products. The response to the injection of λ -carrageenan-induced paw edema is biphasic, and the injected λ -carrageenan releases various substances with time to induce inflammation. In phase I, within 1 to 2 h of λ -carrageenan injection, histamine, serotonin and other mediators are released. During phase II, i. e., within 3 to 5 h of injection, prostaglandin is released. Between the two phases, the substances like kinin are released⁽²⁶⁾. There are also studies showing that λ -carrageenan-induced inflammation is associated with free radicals. Radix Aconiti Kusnezoffii was shown to exert anti-inflammatory activity in both phases of the response, especially at the dose of 40 mg/kg, which presented the best efficacy. It can therefore be deduced that the anti-inflammatory action of Radix Aconiti Kusnezoffii can be attributed to inhibiting the release of the early phase mediators, such as histamine and serotonin, and the late-phase substance prostaglandin.

Reactive oxygen species (ROS) have been thought to play an important role in the onset and progression of many diseases, such as arteriosclerosis, inflammation, cancer, and cardiovascular diseases⁽²⁷⁾. In the inflammatory process, ROS not only causes tissue damage, but also activates arachidonic acid metabolism, cytokines, and heat-shock proteins, and induces apoptosis⁽²⁷⁾. Therefore, the reduction of ROS levels during inflammation can result in an effective anti-inflammatory effect. Many antioxidative enzymes exist in the liver to antagonize free radicals. SOD is an antioxidative enzyme that is able to remove free radical ions and proteins. It is critical in protecting against the toxicity of oxygen radicals, delaying aging, and preventing tumor development and inflammation. Its activity indirectly reflects the ability of the body to clear oxidative free radicals⁽²⁷⁾. MDA is the decomposed product of lipid peroxide, generated from the free radical-damaged unsaturated fatty acids, and is one of the most important products in lipid peroxidative reaction. MDA levels in tissues can be directly linked to the rate and degree of lipid peroxidation⁽²⁸⁾. GPx exist in all kinds of human tissues and cells. It can induce GSH to reduce intracellular superoxide, leading to the elimination of peroxides from the body, thereby preventing lipid oxidation⁽²⁷⁾. GSH can be further reduced to GSSH by glutathione reductase⁽²⁷⁾. Our results showed that the anti-inflammatory mechanism

of Radix *Aconiti kusnezoffii* may be attributed to the elevation of anti-oxidative enzymes in the liver to eradicate free radicals. Additionally, there is also a suppression of MAD levels in the inflammatory footpad, reducing lipid oxidation and thus contributing to the anti-inflammatory effect.

In many traditional medicines in China and Japan, the genus *Aconitum* is widely used for its analgesic, anti-inflammation, and blood sugar-lowering properties and for treating diseases of the nervous system. The results of this investigation indicate that Radix *Aconiti kusnezoffii* exhibits an antinociceptive activity at both central and peripheral levels and an expressive anti-inflammatory effect. Besides, care has to be taken while using *Aconitum* in the clinical administration due to its lower therapeutic range. This represents an example of the use of modern scientific methodology to verify traditional Chinese medicine theory, and studies like this should facilitate the incorporation of Chinese herbal medicines into clinical applications.

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REFERENCES

1. Akeroyd, J. R. and Charter, A. O. 1993. *Aconitum* L. In "Flora Europaea". 2nd ed. pp. 254-256, Tutin, T. G., Burges, N. A., Charter, A. O., Edmondson, J. R., Heywood, V. H., Moore, D. M., Valentie, D. H., Walters, S. M. and Webb, D. A. ed., Cambridge University Press, Cambridge, UK.
2. Bisset, N. G. 1981. Arrow poisons in China. Part II: *Aconitum*-botany, chemistry, and pharmacology. *J. Ethnopharmacol.* 4 : 247-336.
3. Hikino, H., Konno, C., Takata, H., Yamada, Y., Yamada, C., Ohizumi, Y., Sugio, K. and Fujimura, H. 1980. Anti-inflammatory principles of *Aconitum* roots. *J. Pharmacobiodyn.* 3 : 514-525.
4. Xu, H., Arita, H., Hayashida, M., Zhang, L., Sekiyama, H. and Hanaka, K. 2006. Pain-relieving effects of processed *Aconiti tuber* in CCI-neuropathic rats. *J. Ethnopharmacol.* 103: 392-397.
5. Tu, Y., Yang, P., Sun, S. Q., Zhou, Q., Bao, X. H. and Isao, N. 2010. Analysis of fingerprints features of infrared spectra of various processed products of Radix *Aconiti Kusnezoffii*. *J. Mol. Struct.* 974: 103-107.
6. Yamada, K., Suzuki, E., Nakaki, T., Watanabe, S. and Kanba, S. 2005. *Aconiti tuber* increases plasma nitrite and nitrate levels in humans. *J. Ethnopharmacol.* 96: 165-169.
7. Li, S. Z. 1578. Vol. 17. Bencao Gangmu. Taiwan.
8. Anonymous. 200 B C. vol 3. Shen Nong Ben Cao Jing. Taiwan.
9. Vane, J. R. and Botting R. M. 1995. New insights into the mode of action of anti-inflammatory drugs. *Inflamm. Res.* 44: 1-10.
10. Lu, T. C., Ko, Y. Z., Huang, Y. C., Lin, Y. C. and Peng, W. H. 2007. Analgesic and antiinflammematory activities of aqueous extract from *Glycine tomentella* root in mice. *J. Ethnopharmacol.* 113: 142-148.
11. Zimmermann, M. 1983. Ethical guidelines for investigations of experimental pain in conscious animals. *Pain* 16: 109-110.
12. Ecobichon, D. J. 1997. The basis of toxicology testing. pp. 43-86. CRC Press. U.S.A.
13. Winter, C. A., Risley, E. A. and Nuss, G. W. 1962. Carrageenan-induced oedema in hind paw of the rats as an assay for antiinflammatory dugs. *Proc. Soc. Exp. Biol. Med.* 111: 544-547.
14. Misra, H. P. and Fridovich, I. 1972. The role of superoxide anion in the autooxidation of epinephrine and a simple assay for superoxide dismutase. *J. Biol. Chem.* 247: 3170-3175.
15. Guntherberg, H. and Rost, J. 1966. The true oxidized glutathione content of red blood cells obtained by new enzymic and paper chromatographic methods. *Anal. Biochem.* 15: 205-210.
16. Kraus, R. J. and Ganther, H. E. 1980. Reaction of cyanide with glutathione peroxidase. *Biochemical and Biophysical Research* 74: 64-71.
17. Tatum, V. L., Changchit, C. and Chow, C. K. 1990. Measurement of malondialdehyde by high performance liquid chromatography with fluorescence detection. *Lipids.* 25: 226-229.
18. Wang, Z., Wen, J., Xing, J. and He, Y. 2006. Quantitative determination of diterpenoid alkaloids in four species of *Aconitum* by HPLC. *J. Pharm. Biomed. Anal.* 40: 1031-1034.
19. Koster, R., Anderson, M. and Beer, E. J. 1959. Acetic acid for analgesic screening. *Fed. Proc.* 18: 412-417.
20. Hunskaar, S. and Hole, K. 1987. The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. *Pain.* 30: 103-114.
21. Valerie, G. H. 2000. Psychological mechanisms of pain and analgesia. *Trends. Neurosci.* 23: 587-588.
22. Steen, P. F. and Lars, A. N. 2002. From pain research to pain treatment: the role of human experimental pain models. *Clinical. An esthesiol.* 16: 667-680.
23. Tjolsen, A., Berge, O. G. and Hunskaar, S. 1992. The formalin test: an evaluation of the method. *Pain* 5: 15-17.
24. Di Rosa, M. 1972. Biological properties of carrageenan. *J. Pharm. Pharmacol.* 24: 89-102.
25. Vinegar, R., Schreiber, W. and Hugo, R. 1969. Biphasic development of carrageenin odema in rats. *J. Pharmacol. Exp. Ther.* 166: 96-103.
26. Cullen, J. J., Conklin, J. L., Ephgrave, K. S. and Oberley, L. W. 1999. The role of antioxidant enzymes in the control of opossum gallbladder motility. *J. Surg. Res.* 86: 155-161.

27. Paroni, R., Ferma, I. and Cighetti, G. 2002. Validation of methyl malondialdehyde as internal standard for malondialdehyde detection by capillary electrophoresis. *Anal. Biochem.* 307: 92-98.
28. Duh, P. D., Wu, S. C., Chang, L. W., Chu, H. L., Yen, W. J. and Wang, B. S. 2009. Effects of three biological thiols on antimutagenic and antioxidant enzyme activities. *Food Chem.* 114: 87-92.