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Original Article

# Anti-inflammatory and antinociceptive activities of non-alkaloids fractions from *Aconitum flavum in vivo*

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ABSTRACT

*Aconitum flavum* Hand.-Mazz., Ranunculaceae, has been used for the treatment of rheumatism, traumatic injury in folk and clinical medicine, but the alkaloids has high toxicity. This study was designed to investigate the acute toxicity, anti-inflammatory and antinociceptive activities of non-alkaloids fractions from *A. flavum* in rodents. The anti-inflammatory activity was evaluated by inflammatory models of dimethylbenzene-induced ear vasodilatation and acetic acid-induced capillary permeability enhancement test in mice and carrageenan-induced paw edema in rats whereas the antinociceptive activity was evaluated using acetic acid-induced writhes, hot plate test and formalin test in mice. The result showed that the $LD_{50}$ value of BtOH and EtOAc fractions could not be determined as no lethality was observed up to 40 g/kg (*p.o.*) in mice. BtOH fraction significantly decreased the dimethylbenzene-induced ear vasodilatation, carrageenan-induced paw edema and acetic acid-induced capillary permeability. EtOAc fraction only significantly attenuated paw edema and capillary permeability at the dose of 500 mg/kg. In antinociceptive test, BtOH and EtOAc fractions significantly reduced the writhing number evoked by acetic acid injection and the licking time in both phases of the formalin test. Meanwhile BtOH and EtOAc fractions had significant effect on hot plate test after 90 min. Our data indicate that the BtOH and EtOAc fractions of NAF are no toxicity. BtOH and EtOAc fractions not only inhibit inflammatory and peripheral inflammatory pain but also have central antinociceptive effect.



## Introduction

The *Aconitum* consisting of over 300 species are widely distributed in Asia, Europe and North America. Most of them grow among high altitudes in the northern hemisphere. There are 200 species grow in China such as *Aconitum kusnezoffii* Reichb., *Aconitum carmichaelithe* Debx. and *Aconitum flavum* Hand. –Mazz. (Xiao, 2006). In traditional Chinese medicine, *Aconitum* have similar pharmacological actions and are commonly applied for various inflammatory diseases, such as rheumatic fever, painful joints, gastroenteritis, diarrhea, edema, bronchial asthma and various tumors (Qin et al., 2012; Singhuber et al., 2009). However, with the increasing popularity, *Aconitum* poisoning can occur in many parts of the world. Because the highly toxic and narrow margin of safety between therapeutic and toxic doses of these herbs. Lots of researches showed that the effects and severe toxicity of *Aconitum* are mainly attributed to alkaloids (Chan, 2012; Singhuber et al., 2009). Aconitine, mesaconitine, hypaconitine and other alkaloids have potent cardiotoxins and neurotoxins found in all parts of the *Aconitum* species, especially in the tubers and roots. Patients with *Aconitum* poisoning often present with a combination of cardiovascular, neurological, gastrointestinal and other signs and symptoms. The estimated lethal dose is 2 mg of aconitine, 5 ml of aconite tincture (herbal medicinal wine) and 1 g of the raw aconite plant (Chan, 2012; Qin et al., 2012). Facing this dangerous situation, the effects of non-alkaloids fraction are worth to explore, although the alkaloids of *Aconitum* species were been seen as important components in disease treatment. Modern Chinese medicine theory has been suggested that the chemical constituents of traditional Chinese medicine are complex and many different kinds of chemical compositions may treat the diseases in similar way by additive and synergy effect (Tu et al., 2012; Xu et al., 2014a). This theory urged us to explore the effects of non-alkaloids fraction from *Aconitum.*

*A. flavum* Hand.-Mazz. from the family Ranunculaceae, is widely used in western China (Shanxi, Ningxia, Gansu, Inner Mongolia,

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Qinghai and Tibet). It has been used for the treatment of rheumatism, traumatic injury and toothache in folk and clinical medicine (Ting et al., 2008). To date, Chinese modern research about *A. flavum* is mainly concentrated in pharmacological functions and quality control of alkaloids and different processed products (the toxicity of these products was reduced by decomposing the diester diterpene alkaloids to the less toxic monoester diterpene alkaloids). However, there has been little research on anti-inflammatory and antinociceptive activity of non-alkaloids fractions from *A. flavum*. The aim of the present study is to evaluate the anti-inflammatory and analgesic effects of non-alkaloids fractions from *A. flavum* in standard rodent models of inflammation and pain. Additionally, we also studied acute oral toxicity of the non-alkaloids fractions. These studies will show a direction of finding low toxic compounds from *Aconitum*.

## Materials and methods

### *Herbal preparation and extraction*

The roots of *Aconitum flavum* Hand.-Mazz., Ranunculaceae, were purchased from an Herbal Medicinal Materials Company of Mingde Pharmaceutical Co., Ltd (Ningxia, China). The Plant is growing in Liupan mountain, Longde, Guyuan, Ningxia, China. The roots were harvest in August to September. The plant sample was authenticated by Lin Dong at Pharmacognosy Department, College of Pharmacy, Ningxia Medical University, and a voucher specimen was deposited in the same unit (Herbarium number: 20130924).

Sliced dried roots (3.7 kg) were extracted three times with 70% ethanol (mass ratio of solid to liquid was 1:10). The extract was then combined and evaporated to dryness under reduced pressure, which yielded 925 g crude extract. The crude extract was dissolved in 20% (v/v) ethanol aqueous (2 l), and then diluted with a solution (5000 l) of hydrochloric acid 1% in 20% ethanol aqueous. The 001 × 7 strong acid polystyrene cation exchange resin (Anhui Sanxing Resin Technology Co., Ltd., China) were selected for further work. The sample solution was absorbed in column with a 20 ml/min flow rate. Then column was washed with deionized water until the effluent was colorless. The combined effluent was adjusted to pH 7 with a solution of sodium hydroxide and evaporated using a rotary evaporator to give a non-alkaloids fraction (NAF). The NAF was then suspended in distilled water and successively partitioned with petroleum ether, ethyl acetate (EtOAc) and *n*-butanol (BtOH) to yield EtOAc (18.25 g) and BtOH (77.33 g) fractions, as well as an $H_2O$ residue.

### *Estimation of the total phenolic, flavonoid and saponin content*

The total amount of phenolic and flavonoid contents were determined in EtOAc and BtOH fractions using the Folin–Ciocalteu colorimetric method and aluminum chloride colorimetric assay, respectively. The percentage of total phenolic content expressed as gallic acid equivalents for EtOAc and BtOH fractions were 15.08% and 3.05%, respectively. But there have no flavonoid in NAF. In addition, the saponins were measured by vanillin–perchloric acid assay. The saponin contents expressed as oleanolic acid equivalents were 9.37 and 3.83% respectively.

### *Drugs and chemicals*

The following drugs and chemicals were used: acetic acid, dimethylbenzene and formaldehyde solution were purchased from Damao Chemical Company (Tianjin, China). Carrageenan (BR; no. Q1555) was from Shanghai Wanjiang Biological Technology Co., Ltd. Indomethacin (no. A130602) was obtained from Shanxi Yunpeng Pharmaceutical Co., Ltd. Evans blue (no. E2129) was purchased from Sigma Co., Ltd. The fractions of NAF were suspended in water with 0.5% (w/v) sodium carboxyl methyl cellulose (Na-CMC).

### *Animal preparation*

The animals used in this study, including ICR mice (18–22 g; License No.: SYXK (NING) 2011-0001) and Sprague-Dawley rats (180–220 g; License No.: SCXK (NING) 2012-0001) were purchased from the Experimental Animal Center of Ningxia Medical University (Ningxia, China). They were maintained in standard laboratory cages, in moderate humidity (50 ± 5%), at constant temperature (22 ± 1 °C) in a 12-h light–dark cycle room. All animals had free access to food and water during the experimental period. The experiment protocol was approved by the Ethics Committee of Ningxia Medical University, Ningxia (Ethics approval: 2013-146).

### *Toxicity study*

The acute toxicity study of the non-alkaloids fractions was performed according to OECD guidelines (2011). The EtOAc and BtOH fractions was suspended in water with 0.5% (w/v) sodium carboxyl methyl cellulose (Na-CMC) in the dose of 120, 1200, 6000, 20,000 and 40,000 mg/kg body weight were orally administered to overnight-fasted, healthy mice ($n = 8$) and the animals were observed continuously for 24 h for mortality.

### *Anti-inflammatory activity*

#### *Dimethylbenzene (DMB)-induced ear vasodilatation assay*

The test was carried out according to the previously described method (Carlson et al., 1985). Mice of either sex were randomly divided in eight groups ($n = 8$ per group). Control group, indomethacin (10 mg/kg, positive group), EtOAc (500 and 250 mg/kg), BtOH (500 and 250 mg/kg) and $H_2O$ (500 and 250 mg/kg) were administered via oral gavage. 60 min after via gavage of test samples, the mice were topical applied 30 μl DMB to both inner and outer surface of right ear. Mice were sacrificed by cervical dislocation 30 min later then the ear biopsies of both ears were obtained with a punch (a diameter of 8 mm) and weighed. Weight-increase-rate of the right ear over the left one indicated the vasodilatation.

#### *Acetic acid-induced vascular permeability*

The acetic acid-induced increased vascular permeability in mice was carried out using the reported technique (Whittle, 1964). Briefly, grouping and administration were the same as mentioned in ear vasodilatation assay. 60 min after via gavage, 10 ml/kg body weight of 2% Evans blue in normal saline solution to the tail vein then applied 20 ml/kg body weight of 0.6% (v/v) acetic acid immediately (*i.p.*). 20 min after the administration of acetic acid, the mice were sacrificed by cervical dislocation. The peritoneal cavity was rinsed by 10 ml of saline. The washing solutions were collected in centrifuge tube and centrifuged at 1000 × *g* for 5 min. The supernatant was measured at 590 nm by spectrometry and the absorbance of Evans blue in the exudates under 590 nm represented the capillary permeability.

#### *Carrageenan-induced paw edema*

The paw edema was induced according to Winter et al. (1962). Animal of each sex were divided into seven groups of seven each. They were pretreated orally with the vehicle (0.5%, w/v sodium carboxyl methyl cellulose, control group), EtOAc (500 and 250 mg/kg), BtOH (500 and 250 mg/kg), $H_2O$ (500 mg/kg) and indomethacin (10 mg/kg, *p.o.*). After 60 min, edema was induced with the injection of 0.1 ml of 3% (w/v) freshly prepared carrageenan suspension in saline into the right hind paw of each rat. The inflammation

was quantified by measuring the volume (ml) displaced by the paw using a plethysmometer (Beijing Zhongshidichuang Science and Technology Development Co., Ltd, model YLS-7C, China) at 0, 1, 2, 3, 4, 5 h after carrageenan injection. The edema volume was expressed by the variation in volume between each time (1, 2, 3, 4 and 5 h) and basal time (0 h).

*Antinociceptive activity*

*Acetic acid-induced writhing response*

Mice were used according to the method previously reported (Koster et al., 1959). The doses and the routes of administration were provided in ear vasodilatation assay. 60 min after the via gavage, the responses to an intraperitoneal injection of acetic acid solution, manifesting as a contraction of the abdominal muscles stretching of hind limbs, were counted cumulatively after 5 min of stimulus over a period of 20 min. Nociception was induced by intraperitoneal injection of 0.6% acetic acid solution at the dose of 20 ml/kg body weight.

*Formalin test*

Formalin-induced nociception was induced in mice according to a previously described procedure (Hunskaar et al., 1985). A volume of 20 μl of a 2.5% formalin solution in saline was injected intraplantarly (*i.pl.*) in the plantar surface of the right hind paw to mice. After formalin injection, the mice were individually placed in a glass cylinder of 22 cm diameter and were observed from 0 to 5 min (neurogenic pain response) and 15–30 min (inflammatory pain response). The time spent licking the injected paw was recorded with a chronometer for both phases and considered as indicative of nociception. The animals were pretreated with oral doses of vehicle, indomethacin, EtOAc (500 and 250 mg/kg), BtOH (500 and 250 mg/kg) and $H_2O$ (500 and 250 mg/kg) 60 min before formalin administration.

*Hot plate test*

Mice were subjected to the hot plate test (MacDonald et al., 1946; Sahley and Berntson, 1979). A glass cylinder was placed on a hot-plate with adjustable temperature. The temperature of the hot-plate was then regulated to 55 ± 1 °C. The female mice with baseline latencies of more than 20 s were eliminated from the study and the cut-off time of 40 s was fixed to avoid damage to the paws. Each mouse was placed in the glass on the hot-plate in order to obtain the animal's response to heat-induced nociceptive pain stimulus (linking of the hindpaws, jumping or shaking). The reaction times obtained at 0, 30, 60, 90 and 120 min prior to via gavage of vehicle and drugs.

*Statistical analysis*

The results were analyzed using a statistical program SPSS Statistics, version 17.0. One-way ANOVA followed by Dunnett's test was used for determining the statistically significant differences between the values of various experimental groups. Data are expressed as means ± SD and $p < 0.05$ was considered statistically significant.

## Results

*Acute toxicity studies*

The EtOAc and BtOH fractions from NAF at various dose levels did not show any mortality, any morbid symptoms or deleterious effects even at the highest dose (40 g/kg, orally). The $LD_{50}$ value by oral route could not be determined as no lethality was observed up to 40 g/kg in mice.

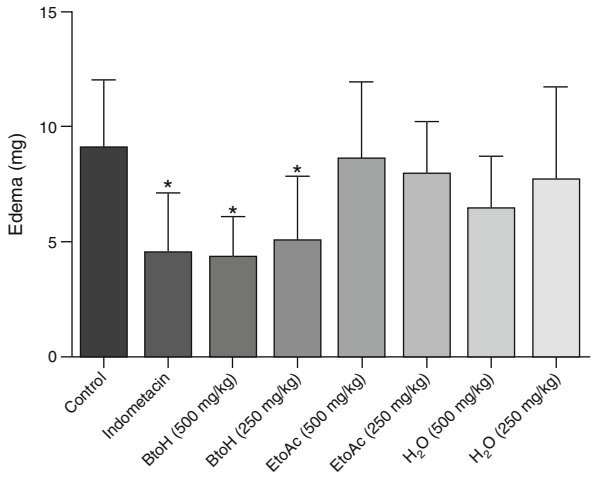


**Fig. 1.** Effects of oral pretreatment with BtOH (500 and 250 mg/kg), EtOAc (500 and 250 mg/kg), $H_2O$ (500 and 250 mg/kg) or indomethacin (10 mg/kg) on DMB-induced ear vasodilatation. The result of ear edema represents mean ± SD ($n = 8$). Differences between the groups were determined by an ANOVA followed by Dunnett's test. $^{*}p < 0.05$, $^{**}p < 0.01$ when compared to the control group.

*Anti-inflammatory effects on DMB-induced ear vasodilatation*

Treatment with BtOH (500 and 250 mg/kg) 1 h before applied DMB in mice ears significantly (Fig. 1; $p < 0.05$) inhibited the edema formation when compared to the control group. Dose-dependent manner was observed at BtOH groups. BtOH group at the dose of 500 mg/kg could remarkably decrease the vasodilatation with inhibition rate over 50%.

*Anti-inflammatory effects on acetic acid-induced vascular permeability enhancement*

Fig. 2 showed acid-induced vascular permeability enhancement was significantly ($p < 0.05$, compared to control group) inhibited in mice pretreated orally with BtOH fraction and EtOAc fraction

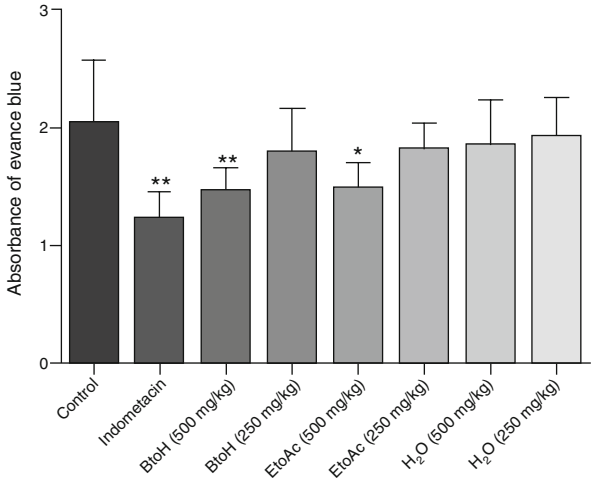


**Fig. 2.** Effects of oral pretreatment with BtOH (500 and 250 mg/kg), EtOAc (500 and 250 mg/kg), $H_2O$ (500 and 250 mg/kg) or indomethacin (10 mg/kg) on the capillary permeability under acetic acid challenge. The absorbance of Evans Blue in the leakage under 590 nm represented indicated the capillary permeability. Date represents mean ± SD ($n = 8$). Differences between the groups were determined by an ANOVA followed by Dunnett's test. $^{*}p < 0.05$, $^{**}p < 0.01$ when compared to the control group.

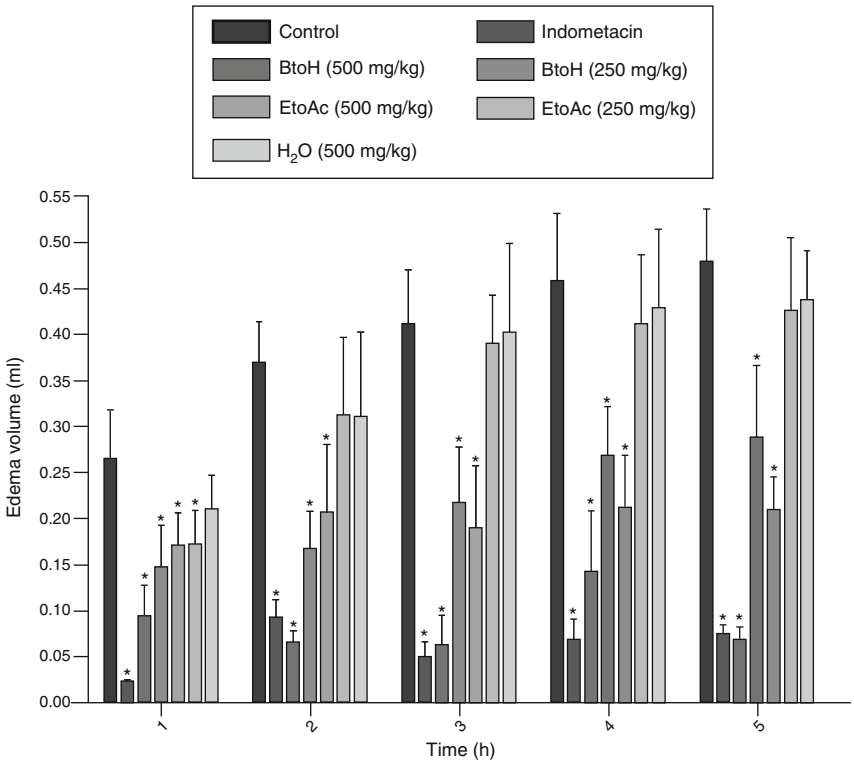


**Fig. 3.** Anti-inflammatory activity of BtOH (500 and 250 mg/kg), EtOAc (500 and 250 mg/kg), $H_2O$ (500 mg/kg) or indomethacin (10 mg/kg) in carrageenan induced hind paw edema. Results were obtained by oral administration of NAF fractions, indomethacin and vehicle. Each value represents the mean ± SD of results from seven rats. Differences from the control group were determined by ANOVA followed by Dunnett's test. $^{*}p < 0.01$.

(500 mg/kg, *p.o.*), with an inhibition of 32.13 and 27.30%, respectively. The standard drug reference indomethacin resulted in a significant reduction (39.46%) of the control number.

*Anti-inflammatory effects on carrageenan-induced paw edema*

Treatment of animals with BtOH fractions (500, 250 mg/kg, *p.o.*) and EtOAc fraction (500 mg/kg) 1 h before injection of carrageenan significantly (Fig. 3; $p < 0.01$) inhibited the edema formation at 1–5 h when compared to control group. But EtOAc fraction at dose of 250 mg/kg only significantly decreased the edema at 1 h. Indomethacin (10 mg/kg, *p.o.*) also produced a significant inhibition of the carrageenan-induced paw edema at 1, 2, 3, 4 and 5 h when compared to control (Fig. 3; $p < 0.01$).

*Anti-nociceptive activity on acetic acid-induced writhes*

In the acetic acid-induced writhing mice (shown in Fig. 4), EtOAc fractions (250–500 mg/kg, *p.o.*) evoked a dose-dependent inhibition, the inhibitory percentage of 44.67% and 28.11%, respectively. All doses of EtOAc fractions significantly ($p < 0.01$ and $p < 0.05$) inhibited the frequency induced abdominal constrictions by acetic acid when compared to the control group. Treatment with BtOH fraction at dose of 500 mg/kg also showed antinociceptive effect in comparison with the control ($p < 0.01$).

*Anti-nociceptive activity on formalin test*

Fig. 5 shows that both first (neurogenic pain) and second (inflammatory pain) phases of formalin-induced nociception were significantly ($p < 0.01$, compared to control group) inhibited in mice pretreated orally with BtOH fractions (500, 250 mg/kg, *p.o.*) and EtOAc fraction (500 mg/kg). EtOAC fraction at dose of 250 mg/kg only significantly ($p < 0.05$) inhibited the nociception at second

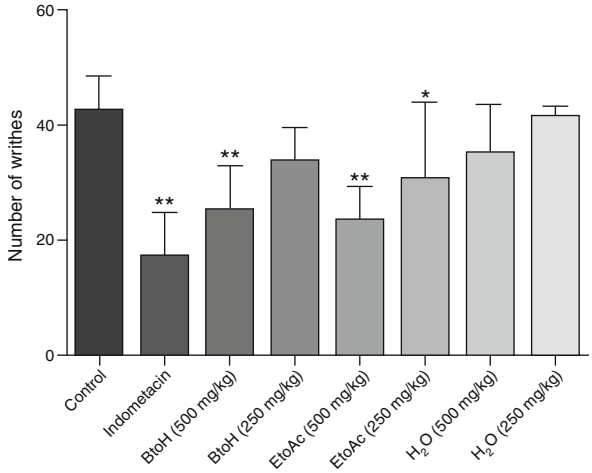


**Fig. 4.** Effects of oral pretreatment with BtOH (500 and 250 mg/kg), EtOAc (500 and 250 mg/kg), $H_2O$ (500 and 250 mg/kg) or indomethacin (10 mg/kg) on acetic acid-induced writhing in mice. Each value represents mean ± SD ($n = 8$). $^{*}p < 0.05$, $^{**}p < 0.01$ when compared to the control group.

phase compared to control group. The inhibitions were 50.8 and 80.3% in BtOH group at the dose of 500 mg/kg, for the first and second phases, respectively. In contrast, indomethacin (10 mg/kg, *p.o.*) only significantly reduced the inflammatory (75.8%) phase of formalin-induced nociception.

*Anti-nociceptive activity on hot plate test*

In the hot plate test, the results presented in Fig. 6 showed that significant ($p < 0.01$ and $p < 0.05$) antinociceptive effect in BtOH fraction and EtOAc fraction groups (500 mg/kg) at 90 and 120 min after administration when compared to control groups.

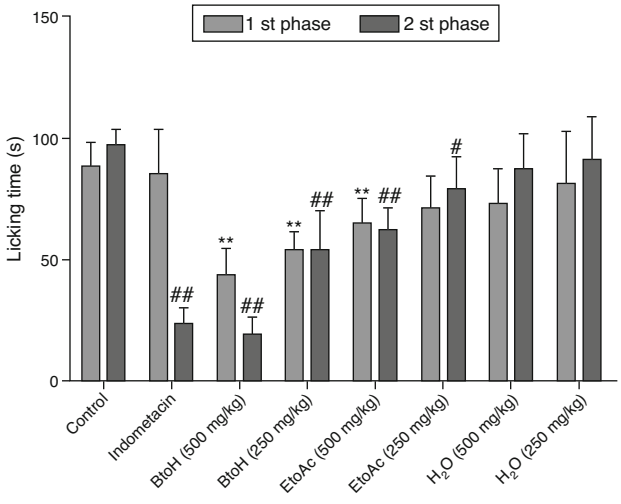


**Fig. 5.** The antinociceptive action of BtOH (500 and 250 mg/kg), EtOAc (500 and 250 mg/kg) and $H_2O$ (500 and 250 mg/kg) on both first (0–5 min) and second (15–30 min) phases of the formalin-induced nociception. Each value represents mean ± SD ($n = 8$). $^{*}p < 0.05$, $^{**}p < 0.01$ by an ANOVA followed by Dunnett's test compared to control group at first phase. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ by an ANOVA followed by Dunnett's test compared to control group at second phase.

## Discussion

The toxicity of alkaloids from *Aconitum flavum* Hand.-Mazz, Ranunculaceae, has made us worried, so to obtain nature products with favorable effects and low toxicity from *A. flavum* become a meaningful research direction. In order to realize this objective, we studied the non-alkaloids fractions from this herb. In our study, 001 × 7 strong acid polystyrene cation exchange resin which is a low cross-linking resin and has porous structure was selected for preparing NAF. This resin may be beneficial to adsorb alkaline high molecular weight components such as ions of alkaloids (Yang and Kong, 2009). The ions of alkaloids were retained in chromatographic column, but the non-alkaloids fractions could be washed using deionized water. In our study, no deaths or serious clinical signs in both EtOAc and BtOH fractions at the highest dose of 40 g/kg (this dose is much higher than the dose of treatment) in acute toxicity study. Although the evidence of risk in longer use is unknown, but the acute toxic results sufficiently support the fact that BtOH and EtOAc fractions of NAF have no toxicity.

Xylene-induced ear edema in mice is a preliminary and simple acute inflammation model for evaluating potential anti-inflammatory agents (Cheng et al., 2005). Ear edema may involve inflammatory mediators such as histamine, kinin, fibrinolysin, phospholipase A2 and PLA2. These mediators induce edema by promoting vasodilation and increasing vascular permeability (Li et al., 2011; Xu et al., 2014b). The BtOH fraction of NAF was able to reduce inflammation in this model and acetic acid-induced vascular permeability enhancement model. These results suggest that BtOH fraction may interfere with the actions of inflammatory mediators and produce the anti-inflammatory effect. Carrageenan-induced paw edema is a largely used test for screening both steroidal anti-inflammatory drugs and NSAID. The inflammatory response involves three phases through sequential release of several mediators. The early phase (the first 90 min) involves the release of histamine and serotonin; the second phase (90–150 min) is mediated by kinin and the third phase (after 180 min) is mediated by prostaglandin (Di Rosa et al., 1971). The results from this study suggest that the BtOH and EtOAc fractions of NAF possibly act by inhibiting the release or action of histamine, serotonin and kinin and prostaglandin of the edema development.

The analgesic activity of NAF fractions in this study was investigated using the abdominal writhing, formalin and hot plate tests in mice. The writhing model induced by acetic acid in mice was

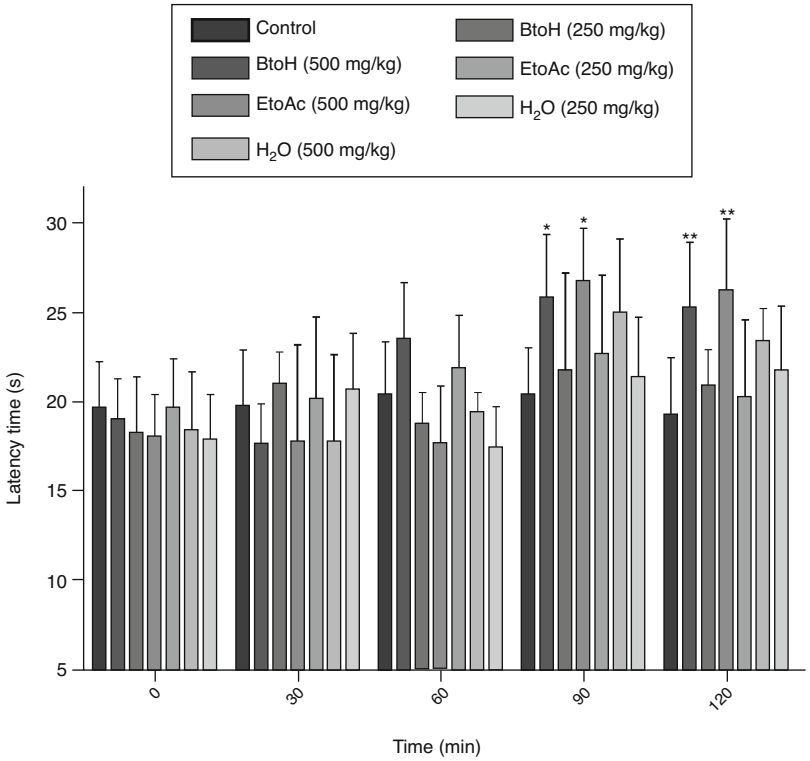


**Fig. 6.** Effects of oral pretreatment with BtOH (500 and 250 mg/kg), EtOAc (500 and 250 mg/kg) and $H_2O$ (500 and 250 mg/kg) on hot-plate test in mice. Date represents mean ± SD ($n = 8$). Differences between the groups were determined by an ANOVA followed by Dunnett's test. $^{*}p < 0.05$, $^{**}p < 0.01$ when compared to the control group.

commonly considered as classical peripheral inflammatory pain animal model for evaluation of analgestic or anti-inflammatory drugs (Negus et al., 2006). The peripheral analgesic is due to the liberation of several inflammatory mediators such as bradykinin, substance P, prostaglandins and cyclo-oxygenases, lipoxygenases, as well as some cytokines such as IL-1β, TNF-α and IL-8 (Ikeda et al., 2001; Ribeiro and Poole, 2000). The hot plate test has been found suitable to evaluate centrally antinociceptive (Hiruma-Lima et al., 2000). In order to further clarify the antinociceptive effect of NAF fractions, the formalin test was carried out. This model is validly used in analgesia research (Tjølsen et al., 1992), it involves two phases (Zakaria et al., 2008). The first phase (0–5 min) is characterized by neurogenic pain caused by a direct stimulation of nociceptors. Substance P and bradykinin are thought to participate in this phase (Hunskaar and Hole, 1987). The second phase (15–30 min) is characterized by inflammatory pain, a process in which several inflammatory mediators are believed to be involved, including histamine, serotonin, prostaglandins and bradykinin (Tjølsen et al., 1992). In general, centrally acting drugs inhibit both phases equally, while peripherally acting drugs inhibit the second phase (Xu et al., 2014b). As presented in Fig. 5, the BtOH and EtOAc fractions of NAF suppressed the pain in two phases. The results obtained from the formalin test were therefore in good agreement with the results from the hot-plate test and writhing test, thereby indicating that BtOH and EtOAc fractions of NAF had central and peripheral analgesic property. These results obtained from inflammation and pain animal model confirm that BtOH and EtOAc fractions could inhibition of the production of inflammatory mediators such as histamine, serotonin, prostaglandins and bradykinin.

Although this research obtained meaningful results, it still existed many problems worth us thinking and improving. The chemical compositions of BtOH and EtOAc fractions are not very clear. Together with previous studies, the constituents of *Aconitum* also include flavonoids, phenolic acids and saponins besides alkaloids (Shrestha et al., 2006; Wuljitegus et al., 2008). The flavonoids, saponins and phenolic acids in those two NAF fractions were analyzed by ultraviolet spectrophotometry in our study. No flavonoids were identified in those two fractions. However the phenolic acids and saponins gave a 15.08 and 9.73 percent in EtOAc fractions. At the same time, the phenolic acids and saponins percentage of BtOH fraction were 3.05% and 3.83%. With reference to the results that EtOAc and BtOH fractions of NAF have anti-inflammatory and antinociceptive effects at high dose, we can speculate that the anti-inflammatory and antinociceptive effects of EtOAc and BtOH fractions may be attributed to chemical components with low concentration, such as phenolic acids and saponins.

## Authors' contributions

YZ, ZS and YL contributed in collecting plant samples and running the laboratory work. LM contributed to estimation of the chemical composition. LY contributed in analysis of the data. YZ wrote manuscript. XW contributed to critical reading of the manuscript. XF designed the study, supervised the laboratory work and contributed to the critical reading of the manuscript. All the authors have read the final manuscript and approved submission.

## Conflict of interest

The authors have declared that there is no conflict of interest.

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