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The anti-arthritic effects of *Aconitum vilmorinianum*, a folk herbal medicine in Southwestern China

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ABSTRACT

Ethnopharmacological relevance: Aconiti Radix (AC) and Aconiti Kusnezoffii Radix (AK) are two traditional Chinese medicines commonly used to treat joint pain and arthritis. In Southwestern China, Huangcaowu (AV), the root of *Aconitum vilmorinianum* Kom., has long been used as a local substitute for these herbs for analgesia and anti-inflammation. However, its anti-arthritic effects have not been investigated.

Aim of study: To investigate the anti-arthritic effects of Huangcaowu (AV).

Materials and methods: Mono-arthritis in SD rats was induced by unilateral intra-articular injection of Freund's complete adjuvant. Physiological saline was injected in the contralateral knee. Seventy five percent ethanol extracts of AV (10 mg/kg/day and 100 mg/kg/day), AC (100 mg/kg/day) and AK (100 mg/kg/day) were administered to rats by oral gavage for 14 consecutive days (Day -6 to Day 7) while arthritis was induced at the seventh day (Day 0). The anti-arthritic effects of the herbs were assessed by measuring allodynia, swelling, hyperaemia and the vascular permeability of the knee joints.

Results: AV (10 mg/kg/day and 100 mg/kg/day), AC (100 mg/kg/day) and AK (100 mg/kg/day) suppressed joint allodynia. AV (10 mg/kg/day and 100 mg/kg/day) and AK (100 mg/kg/day) significantly reduced join swelling and hyperaemia while AC (100 mg/kg/day) did not. AV (100 mg/kg/day) attenuated vascular permeability while AC (100 mg/kg/day) and AK (100 mg/kg/day) showed no improvement.

Conclusions: Huangcaowu (AV) significantly improved allodynia, swelling, hyperaemia and vascular permeability in arthritic knee joints. It showed the highest anti-arthritic effects among the three tested *Aconitum* herbs.

1. Introduction

The *Aconitum* L. species (Ranunculaceae) have been used as traditional herbal medicines in China since the seventh century (Chen and Jiang, 1988). The tuberous mother roots of *A. carmichaelii* Debx. and *A. kusnezoffii* Reichb. are used as the popular Chinese medicines Aconiti Radix and Aconiti Kusnezoffii Radix, respectively, for analgesic treatment of joint pain and arthritis (Chinese Pharmacopoeia Commission, 2010; Kubo et al., 1990). The major components in Aconiti Radix and Aconiti Kusnezoffii Radix are alkaloids including aconitine, hypaconitine, mesaconitine and related diterpenoid alkaloids (Csupor et al., 2009; Liu et al., 2010). These alkaloids are the bioactive compounds for their anti-arthritic and anti-nociceptive effects (Oyama et al., 1994; Shi et al., 1990). In Southwestern China (including Yunnan, Guizhou and Sichuan Provinces), the folk herbal medicine Huangcaowu, derived from the tuberous mother roots of *A. vilmorinianum* Kom., has been used as a local substitute for Aconiti Radix and Aconiti Kusnezoffii Radix (Fig. 1) (National Institute for the Control of Pharmaceutical and Biological Products and Guangdong Institute for Food and Drug Control, 1995; Wan et al., 2007). Although *A. vilmorinianum* is closely related to *A. carmichaelii* and *A. kusnezoffii* as revealed by DNA barcoding analysis using *trnH-psbA* intergenic spacer (He et al., 2010), their chemical components are quite different. For example, Huangcaowu contains two diterpenoid alkaloids yunaconitine and vilmorrianine A which were undetectable in Aconiti Radix and

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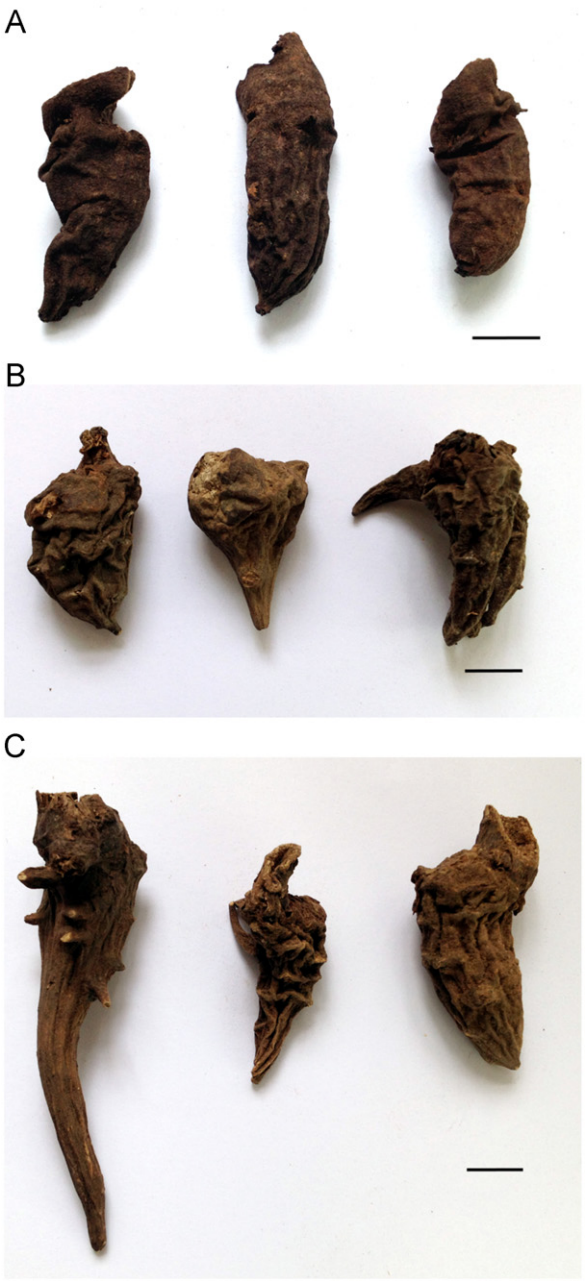

Fig. 1. Samples of (A) Huangcaowu, (B) Aconiti Radix and (C) Aconiti Kusnezoffii Radix. Scale bars represent 10 mm.

Aconiti Kusnezoffii Radix (Xiao et al., 2006). Although Huangcaowu has long been used as a popular analgesic and anti-inflammatory agent in some local areas, its anti-arthritic effects have not been studied. Consequently, the objective of this study is to investigate the anti-arthritic effects of Huangcaowu. The findings in this study have provided scientific bases for its use in treating arthritis.

2. Materials and methods

2.1. Preparation of herbal extracts

Samples of Huangcaowu (AV), Aconiti Radix (AC) and Aconiti Kusnezoffii Radix (AK) were collected from Yunnan Province, PR China, and were authenticated by experts in the Herbarium of the Kunming Institute of Botany, PR China. One hundred grams of each herbal material were extracted with 1 L of 75% ethanol thrice by ultrasonication at room temperature for 1 h. The three extracts of each herb were pooled, dried by rotary evaporation and lyophilized under a reduced pressure. The dried extracts were stored at −20 °C and freshly prepared in distilled water to designated dosages before use. The maximum dosage for rats (100 mg/kg/day) was translated from the human equivalent dosage according the conversion equation as described previously (Reagan-Shaw et al., 2008). AV (10 mg/kg/day) was also included in this study to explore the anti-arthritic effects of AV at a lower dose.

2.2. Induction of arthritis

The experiment was conducted in accordance with the Animal Research Ethics Committee of The Chinese University of Hong Kong. Induction of arthritis was performed as described previously (Lam and Ng, 2010). In brief, male Sprague-Dawley rats (250–300 g) were anesthetized with intraperitoneal injections of thiopentone (40 mg/kg/day). Mono-arthritis was induced by a single injection of 125 μl of Freund's complete adjuvant (FCA) into the synovial cavities of the ipsilateral knee. An equivalent amount of physiological saline was injected into the contralateral knee as an internal control. The rats were returned to their cages with free access to water and food.

2.3. General procedures and treatments

The general procedures of this anti-arthritis experiment were performed as described previously, with a minor modification in that animals were treated for 6 days before arthritis induction and 7 days after induction (Lam et al., 2008). Rats were given 1.5 ml of AV (10 mg/kg/day and 100 mg/kg/day), AC (100 mg/kg/day) or AK (100 mg/kg/day) daily by oral gavage for 14 consecutive days (Day -6 to Day 7). AC and AK were served as the control herbs. An equivalent amount of distilled water (1.5 ml) was used as the vehicle control. Mono-arthritis was induced as described in Section 2.2 on Day 0. The body weight of the animals was monitored during the treatment. The change in body weight between designated days (Day 0 to Day 7) and Day -6 in each treatment were compared to the vehicle control. The anti-arthritic effects of each herb were assessed by knee joint allodynia, swelling, hyperaemia and vascular permeability as described below. The duration of treatment was adopted from the model developed by Lam et al. (2008) which provides sufficient time to demonstrate the anti-arthritic effects of herbal extracts but avoids the influence of spontaneous remission of animals beyond 14 days after arthritis induction.

2.4. Assessment of knee joint allodynia

Knee joint allodynia was assessed as previously described with a minor modification (Yu et al., 2002). In brief, the conscious animal was restrained gently with a towel. The thigh was held with the thumb and the second finger of one hand. The leg was then gently extended until the rat showed struggling behavior and the knee extension angle was measured using a protractor. Each joint was measured thrice and the average knee extension angle was recorded. A reduction of the knee joint extension angle was calculated by subtracting the knee extension angle at each time point (Day 1 to Day 7) by the knee extension angle just before arthritis induction (Day 0).

2.5. Assessment of knee joint swelling

Knee joint swelling was assessed as previously described (Lam et al., 2004). The animal was anaesthetized by intraperitoneal

injection of thiopentone (40 mg/kg/day). The knee joint was placed in a digital micrometer (Mitutoyo, Kawasaki, Japan) so that it was just touching skin without pressing it. The reading of the micrometer was then recorded. Knee joint swelling was assessed just before arthritis induction (Day 0) and daily after arthritis induction (Day 1 to Day 7).

2.6. Assessment of knee joint hyperaemia

Knee joint hyperaemia was assessed as described previously (Lam et al., 1993). The animal was anaesthetized by intraperitoneal injection of urethane (2 g/kg). The anteromedial aspect of the knee joint capsule was exposed by removing an ellipse of skin. Physiological saline was added to the joint capsule to prevent dehydration before measurement. A laser Doppler perfusion imager (Moor Instruments, Axminster, UK) was placed approximately 30 cm above the exposed knee joint and the surface of the joint was scanned by a helium-neon laser (633 nm) in a rectangular pattern (3 cm × 4 cm) for 30 s. An infrared color-coded perfusion image and actual perfusion value (flux) were recorded for the subsequent calculation of mean perfusion using image processing software (Moor Instruments). Knee joint hyperaemia was assessed at the end of the treatment period (Day 7).

2.7. Assessment of knee joint vascular permeability

Knee joint vascular permeability was assessed as described previously (Lam and Ferrell, 1991). The animal was anaesthetized by intraperitoneal injection of urethane (2 g/kg). Evans blue (50 mg/kg/day) was injected via the external jugular vein. After 4 h, the anterior and posterior parts of the knee joint capsule of the ipsilateral and contralateral knees were collected and weighted. The capsule tissue was cut into small pieces and Evans blue was extracted by acetone in 1% $NaSO_4$ at a ratio of 7:3 with gentle shaking for 24 h at room temperature. A clear supernatant was obtained by centrifugation and subjected to spectrophotometric measurement at 620 nm. Knee joint vascular permeability was assessed at the end of the treatment period (Day 7).

2.8. High performance liquid chromatography (HPLC)

One gram of dried and pulverized AV was extracted with 10 ml of 75% ethanol thrice by ultrasonication at room temperature for 10 min. The extracts were pooled, filtered and then concentrated using a rotary evaporator. The concentrated extract was partitioned with a double volume of chloroform thrice. The chloroform partitions were combined, evaporated until fully dry, and dissolved in 3 ml acetonitrile. The acetonitrile solution was filtered and directly used for HPLC analysis using an Agilent 1100 series LC system (Hewlett-Packard, US) equipped with a diode array detector. An aliquot of 5 µl samples were eluted with a mobile phase of acetonitrile: ammonium acetate (40 mM, pH 9.5) at the ratio 3:7 and a flow rate of 1 ml/min on a Waters Xterra RP18 column (4.6 × 250 mm^2, 5 µm, Waters, USA) at room temperature. Signals were detected at 230 nm and 260 nm. Standard compounds of vilmorrianine A and yunaconitine were dissolved in acetonitrile to 0.02–0.1 mg/ml for HPLC analysis.

2.9. Statistical analysis

The results are presented as mean ± standard error of mean (SEM). The difference between the knees within a treatment group was analyzed by a paired t-test, while differences among groups were analyzed by t-test and two-way analysis of variance (ANOVA). *P*-value less than 0.05 was considered statistically significant.

3. Results and discussion

Aconitum herbs including AC and AK have been commonly used to treat joint pain and arthritis in China (Chinese Pharmacopoeia Commission, 2010; Kubo et al., 1990). In this study, we investigated the anti-arthritic effects of AV, a local substitute used in Southwestern China. The bioactive compounds of these *Aconitum* herbs included C_{18}-, C_{19}- and C_{20}-diterpenoid alkaloids (Xiao et al., 2006), which showed significant analgesic and anti-inflammatory effects (Hikino et al., 1980; Hikino et al., 1982; Oyama et al., 1994). However, these alkaloids also showed toxic effects which activated voltage-dependent sodium channels and rapidly paralyzed neural, cardiac and muscular tissues (Fu et al., 2006). This study showed that there was no significant difference in change in body weight between the vehicle control and the treatments of 10 mg/kg/day and 100 mg/kg/day of AV, 100 mg/kg/day of AC and 100 mg/kg/day of AK ($p > 0.05$) (Fig. 2). However, mild toxic effects may not be reflected by the change in body weight.

The anti-arthritic effects of AV were assessed by joint allodynia and swelling in the treatment period after arthritic induction (Day 0 to Day 7). Firstly, substantial alloydonia was induced in the ipsilateral arthritic knees as demonstrated by the marked 57% reduction in the extension angle on Day 1, which gradually reduced to 45% on Day 7 in the vehicle control (Fig. 3A). Oral administration of the control herbs AC (100 mg/kg/day) and AK (100 mg/kg/day) significantly suppressed alloydonia ($p < 0.001$) and the reductions were 31% and 23%, respectively, at the end of treatment (Day 7). Oral administration of AV (10 mg/kg/day and 100 mg/kg/day) significantly inhibited alloydonia ($p < 0.001$) and the reductions were 26% for both doses on Day 7. Secondly, pronounced joint swelling was also induced in the ipsilateral knees, demonstrated by the marked 52% increase in knee joint size on Day 1, which gradually diminished to 30% on Day 7 in the vehicle control (Fig. 3B). AK (100 mg/kg/day) significantly attenuated the knee joint swelling ($p < 0.001$) and reduced it to 16% on Day 7. AC (100 mg/kg/day) showed a mild reduction in knee joint swelling but no statistical significance was observed ($p > 0.05$). On the contrary, AV (10 mg/kg/day and 100 mg/kg/day) significantly suppressed joint swelling ($p < 0.01$ and $p < 0.001$, respectively) to 14% and 16%, respectively (Fig. 3B).

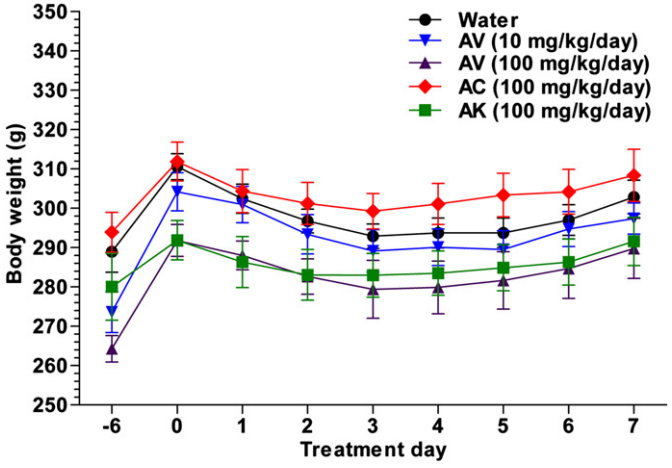

Fig. 2. Effect of oral administration of 75% ethanol extracts of Huangcaowu (AV; 10 mg/kg/day and 100 mg/kg/day), Aconiti Radix (AC; 100 mg/kg/day) and Aconiti Kusnezoffii Radix (AK; 100 mg/kg/day) on the body weight of FCA-induced mono-arthritic rats during the treatment period. Oral administration of distilled water was applied as the vehicle control. Data represent mean ± SEM (n=7–13). There was no significant difference in change in body weight between the treatments and the vehicle control ($p > 0.05$, two-way ANOVA).

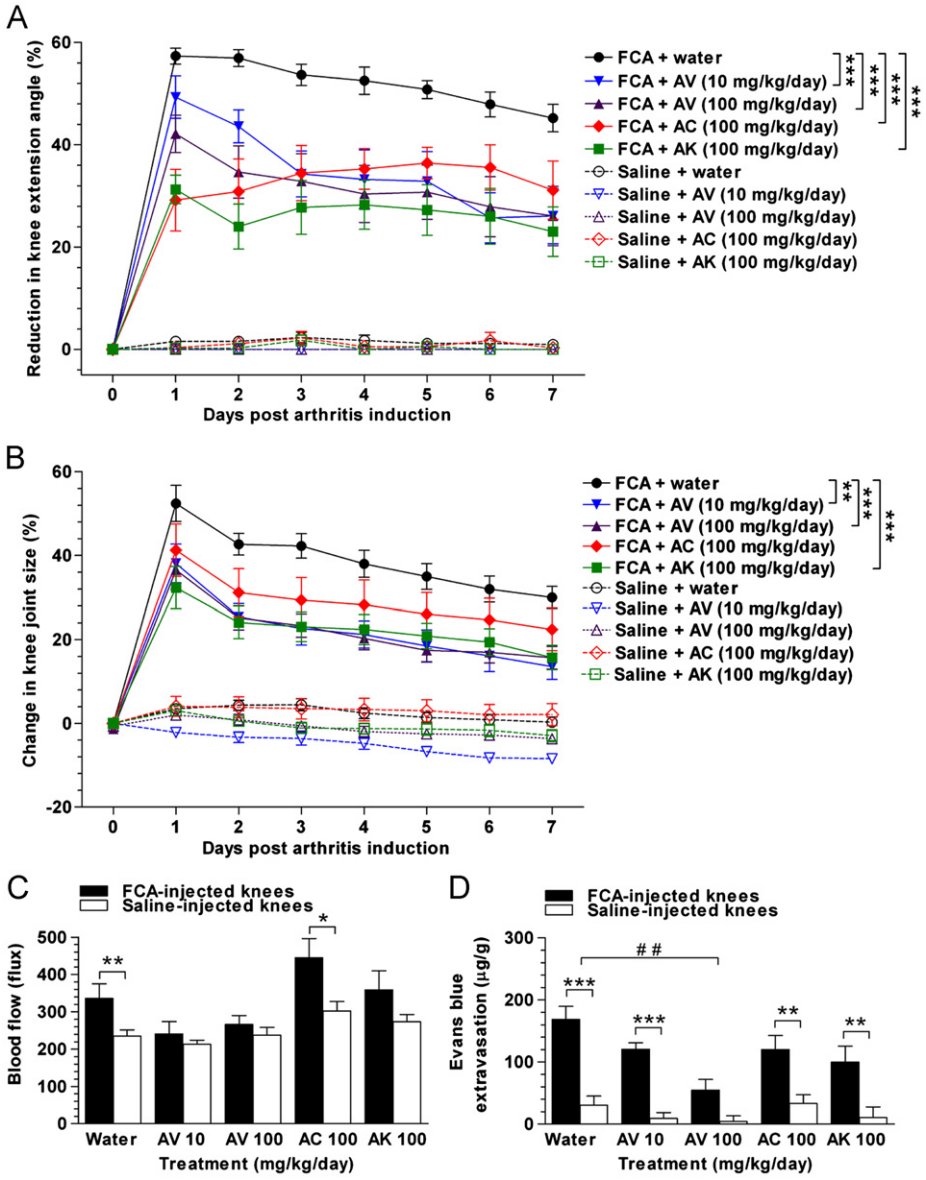

Fig. 3. Anti-arthritic effects of Huangcaowu (AV; 10 and 100 mg/kg/day), Aconiti Radix (AC; 100 mg/kg/day), Aconiti Kusnezoffii Radix (AK; 100 mg/kg/day) and distilled water (vehicle control) on FCA-induced arthritic rat model. Mono-arthritis was induced by unilateral injection of FCA in knee joints of rats. Physiological saline was injected in the contralateral control knee joints. Data represent mean ± SEM. (A) Assessment of knee joint allodynia was presented as the percentage reduction in knee extension angle (n=7–13). All herbal treatments significantly attenuated allodynia in arthritic knees when compared with the vehicle control (***$p < 0.001$, two-way ANOVA). (B) Assessment of knee joint swelling was presented as the percentage change in the size of arthritic knees and control knees (n=7–13). Significant differences were found between AV (10 and 100 mg/kg/day), AK (100 mg/kg/day) and the vehicle control (**$p < 0.01$, ***$p < 0.001$, two-way ANOVA). (C) Knee joint hyperaemia was assessed by measuring the blood flow in both arthritic and control knees on Day 7 (n=6–13). A significant difference between arthritic and control knees was observed in AC (100 mg/kg/day) and the vehicle control (*$p < 0.05$, **$p < 0.01$, paired t-test). (D) Knee joint vascular permeability was assessed by Evans blue extravasation in both arthritic and control knees on Day 7 (n=7–11). Significant difference between arthritic and control knees was observed in AV (10 mg/kg/day), AC (100 mg/kg/day), AK (100 mg/kg/day) and the vehicle control (**$p < 0.01$, ***$p < 0.001$, paired t-test). The difference of Evans blue extravasation between arthritic and control knees in AV (100 mg/kg/day) was significantly smaller then and the vehicle control (##$p < 0.01$, t-test).

Joint hyperaemia and vascular permeability were assessed at the end of the treatment period (Day 7). Joint hyperaemia was measured by comparing the blood flow in the ipsilateral arthritic knees and the contraliateral control knees. The blood flow in arthritic knees (336 flux) was significantly increased by 43% ($p < 0.01$) when compared with the control knees (235 flux) in the vehicle control (Fig. 3C). AC (100 mg/kg/day) did not relieve arthritis hyperaemia as blood flow in the arthritic knees (445 flux) remained significantly higher ($p < 0.05$) than the control knees (302 flux) accounting for a 47% increase in blood flow. In contrast, AK (100 mg/kg/day) and AV (10 mg/kg/day and 100 mg/kg/day) reduced the blood flood difference between the arthritic and control knees and no significant difference was found between the knees ($p > 0.05$) (Fig. 3C). AK (100 mg/kg/day), AV (10 mg/kg/day) and AV (100 mg/kg/day) reduced the hyperaemia response by 26%, 70% and 72%, respectively, when compared with the vehicle control (Fig. 3C). Vascular permeability was measured by Evans blue extravasation in the knee joint capsule. Evans blue bound to plasma proteins restricted in vascular vessels. Consequently, its presence in the knee joint capsule suggested a change in vascular permeability in an arthritic knee. Our data demonstrated that arthritic knees showed a significant increase of 138 μg/g of Evans blue extravasation when compared with the control knees in the vehicle control ($p < 0.001$) (Fig. 3D). AC (100 mg/kg/day), AK (100 mg/kg/day) and AV (10 mg/kg/day) did

not reduce this difference between the knees ($p < 0.01$, $p < 0.01$ and $p < 0.001$, respectively). The differences of Evans blue extravasation were 86 μg/g, 89 μg/g and 111 μg/g for AC (100 mg/kg/day), AK (100 mg/kg/day) and AV (10 mg/kg/day), respectively. In contrast, AV (100 mg/kg/day) reduced the difference between the arthritic knees and the control knees (51 μg/g) and there was no statistical significance difference between the knees ($p > 0.05$). The difference of Evans blue extravasation between arthritic and control knees in AV (100 mg/kg/day) (51 μg/g) was significantly smaller than the vehicle control (138 μg/g) ($p < 0.01$) (Fig. 3D). Among the three *Aconitum* herbs included in this study, AV (100 mg/kg/day) showed the highest anti-arthritic effects which improved all of the allodynia, swelling, hyperaemia and vascular permeability in arthritic knees. In contrast, AC (100 mg/kg/day) and AK (100 mg/kg/day) showed improvements on some of these symptoms (Table 1). AK (100 mg/kg/day) improved joint swelling, hyperaemia and vascular permeability while AC (100 mg/kg/day) only improved joint allodynia. This is in line with a previous study showing that AC had no effect on vascular permeability in arthritis models (Kubo et al., 1990). Besides, its effect on joint edema varied depending on the periods of treatment (Kubo et al., 1990).

HPLC analysis showed that the major alkaloids in AV were vilmorrianine A and yunaconitine which were detected at 48.1 min and 31.5 min, respectively (Fig. 4). Their identities were confirmed by liquid chromatography/mass spectrometry (data not shown). These two alkaloids were chemical markers of AV and were undetectable in AC and AK (Xiao et al., 2006; Yang et al., 1981). Yunaconitine showed significantly analgesic effects against inflammation in mice (Lin et al., 1987; Yu et al., 1996). In contrast, study of the analgesic and anti-inflammatory effects of vilmorrianine A was rarely reported. A previous study showed that the subcutaneous injection and oral administration of 0.1 mg/kg/day yunaconitine also significantly inhibited the inflammation-induced vascular permeability and hind paw swelling in mice (Lin et al., 1987). Consequently, the presence of yunaconitine and other alkaloids might contribute to the anti-arthritic effect of AV. Further study using a bioassay-guided fractionation approach to identify the bioactive components in AV would be most rewarding.

In conclusion, AV was an effective anti-arthritic herb showing significant improvements of join allodynia, swelling, hyperaemia and vascular permeability in arthritic knees. It showed the highest anti-arthritic effects among the three tested *Aconitum* herbs. To the best of our knowledge, this is the first report on the anti-arthritic effects of the folk herbal medicine Huangcaowu derived from *A. vilmorinianum*. This work provided scientific evidence on the anti-arthritic effects of Huangcaowu. The next step is to carry out an in-depth safety evaluation and to investigate the preventive/prophylactic and therapeutic anti-arthritic effects of Hunagcaowu.

Table 1
Summary of the anti-arthritic effects of Aconiti Radix (AC; 100 mg/kg/day), Aconiti Kusnezoffii Radix (AK; 100 mg/kg/day) and Huangcaowu (AV; 10 mg/kg/day and 100 mg/kg/day) on FCA-induced arthritis in rats.

	AC (100 mg/kg/day)	AK (100 mg/kg/day)	AV (10 mg/kg/day)	AV (100 mg/kg/day)
Knee joint allodynia	+	+	+	+
Knee joint swelling	−	+	+	+
Knee joint hyperaemia	−	+	+	+
Knee joint vascular permeability	–	–	–	+

'+' represents significant improvement; '−' represents no significant effect.

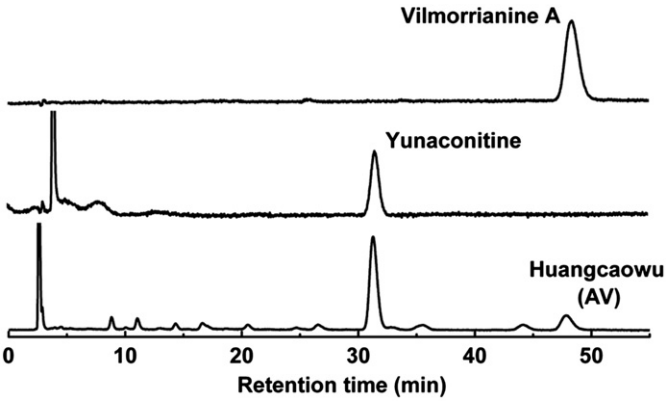

Fig. 4. High performance liquid chromatography profiles of Hunagcaowu (AV) extract were detected at 260 nm. Vilmorrianine A (48.1 min) and yunaconitine (31.5 min) were included as standard compounds.

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