Analgesic and Anti-inflammatory Activities of an Aqueous Extract of *Hydrocotyle batrachium* Hance in Mice

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Background/Purpose. To investigate the analgesic and anti-inflammatory effects of a water extract of *Hydrocotyle batrachium* Hance (HBW) in mice.

Methods. The analgesic effects of HBW were investigated by measuring the acetic acid-induced writhing response and the hind paw licking time following formalin injection. λ-Carrageenan (CARR)-induced paw edema was studied to explore the anti-inflammatory effect of HBW. The chromatograms of rutin, quercetin and HBW were obtained by high-performance liquid chromatography (HPLC).

Results. Treatment of male ICR mice with HBW (100, 500, 1000 mg/kg) inhibited the writhing response in a dose-dependent manner. The inhibitory effect of HBW at a dose of 1000 mg/kg was similar to that of indomethacin at a dose of 10 mg/kg. HBW significantly inhibited the degree of formalin-induced pain in the late phase. HBW (500, 1000 mg/kg) also inhibited the development of paw edema induced by CARR. The HPLC analysis revealed that rutin was an important bioactive compound in HBW.

Conclusion. HBW appears to have analgesic and ant-inflammatory activities. **(Mid Taiwan J Med 2008;13:179-85)**

Key words

ant-inflammation, formalin, high-performance liquid chromatography, *Hydrocotyle batrachium* Hance, writhing response, λ-carrageenan

INTRODUCTION

Hydrocotyle batrachium Hance, previously known as *H. formosana* Masamune [1], is the major component of Pian-di-jin, a common folk drug in Taiwan. The entire plant of *H. batrachium* Hance is used to treat the common cold, tonsillitis, nephrolith, cephalitis, enteritis and contusion [2]. Only a few studies have confirmed the pharmacological activity of members of the genus *Hydrocotyle*. For example, *H. sibthorpioides* Lam. has been shown to inhibit the growth of transplanted tumors in mice, such as hepatic carcinoma (Hep), sarcoma (S_{180}) and uterine cervical carcinoma (U_{14}). Both *H. leucocephala* Cham. & Schlecht. and *H. sibthorpioides* Lam. have been reported to have immunomodulatory effects [3,4].

In our previous (unpublished) study, the

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results from a series of in vitro tests, including the 2,2'-azinobis-(3-ethylbenzothiazoline)-6-sulphonic acid (ABTS) radical monocation scavenging test, the ferric reducing antioxidant power (FRAP) method, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging test and reducing power method, revealed that members of the genus *Hydrocotyle* displayed significant antioxidant activities. We also found that *H. nepalensis* Hook exhibited hepatoprotective and antioxidant effects in rats with carbon tetrachloride-induced liver damage.

However, little information is available on the analgesic and anti-inflammatory effects of *Hydrocotyle* species. Therefore, we examined the effects of *H. batrachium* Hance on acetic acid- and formalin-induced nociception in mice. We also evaluated the anti-inflammatory effects of *H. batrachium* Hance on paw edema induced by λ-carrageenan (CARR).

MATERIALS AND METHODS

Material

H. batrachium Hance was collected from Taichung, Nantou, and Hsinchu counties in Taiwan in 2006. The plants were identified and authenticated by Dr. Chao-Lin Kuo, Associate professor and Chairman, Department of Chinese Medicine Recourses, China Medical University, Taichung, Taiwan. A voucher specimen (No. CMU20060824) was deposited in the Graduate Institute of Chinese Pharmaceutical Sciences, China Medical University, Taichung, Taiwan.

Chemicals

Acetic acid and formalin were purchased from Merck (Darmstadt, Germany). λ-Carrageenan, indomethacin, rutin and quercetin were obtained from Sigma (St. Louis, MO, USA).

Extraction

The dried whole herb (100 g) was boiled in distilled water (1000 mL) for 60 min. The extracts were filtered and collected three times. The decoction (about 1000 mL) was evaporated to 10 mL and dried in a vacuum at 50°C. The yield obtained was 26.37% (w/w) from the water extract of *H. batrachium* Hance.

Animals

Male ICR mice (about 18 g) were obtained from the BioLASCO Taiwan Co., Ltd. The animals were kept in plexiglass cages at a constant temperature of 22 ± 1°C, relative humidity 55 ± 5% on a 12 h dark-light cycle for at least 2 weeks before the experiment. They were given food and water ad libitum. All experimental procedures were performed according to the NIH Guide for the Care and Use of Laboratory Animals. All tests were conducted under the guidelines of the International Association for the Study of Pain [5].

Acetic acid-induced writhing response

After a 2-week adaptation period, the mice (25 to 28 g) were randomly assigned to five groups (n = 6), including a normal group, a positive control group, and three HBW-treated groups, respectively. All drugs were administered intraperitoneally. The normal control group received only 0.1 mL/10 g normal saline [6,7]. The positive control animals were pretreated with indomethacin (10 mg/kg, i.p.) [8] 30 min before 1% acetic acid (0.1 mL/10 g) administration. Mice in each of the HBW-treated groups were pretreated with 100, 500 or 1000 mg/kg HBW p.o. 60 min before acetic acid (0.1 mL/10 g) administration. Five minutes after the i.p. injection of 1% acetic acid, the number of writhings during the following 10-min period was counted.

Formalin test

The antinociceptive activity of the drugs was determined using the formalin test as described by Dubuisson and Dennis [9]. The mice were randomly assigned to five groups (n = 6), including a normal, a positive control, and three HBW-treated groups. The normal control group received only normal saline. The animals were kept in a standard observation cage (30 cm × 12 cm × 13 cm) for 60 min before the test began. At the end of the one-hour acclimation period, 20 μL of 5% formalin was injected into the dorsal surface of the right hind-paw. The mice were then observed for 30 min and the amount of time spent licking the injected hind-paw was recorded. The first 5 min after formalin injection

was referred to as the early phase, and the period from 20 min to 30 min after injection was referred to as the late phase. HBW (100, 500, 1000 mg/kg, p.o.) was administered to mice in the HBW groups 60 min before formalin injection. Indomethacin (10 mg/kg, i.p.) was administered to mice in the positive control group 30 min before formalin injection. The total time spent licking or biting the injured paw (pain behavior) was measured with a stopwatch.

λ-Carrageenan (CARR)-induced paw edema

The anti-inflammatory activity of HBW was determined by the CARR-induced edema test [8,10]. Male ICR mice were randomly assigned to five groups (n = 6) and then fasted with free access to water for 24 hours before the experiment. Fifty microliters of a 1% suspension of CARR in saline, which had been prepared 30 min before each experiment, was injected into the plantar side of the right hind paws of the mice. After 60 min, HBW at doses of 100, 500 and 1000 mg/kg were administered orally, and after 90 min, indomethacin at the dose of 10 mg/kg was administered via an intraperitoneal route after the CARR treatment. Paw volume was measured prior to CARR injection and at 60, 120, 180, 240, 300 and 360 min intervals after the administration of the edematogenic agent using a plethysmometer (model 7159, Ugo Basile, Varese, Italy). The degree of swelling was evaluated according to the a-b value, where a is the volume of the right hind paw after CARR treatment and b is the volume of the right hind paw before CARR treatment. Indomethacin was used as a positive control compound [11].

Analysis of rutin, quercetin and HBW by HPLC

Five milligrams of HBW was weighed and dissolved in 5 mL methanol. The solutions were filtered through 0.45 μm PVDF filters. The HPLC (Waters 2695 separations module; detector: Waters 996 photodiode array detector) analysis was carried out under the following conditions: a Waters XTerra RP_{18} column (5 μm, 4.6×250 mm) was used with 0.05% phosphate buffer as mobile phase A, and acetonitrile was used as mobile phase B; the gradient elution was run with 30% solution B at 0 min, and 35% solution B at 30 min at a flow rate of 0.8 mL/min. The injection volume was 10 μL, and a wavelength of 254 nm was used for detection. Pure compounds, including rutin and quercetin, were also analyzed by HPLC under the same conditions. The retention time was used to identify the flavonoids in the samples.

Statistical analysis

Data are expressed as mean ± S.E.M. Statistical evaluation was carried out by one-way or two-way analysis of variance (ANOVA), followed by Scheffe's multiple range test. Statistical significance is expressed as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

RESULTS

The cumulative amount of abdominal stretching correlated with the level of acetic acid-induced pain. HBW treatment (100, 500 and 1000 mg/kg) significantly ($p < 0.001$) inhibited the control writhes in a dose-dependent manner (Fig. 1). The inhibitory effect of HBW (1000 mg/kg) was similar to that of indomethacin (10 mg/kg) ($p < 0.001$).

HBW (500 and 1000 mg/kg) significantly inhibited ($p < 0.001$) the formalin-induced pain in the late phase (Fig. 2B). The positive control

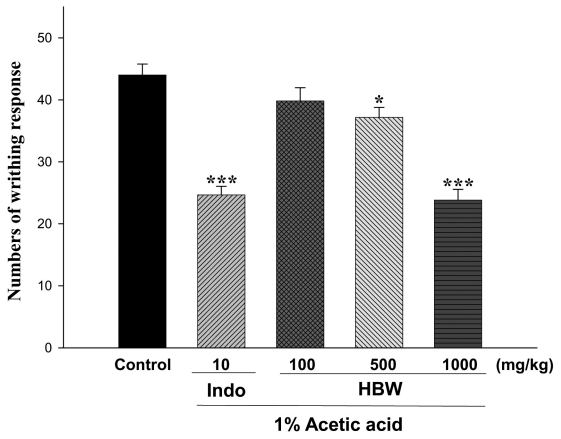

Fig. 1. The effect of HBW on 1% acetic acid-induced writhing response in mice. HBW (100, 500, 1000 mg/kg, p.o.) for 60 min or indomethacin (10 mg/kg, i.p.) for 30 min. Data are presented as mean ± SEM (n = 6). $*p < 0.05$, $***p < 0.001$ as compared with the control group. (One-way ANOVA followed by Scheffe's test).

indomethacin also significantly inhibited ($p < 0.001$) the formalin-induced pain in the late phase. Neither HBW nor indomethacin inhibited the formalin-induced pain in the early phase (Fig. 2A).

As shown in Fig. 3, CARR induced paw edema. HBW (500, 1000 mg/kg) significantly inhibited ($p < 0.001$) the development of paw edema induced by CARR at 180, 240, 300 and 360 min after the CARR treatment. However, HBW (100 mg/kg) did not significantly inhibit the development of edema. Indometacin (10 mg/kg) significantly decreased the CARR-induced paw edema at 120 ($p < 0.05$), 180, 240, 300 and 360 min after the CARR treatment ($p < 0.001$).

HBW was analyzed by HPLC. Its chromatogram is shown in Fig. 4. Both the standard (rutin) and HBW showed similar peaks at the retention time of 4.6 min. The other standard (quercetin) and HBW, however, did not show similar peaks at the same retention time. The chromatogram indicates that HBW contains the active ingredient rutin. The HPLC fingerprint of HBW could provide the chemical basis for future repetitive trials.

DISCUSSION

The acetic writhing test is normally used to study the peripheral analgesic effects of drugs. Although this test is nonspecific (eg, anti-cholinergic, antihistaminic and other agents also show activity in the test), it is widely used for analgesic screening and involves local cholinergic and histaminic receptors [12]. In this study, we found that HBW exhibited an antinociceptive effect on acetic acid-induced writhing response.

The formalin test is a valid and reliable model of nociception and is sensitive for testing various classes of analgesic drugs. The formalin test produces a distinct biphasic response and different analgesics might act differently in the early and late phases of this test. Therefore, the test can be used to clarify the possible antinociceptive mechanism of action of a proposed analgesic [13]. Central acting drugs such as opioids have been shown to exhibit inhibitory effects in both phases equally [12], whereas peripherally acting drugs such as aspirin,

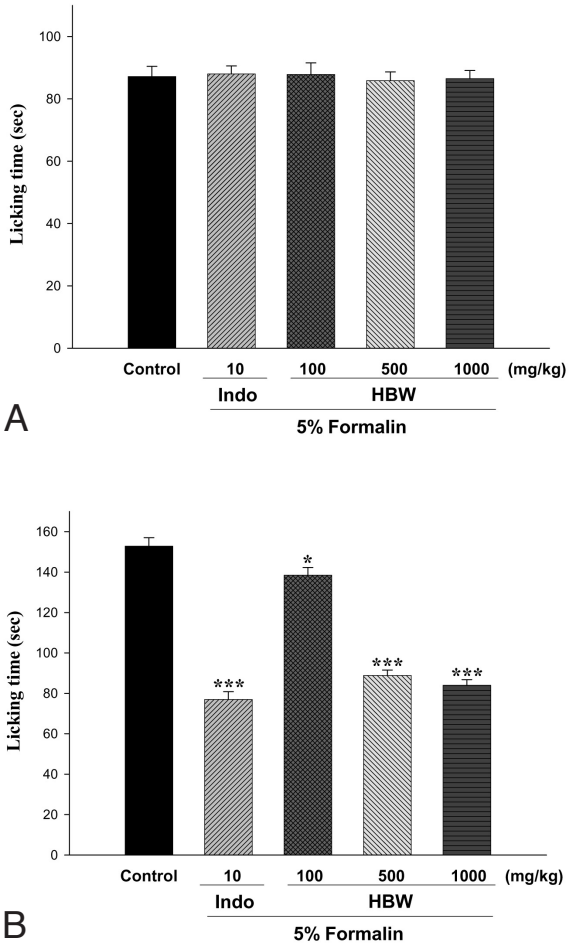

Fig. 2. The effects of HBW. A: Early phase (0 to 5 min). B: Late phase (20 to 30 min) of 5% formalin-induced inflammation in mice. Data are presented as mean ± SEM (n = 6). *$p < 0.05$, ***$p < 0.001$ as compared with the control group. (One-way ANOVA followed by Scheffe's test).

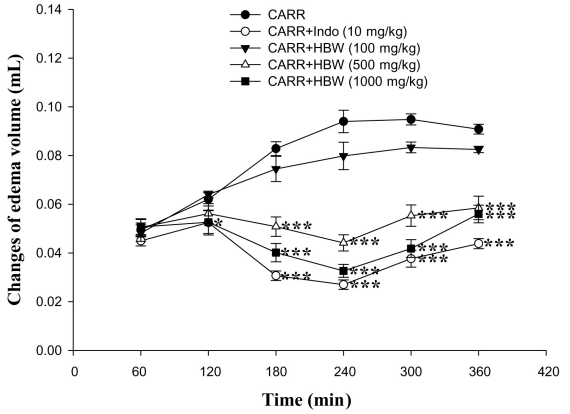

Fig. 3. The effects of HBW and indomethacin on mice hind paw edema induced by λ-carrageenan. Data are presented as mean ± SEM (n = 6). *$p < 0.05$, ***$p < 0.001$ as compared with the control group. (One-way ANOVA followed by Scheffe's test).

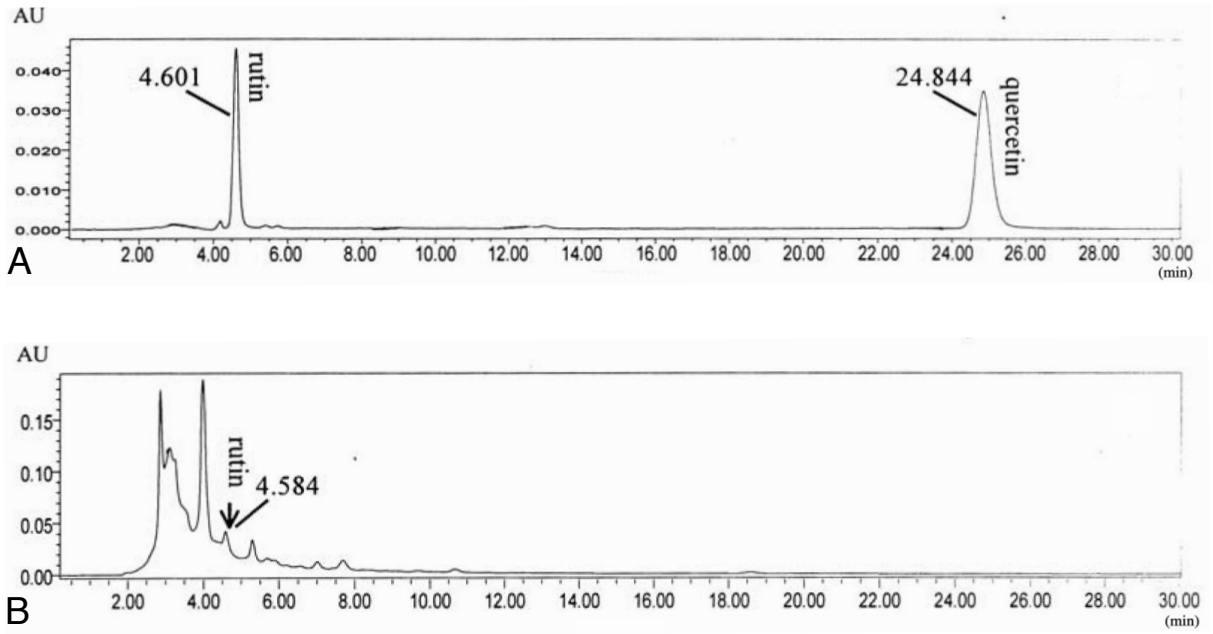

Fig. 4. A: HPLC chromatograms of standards (rutin and quercetin). B: HPLC chromatograms of HBW. Instrument: Waters 996; Column: the Waters XTerra RP_{18} column, 5 μm, 4.6 ×250 mm; Mobile phase: 0.05% phosphate buffer and acetonitrile (the gradient elution); Flow rate: 0.8 mL/min; detection: 254 nm. Retention time: 4.601 (rutin), 24.844 (quercetin). Sample (10 mg/mL water); rutin and quercetin (0.1 mg/mL ethanol); Inj. vol. 10 μL.

indomethacin and dexamethasone have been reported to exhibit inhibitory effects only in the late phase. The late phase seems to represent an inflammatory response to pain that can be inhibited by anti-inflammatory drugs [14,15]. The inhibitory effect of HBW on the nociceptive response in the late phase of the formalin test suggests that the antinociceptive effect of HBW might be due to its peripheral action.

The CARR test is highly sensitive to nonsteroidal anti-inflammatory drugs, and has long been accepted as a useful phlogistic tool for investigating new drug therapies [16]. The maximum degree of swelling of the CARR-injected paws occurred 240 min after injection. Statistical analysis revealed that HBW at doses of 500 and 1000 mg/kg significantly inhibited the development of edema at 180, 240, 300 and 360 min after treatment.

Polyphenols have been reported to exhibit analgesic and ant-inflammatory effects [17,18]. The HPLC analysis showed that HBW contained rutin, but not quercetin. Rutin, a glycoside comprising the flavonol quercetin and the disaccharide rutinose, is widely distributed in the plant kingdom and shows remarkable antioxidant, anti-inflammatory, and anticancer activities. It also has relaxing effects on smooth muscles [19]. Moreover, Galati et al showed that rutin was one of the components in the active anti-inflammatory methanol extract of *Hypericum rumeliacum* Boiss. subsp. *apollinis* (Boiss. & Heldr.) [20]. Rutin might be an important component in the analgesic and anti-inflammatory activities of the HBW.

Studies of members of the genus *Hydrocotyle* resulted in isolation of trans-β-farnesene, α-terpinenes, and thymol methyl ether from *H. sibthorpioides* Lam. and *H. maritime* Honda [21], quercetin-3-O-galactoside from *H. umbellata* L. [22], monogalactosylmonoacyl-glycerol from *H. ramiflora* Maxim. [23], oleane and ursane type glycosides from *H. ranunculoides* Blume [24,25] and *H. sibthorpioides* [26], diacetylene from *H. leucocephala* Cham. and Schlecht. [4]. However, no chemical studies on *H. batrachium* Hance have been reported. Furthermore, only a few reports on the flavonoid compounds of the genus *Hydrocotyle* have been published. Therefore, further studies of the phytochemicals of the genus Hydrocotyle are needed.

In conclusion, this study demonstrates that HBW possesses analgesic and anti-inflammatory activities. Further studies are necessary to elucidate the mechanisms of action.

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台灣天胡荽水萃取物於小鼠之鎮痛及抗發炎活性

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背景/目的 本研究探討台灣天胡荽水萃取物於小鼠之鎮痛及抗發炎作用。

方法 研究台灣天胡荽水萃取物的鎮痛作用，以測量醋酸所誘導之扭體反應及福馬林注射後足蹠之舔足時間試驗進行。而λ-角叉菜膠(CARR)誘導足蹠浮腫試驗，可用於探討台灣天胡荽水萃取物之抗發炎作用。蘆丁(rutin)、槲皮素(quercetin)及台灣天胡荽水萃取物之高效液相層析分析圖譜被建立。

結果 以台灣天胡荽水萃取物100 、500及1000 mg/kg給藥的雄性ICR小鼠，對小鼠醋酸扭體反應次數產生了一個有意義且劑量依賴的抑制作用，而台灣天胡荽水萃取物在1000 mg/kg濃度時，其抑制效果相同於吲哚美辛(indomethacin) 在10 mg/kg的抑制作用。台灣天胡荽水萃取物有意義的抑制福馬林所誘導的後期疼痛。台灣天胡荽水萃取物於500及1000 mg/kg濃度下，也抑制了λ-角叉菜膠所誘導的足蹠浮腫現象。在高效液相層析分析中，我們則發現蘆丁可能是台灣天胡荽水萃取物中的一個重要活性化合物。

結論 台灣天胡荽水萃取物可能具有鎮痛及抗發炎活性。（中台灣醫誌 **2008;13:179-85**）

關鍵詞

抗發炎，福馬林，高效液相層析法，台灣天胡荽，扭體反應，λ-角叉菜膠

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