Inhibition of Itch-Scratch Response by Fruits of *Cnidium monnieri* in Mice

Purusotam BASNET,[a] Ikuyo YASUDA,[b] Noriko KUMAGAI,[b] Chihiro TOHDA,[a] Hiroshi NOJIMA,[b] Yasushi KURAISHI,[b] and Katsuko KOMATSU*,[a]

Research Center for Ethnomedicines, Institute of Natural Medicine,[a] and Department of Applied Pharmacology, Faculty of Pharmaceutical Sciences,[b] Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930–0194, Japan.
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We previously screened the anti-itching activities of 33 herbal medicines in substance P (SP)-induced itching model mice. One of the most potent antipruritogenic extracts, the methanol extract of fruits of *Cnidium monnieri* (Cnidii Fructus) was studied further. The chloroform-soluble fraction of the methanol extract markedly inhibited SP-induced scratching. Among 10 subfractions of the chloroform-soluble fraction, the CS-3 fraction had the most potent inhibitory effect on scratching. Each of 3 subfractions of CS-3 showed significant anti-scratching activities. However, inhibitory potencies were not different among the three and weaker than that of CS-3 itself at a same dose. These 3 subfractions of CS-3 mainly contained xanthotoxin, isopimpinellin, bergapten, imperatorin and osthol. Single administration of osthol did not inhibit SP-induced scratching, and imperatorin very weakly subsided scratching. These results suggest that the strong antipruritic action was focused on the CS-3 fraction of the *C. monnieri* methanol extract, and it might result from the combined effects of these coumarin derivatives, or by undetermined minor compounds.

Key words *Cnidium monnieri*; substance P; itch-scratch response; coumarin derivative

Itching is an unpleasant sensation in skin diseases such as dermatitis and urticaria, which is not often subsided by presently available medicines. Since inhibition of itching can break off a vicious circle of scratching and worsening of the lesions, novel drugs for severe pruritus are needed. For this purpose, we previously screened the anti-itching activities of 33 herbal medicines in substance P (SP)-induced itching model mice.[1] One of the most potent antipruritogenic extracts of these herbal medicines taken orally (*p.o.*) was the methanol extract of Cnidii Fructus (Chinese name *Shechaungzi* and Japanese name *Jashoshi*). Cnidii Fructus, the dried fruits of *Cnidium monnieri* (L.) CUSSON (Umbelliferae), is an important crude drug and is used in traditional Chinese medicine for the treatment of eczema, cutaneous pruritus and *Trichomonas vaginalis*[2] as an external medicine, as well as for impotence and frigidity as an internal medicine.[3] Recent pharmacological studies have revealed antiallergic,[4,5] antidermatophytic,[6] antiosteoporotic,[7—9] and antibacterial and antifungal[10] activities for the crude extracts or isolated constituents from this drug. Coumarin derivatives such as osthol, imperatorin, xanthotoxin, isopimpinellin, and bergapten, have been reported as the main constituents from *C. monnieri.*[11] However, the inhibitory effect of *C. monnieri* on "itching" has never been studied in detail.

SP is a potent pruritogenic endogenous peptide in humans.[12] Experimental evidences has shown that SP elicits scratching through a histamine-independent mechanism in mice, and that SP-induced scratching was due to an itch sensation.[13] The purpose of this study were to identify candidates for anti-pruritic constituents in the methanol extract of *C. monnieri* using SP-induced itch model mice.

MATERIALS AND METHODS

Plant Material The fruits of *Cnidium monnieri* (L.) CUSSON were purchased from Tochimoto Tenkaido Co., Ltd., Osaka, Japan. A voucher specimen is deposited in the Museum of Materia Medica, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University, Toyama, Japan (TMPW No. 18211).

Extraction and Fractionation Dried fruits (5 kg) of *C. monnieri* were pulverized and extracted three times with methanol (15 l×3) under reflux conditions for 3 h for the first time and two hours for the second and third extractions. The combined extracts were evaporated on a rotatory evaporator under reduced pressure to obtain a viscous alcoholic extract. This was suspended in water and lyophilized to obtain the methanol extract. This lyophilized methanol extract was suspended in water and partitioned with chloroform (3 l×3) to give a chloroform-soluble fraction (575 g) and an aqueous fraction (21.5 g). The chloroform-soluble fraction was found to be the most active fraction and was subjected to further purification.

The chloroform-soluble fraction was subjected to column chromatography on silica gel (Wako gel C 200, Wako Pure Chemical, Ind., Ltd., Osaka, Japan) eluting with the chloroform and gradually increasing methanol concentration to obtain 10 fractions (CS-1 to CS-10). Further preparative HPLC analysis was carried out in the CS-3 fraction. CS-3 was divided into 3 subfractions (3-a, -b and -c) guided by HPLC peak patterns. Subfractions 3-a, 3-b and 3-c were purified by preparative HPLC and shown to contain mainly xanthotoxin, isopimpinellin and bergapten (3-a), imperatorin (3-b) and osthol (3-c) by ^{1}H- and ^{13}C-NMR analyses in comparison with the literature (Chart 1).[14—17]

HPLC Method The HPLC was run with a SIL-10AXL Shimadzu autoinjector with a Shimadzu C-R6A Chromopac recorder. The following conditions were used; column: YMC pack ODS AQ (6 mm×150 mm), flow rate: 1 ml/min; column temperature: 40 °C; detector: UV 320 nm, and eluent: methanol–water–CH_3CN (44 : 42 : 14).

Animals Male ICR mice (Japan SLC, Shizuoka, Japan) 5 to 11 weeks old were used. They were housed under controlled temperature (23—25 °C) and light (lights on from 8 : 00 to 20 : 00). Food and water were given freely.

Behavioral Observation The hair of mice was clipped

* To whom correspondence should be addressed. e-mail: katsukok@ms.toyama-mpu.ac.jp

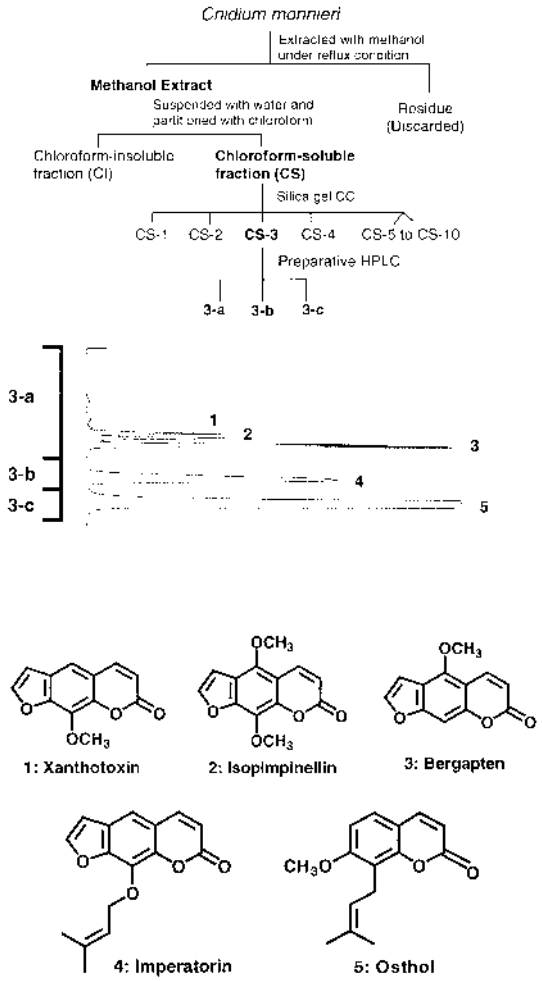

Chart 1. Flow Diagram of Extraction and Fractionations of Cnidii Fructus. HPLC Chromatogram of CS-3 and Chemical Structures of Xanthotoxin (1), Isopimpinellin (2), Bergapten (3), Imperatorin (4) and Osthol (5).

over the rostral part of the back the day before the experiment. Before behavioral recording, the mice were put into an acrylic cage (26×18×30 cm) composed of 4 cells for 1 h acclimation. The extract, fractions or compounds were administered *p.o.* as a suspension in 5% arabic gum. Thirty minutes later, SP (Peptide Institute, Osaka, Japan) at a dose of 100 nmol/site was injected i.d. in 50 μl physiological saline into the rostral part of the mice. Immediately after i.d. injection, the mice were put back into the same cell and the activity of the mice was videotaped for 30 min, in an environment without any experimenter. The number of scratches on the injected site by the hind paws was counted as an index of itch response with the help of a video recorder.[1)]

Locomotion activity was measured using a wheel cage (MODEL SW-20, Toyo Sangyo Co., Ltd., Toyama, Japan). Thirty minutes after *p.o.* administration of the extract, fractions or saline, the mice were placed in a wheel cage and the number of revolutions was counted for 20 min.[1)]

Data Analysis Data were analyzed with the one-way analysis of variance followed by post hoc Dunnett's test; a p value 0.05 or less was considered to be significant. All data are presented as means together with S.E.M.

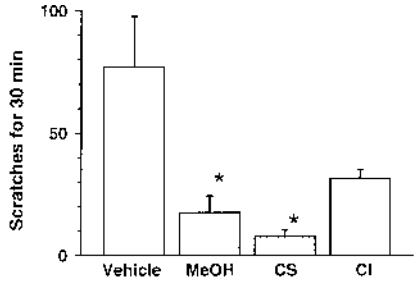

Fig. 1. Effects of Methanol Extract (MeOH), and Chloroform-Soluble (CS) and Chloroform-Insoluble (CI) Fractions of Methanol Extract of Cnidii Fructus on SP-Induced Scratching in Mice

The methanol extract, CS, CI, and/or vehicle (5% arabic gum) were *p.o.* administered at a dose of 200 mg/kg 30 min before the intradermal injection of SP (100 nmol/site). Immediately after the injection of SP, the number of scratches was counted for a period of 30 min. Each value represents the mean±S.E.M. (n=8). $*p<0.05$ when compared with vehicle.

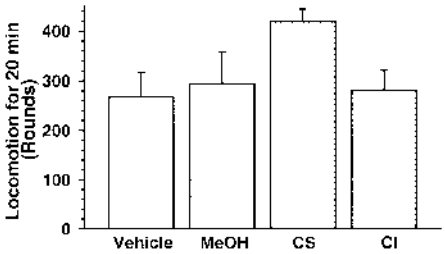

Fig. 2. Effects of Methanol Extract (MeOH), and Chloroform-Soluble (CS) and Chloroform-Insoluble (CI) Fractions of Methanol Extract on the Locomotion Activity

The methanol extract, CS and CI were each *p.o.* administered at a dose of 200 mg/kg. Thirty minutes later, the mice were placed in a wheel cage and the number of revolution was counted. Each value represents the mean±S.E.M. (n=8).

RESULTS

The methanol extract from *C. monnieri* was partitioned with chloroform to obtain chloroform-soluble and -insoluble fractions. The effects of the methanol extract, chloroform-soluble and -insoluble fractions on SP-induced scratching were examined at a dose of 200 mg/kg. The methanol extract and its chloroform-soluble fraction markedly suppressed the scratching response induced by SP, and the effect of the chloroform-soluble fraction was stronger than that of the methanol extract. On the other hand, the chloroform-insoluble fraction tended to suppress the scratching response but not significantly (Fig. 1).

The effect of the methanol extract and its fractions on locomotion activity was studied. Neither the methanol extract nor chloroform-soluble or -insoluble fractions at a dose of 200 mg/kg, affected the locomotion activity (Fig. 2).

The chloroform-soluble fraction obtained as an active fraction was further fractionated into 10 fractions (CS-1 to CS-10) by silica gel column chromatography. We examined the effects of 10 fractions (CS-1 to CS-10) on SP-induced scratching at a dose of 200 mg/kg. The CS-2, CS-3 and CS-4 fractions significantly suppressed the scratching, however among the three, CS-3 was found to be the most active, with 96.0% inhibition (Fig. 3). The CS-3 fraction was further separated by preparative HPLC (Chart 1). Subfractions 3-a (37.1%), 3-b (29.0%) and 3-c (50.8%) significantly inhibited SP-induced scratching at a dose of 100 mg/kg (Fig. 4). However, the potencies of these 3 subfractions were not stronger

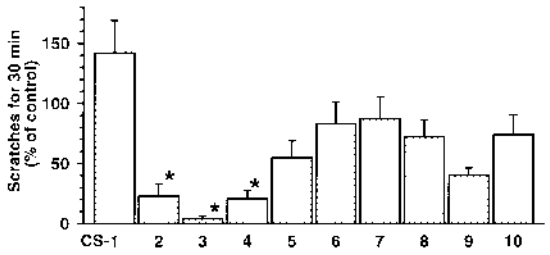

Fig. 3. Effects of the Fractions (CS-1 to CS-10) Obtained after Silica Gel Column Chromatography of the Chloroform-Soluble (CS) Fraction

Each fraction was *p.o.* administered at a dose 200 mg/kg 30 min before an intradermal injection of SP (100 nmol/site). Immediately after the injection of SP, the number of scratches was counted for a period of 30 min. Each value represents the mean±S.E.M. (n=7—8). $*p<0.05$ when compared with control.

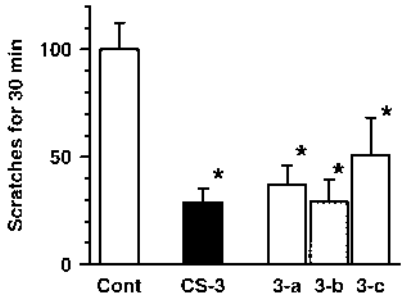

Fig. 4. Effects of the Subfractions (3-a, 3-b and 3-c) of CS-3 Obtained by Preparative HPLC. CS-3 (a Closed Column), Subfractions (3-a, 3-b and 3-c, Hatched Columns) or Vehicle (Cont, an Open Column) was *p.o.* Administered at a Dose of 100 mg/kg 30 min before an Intradermal Injection of SP (100 nmol/Site)

Immediately after the injection of SP, the number of scratches was counted for a period of 30 min. Each value represents the mean±S.E.M. (n=8). $*p<0.05$ when compared with control.

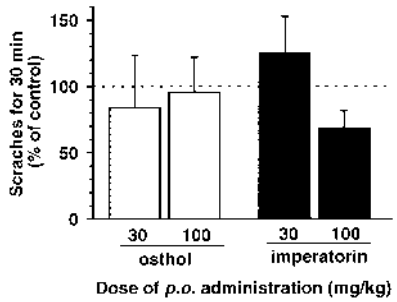

Fig. 5. Effects of Osthol and Imperatorin, Isolated as the Major Compounds from CS-3, on SP-Induced Scratching in Mice

Each drug was *p.o.* administered at a dose of 30 or 100 mg/kg 30 min before an intradermal injection of SP (100 nmol/site). Immediately after the injection of SP, the number of scratches was counted for a period of 20 min. Each value represents the mean± S.E.M. (n=8).

than that of CS-3 fraction (28.6%) at the same dose, therefore a certain subfraction with the most potent activity was not found. Collected peaks in preparative HPLC were analyzed by ^{1}H- and ^{13}C-MNR, and five main compounds were identified. The 3-a subfraction contained xanthotoxin, isopimpinellin and bergapten, and 3-b and 3-c contained mainly imperatorin and osthol, respectively. Among them, osthol, a major component of 3-c, did not inhibit SP-induced itch response at doses of 30 and 100 mg/kg (Fig. 5). Imperatorin, a major component of 3-b, very weakly inhibited the scratching (68.4%) at a dose of 100 mg/kg.

DISCUSSION

In the present experiment, we showed that the chloroform-soluble fraction of the methanol extract of *C. monnieri* fruits decreased SP-induced itch-scratch responses in ICR mice. The methanol extract and its chloroform-soluble fraction did not inhibit the locomotion activity at 200 mg/kg, a dose which was effective against scratch response induced by SP. Therefore, the scratch-inhibiting action of these extract and fraction may be due to their inhibition of the itching sensation and/or something reflex, rather than to sedation or depression of general functions of the central nervous system.

Activity-guided fractionation and purification using the SP-induced scratching model led to the most active fraction, CS-3 of the chloroform-soluble fraction. As the methanol extract was further purified, the inhibitory potencies become stronger the methanol extract, the chloroform-soluble fraction and CS-3 fraction inhibited scratching at 22.7%, 10.1% and 4.0% of the control, respectively at a dose of 200 mg/kg. Each of 3 subfractions of CS-3 (3-a, -b and -c) did not show more stronger inhibitory effects on scratching than that of CS-3 at a dose of 100 mg/kg. Since these subfractions were separated by preparative HPLC peaks, it might not seem that active compounds were divided among the three subfractions. Nevertheless, activity did not become more intense after subfractionation. This suggests that the effect of CS-3 may be due to additive or synergistic action of multiple compounds, and not by a single constituent.

The CS-3 fraction was found to be the most active fraction for anti-itching activity. The 3 subfractions of CS-3 mainly consisted of five coumarin derivatives, xanthotoxin, isopimpinellin, bergapten, imperatorin and osthol. Imperatorin and osthol which were the main constituents of 3-b and 3-c, respectively, did not inhibit scratching significantly at doses 30 and 100 mg/kg, although imperatorin (100 mg/kg) weakly subsided the scratching. This suggests that minor compounds, other than imperatorin and osthol, may be involved in the anti-itching activity of at least the 3-b and 3-c subfractions. Subfraction 3-a mainly contains xanthotoxin, bergapten and imperatorin. Although the pharmacological effects of isopimpinellin are unknown, xanthotoxin and bergapten are known to be used in skin chemotherapy in combination with long-wave ultraviolet radiation,[18] and have inhibitory activity on formalin-induced inflammation in the skin.[19] Imperatorin shows anti-inflammatory effect against inflammation induced by picryl chloride[4] and an inhibitory effect on nitric oxide production.[20] Osthol has an inhibitory effect on formalin-induced inflammation in the skin.[19] Although there are no reports on the use of these coumarin derivatives as anti-pruritus agents, evidences suggests that they may have an anti-inflammatory effect. Since SP is an important neuropeptide eliciting inflammation[21,22] as well as an itch sensation at the cutaneous site, it is possible that coumarin derivatives contained in the CS-3 fraction may inhibit the pathway of SP-triggered signal transduction, and therefore subside the itch sensation.

In the present study, we focused on the CS-3 fraction from the methanol extract of *C. monnier*, which markedly inhibited an itch sensation induced by SP, and showed that the effect of CS-3 might be displayed by the combined effects of coumarin compounds. We will study further which combina-

tion of compounds is necessary and the most effective for anti-itching, and whether there are any other minor active compounds in CS-3.

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