The Anti-inflammatory and Anti-nociceptive Effects of Ethyl Acetate Fraction of *Cynanchi Paniculati Radix*

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The anti-inflammatory and anti-nociceptive effects and sedative activities of the ethyl acetate fraction of *Cynanchum paniculatum* (EACP) were evaluated in mice and rats by acetic acid-induced vascular permeability, arachidonic acid-induced paw edema, cotton pellet-induced granuloma formation, formalin-induced licking time, acetic acid-induced writhing response, and pentobarbital-induced sleeping time. EACP at a dose of 40 mg/kg significantly exhibited anti-inflammatory activities on acetic acid-induced vascular permeability, arachidonic acid-induced paw edema, and the late phase of formalin-induced licking time. Moreover, it showed anti-nociceptive effects on acetic acid-induced writhing responses and significant sedative effects on pentobarbital-induced sleeping time. The results demonstrated that the anti-nociceptive effects are apparently related to the sedative effects of EACP. These results support the use of *Cynanchum paniculatum* in relieving inflammatory pain.

Key words *Cynanchum paniculatum*; Asclepiadaceae; anti-inflammatory effect; anti-nociceptive effect; cotton pellet; writhing response

Cynanchum paniculatum KITAG. (Asclepiadaceae) is a vivacious plant distributed across all of Korea and also cultivated in China. The root of *Cynanchum paniculatum* is used as a Chinese traditional medicine. Recently, the plants of genus *Cynanchum* of Asclepiadaceae, e.g. *Cynanchum atratum*, *Cynanchum auriculatum* and *Cynanchum paniculatum*, were studied for their anticancer and analgesic effects.^{1–3} In addition, *Cynanchum paniculatum* has been traditionally used for gastritis and relieving pain in China. In traditional medicine, the dried root of *Cynanchum paniculatum* relieves rheumatic arthralgia, lumbago, pain due to traumatic injuries, abdominal pain, toothache, and other kinds of pain, as well as skin diseases such as eczema, rubella, neurodermatitis and snake bite.⁴ A number of chemical constituents such as cynapanosides A, B, and C, paeonol, and antopine have been identified from *Cynanchum paniculatum*,^{3,5,6} of which paeonol had been found to have analgesic effects in mice.⁶ These studies concentrated on its analgesic and anticancer effects, without investigating its anti-inflammatory effect. Therefore, the present study employed NSAIDs; indomethacin, which is a non-selective cyclooxygenase (COX) inhibitor, and phenidone, which is a dual blocker of COX and lipoxygenase (LOX), to compare the anti-inflammatory effects with EACP. Pentobarbital sodium was used to study the sedative effect. The present study was undertaken to further elucidate the anti-inflammatory and anti-nociceptive effects of *Cynanchum paniculatum*, using the ethyl acetate (EtOAc)-soluble portion of the 70% methanol extract of its roots.

MATERIALS AND METHODS

Plant Material The roots of *Cynanchum paniculatum* were purchased from Young-chang Co., Seoul, Korea. This plant material was authenticated by Dr. D. Y. Kwon and a

voucher specimen (No. 05-010) was deposited in the laboratory of herbology, College of Pharmacy, Wonkwang University, Iksan, Korea.

Preparation of Fraction The roots of *Cynanchum paniculatum* (1 kg) were extracted with 70% MeOH three times for 2 h under heating mantle-reflux. The resultant extract was condensed to 1 l in a rotary vacuum evaporator (N-1000S, EYELA, Japan) and in succession was partitioned with hexane, EtOAc and 1-butanol (BuOH). After each partition, the solutions were filtered and the solvents were evaporated in a rotary vacuum evaporator. The extract yielded hexane (13.0 g), EtOAc (9.85 g), BuOH (40.4 g) and water (103.7 g) soluble fractions. All of the samples were stored at –4 °C. The samples of the fractions and reference drugs were suspended in 5% Tween 80.

Animals Male Sprague–Dawley rats (100–120, 180–200 g) and male ICR mice (30 ± 3 g) were purchased from SAMTAKO BIOKOREA, Korea and used in the experiments. All animals were kept in a temperature-controlled room with a 12 h light–12 h dark cycle. The animals had free access to commercial solid food (SCF Co., Ltd., Korea) and water *ad libitum*. They were acclimatized for at least 1 week before starting the experiments.

Drugs and Chemicals Evans Blue, acetic acid, arachidonic acid (AA), aspirin, indomethacin, prednisolone, and formalin were purchased from Sigma, U.S.A.; Tween 80 from Duksan, Korea; NaHCO₃ and Na₂CO₃ from Daejung, Korea; and Paeonol standard (No. 169-12871) from Wako, Japan.

Acetic Acid-Induced Vascular Permeability Whittle's⁷⁾ method was used with some modification. In brief, male mice weighing 27–33 g were fasted for 10 h prior to the experiments and were then orally given the test drugs and vehicle. Each animal was given an intravenous injection of a 1%

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solution of Evans blue as 0.1 ml/10 g at 30 min after the oral treatment. The vascular permeability inducer, 0.1 ml/10 g of 0.6% acetic acid in saline, was injected intraperitoneally at 30 min after Evans blue injection. After 20 min, the mice were killed by dislocation of the neck and 10 ml of normal saline was injected intraperitoneally, after which the washing solution was collected in tubes and then centrifuged at 2000 rpm for 10 min. The absorbance of the supernatant was read at 610 nm with a spectrophotometer (UV-2401PC, Shimadzu, Japan). The control group was treated similarly except that they received an oral dose of vehicle alone. The vascular permeability was expressed in terms of the amount of total dye ($\mu\text{g}/\text{mouse}$) which was leaked into the intraperitoneal cavity.

Arachidonic Acid-Induced Paw Edema Paw edema was produced in rats by AA following the methods of Di-Martino *et al.*⁸⁾ Male rats weighing 100–120 g were used. A volume of 0.1 ml of 0.5% AA in 0.2 M carbonate buffer, at pH 8.4, was injected into the plantar surface of the right hind paw of the rats. Test drugs and vehicle were given at 2 h prior to AA injection. The edema volume was measured using a plethysmometer (7140, Ugo Basile, Italy) prior to and 1 h after AA injection. The control group received vehicle only. Results were obtained with the increasing volume of edema and compared with the control group.

Cotton Pellet-Induced Granuloma Formation This was carried out using the method of Swingle and Shideman.⁹⁾ Male rats weighing 180–200 g were used. Test drugs were administered orally on a once daily dosage regimen for 7 d, while the control group received vehicle only. Two sterilized pellets of cotton weighing 20 ± 1 mg were implanted subcutaneously, one on each side of the abdomen of the animal, under anesthesia and sterile technique. The rats were sacrificed on the eighth day. The implanted pellets were dissected out and recorded for wet weight. The thymus was also dissected out. Both pellet and thymus were dried at 60 °C for 18 h and the dry weight was recorded. The transudative weight, granuloma formation and the percent granuloma inhibition of the test drugs were calculated. The body weight and dry weight of the thymus were also recorded.

Formalin-Induced Licking Time The formalin test possesses two distinctive phases, possibly reflecting different types of pain. The method of Hunskaar and Hole¹⁰⁾ was used with some modification. Pain was induced by injecting 20 μl of 2.5% formalin (1% formaldehyde, Sigma) in distilled water into the subplantar area of the right hind paw. Mice were given EACP (20, 40 mg/kg, *p.o.*), aspirin (300 mg/kg), indomethacin (10 mg/kg), and 5% Tween 80 (*p.o.*) at 60 min prior to injecting formalin. These mice were individually placed in a transparent plexiglass cage (25 cm $\times$ 25 cm $\times$ 20 cm) observation chamber. The amount of time spent licking and biting the injected paw was recorded in 0–5 min (early phase) and 15–30 min (late phase) as an indication of pain.

Acetic Acid-Induced Writhing Response Male Swiss albino mice weighing 30 ± 3 g were used. The study was carried out as described by Nakamura *et al.*¹¹⁾ Test drugs and control vehicle were orally administered 60 min before the acetic acid injection. A writhing response was produced by injection of an aqueous solution of 0.75% acetic acid in a volume of 0.1 ml/10 g body weight into the peritoneal cavity

and the animals were then placed in a transparent plexiglass chamber. The number of writhes, a response consisting of contraction of the abdominal wall and pelvic rotation followed by hind limb extension, was counted during continuous observation for 15 min beginning 5 min after the acetic acid injection.

Pentobarbital-Induced Sleeping Time The effect of EACP on pentobarbital-induced sleeping time was studied in mice as described previously by Pieretti *et al.*¹²⁾ Thirty minutes after administration of 5% Tween 80 and EACP (12.5, 25 mg/kg, *i.p.*), all mice received pentobarbital sodium (40 ml/kg, *i.p.*). The time between loss and subsequent recovery of the righting reflex was taken as the sleeping time and was recorded. The amount of sleep time was compared with the control and test drugs group.

High-Performance Liquid Chromatography (HPLC) The chromatographic system consisted of a pump (Shimadzu LC-10AT VP) and a UV detector (Shimadzu LC-SPD-10A VP). A Shim-pack VP-ODS column (4.6 $\times$ 250 mm, Shimadzu) was used. An isocratic of $\text{CH}_3\text{CN} : \text{H}_2\text{O}$ (60 : 40) was used as the mobile phase at a flow rate of 1.0 ml/min. The HPLC was monitored at 275 nm.

Statistical Analysis The data from the experiments are presented as mean $\pm$ S.E. The level of statistical significance was determined by analysis of variance (ANOVA) followed by Dunnett's *t*-test for multiple comparisons. **p* values less than 0.05 were considered to be significant.

RESULTS

Acetic Acid-Induced Vascular Permeability The oral administration of EACP at doses of 10, 20 and 40 mg/kg inhibited the increase of dye leakage into the peritoneal cavity in mice (Fig. 1). Its potency was comparable to that of 10 mg/kg of indomethacin. Among the fractions obtained from the methanol extract, only EACP showed a significant inhibition of vascular permeability (data not shown). Its inhibitory effect at doses of 10, 20 and 40 mg/kg of the ethyl acetate fraction was 10.5%, 24.2% and 28.8%, respectively, in comparison with that of the control group.

Arachidonic Acid-Induced Paw Edema The results for EACP on AA-induced hind paw edema in rats are shown in Table 1. EACP produced a dose-dependent reduction of paw edema. EACP at an oral dose of 40 mg/kg as well as phenidone exerted a significant reduction of paw edema.

Fig. 1. The Effects of EACP on Acetic Acid-Induced Vascular Permeability in Mice

Animals were orally pretreated by vehicle, indomethacin and EtOAc fraction, **p* < 0.05, significant as compared to the control by Dunnett's *t*-test. Indo: Indomethacin.

Cotton Pellet-Induced Granuloma Formation Table 2 shows the effect of EACP on cotton pellet-induced granuloma formation in rats. The results indicate that EACP at an oral dose of 30 mg/kg slightly inhibited the transudate but did not influence granuloma formation, thymus weight or body weight gain. Indomethacin significantly inhibited the transudative weight and granuloma formation but did not influence thymus weight or body weight gain. Prednisolone (5 mg/kg) elicited marked reduction in all parameters.

Formalin-Induced Licking Time The effects of EACP in the early and late phases of the formalin test are shown in Fig. 2. Aspirin (300 mg/kg), indomethacin (10 mg/kg) and EACP (20, 40 mg/kg) did not inhibit licking time in the early phase, but did significantly inhibit licking time in the late phase of the formalin test. In the late phase, EACP (40 mg/kg) possessed an inhibitory effect superior to that of aspirin (300 mg/kg), but weaker than that of indomethacin (10 mg/kg).

Acetic Acid-Induced Writhing Response The effect of EACP on writhing response in mice is shown in Fig. 3. It was found that EACP (40 mg/kg) inhibited the writhing response induced by acetic acid. The other fraction, consisting of hexane (150 mg/kg), BuOH (300 mg/kg) and water (300 mg/kg) did not inhibit the writhing response. Indomethacin (10 mg/kg) significantly inhibited the writhing response.

Pentobarbital-Induced Sleeping Time The values of sleeping time are presented in Fig. 4. EACP at doses of 12.5 and 25 mg/kg significantly extended pentobarbital induced-sleeping time, while 40 mg/kg significantly extended the sleeping time over 8 h (data not shown).

Standardization of *Cynanchum paniculatum* We standardized EACP using HPLC. The structure of paeonol was analyzed by a combination of ^1H - and ^{13}C -NMR (data not shown). HPLC indicated that the principle contained in the ethyl acetate fraction of *Cynanchum paniculatum* contained was paeonol (t_R 21.60 min) (Fig. 5).

Table 1. Effects of EACP on Arachidonic Acid-Induced Hind Paw Edema in Rats

Group	Dose (mg/kg)	EV (ml)	EI (%)
Control	—	0.53 ± 0.01	—
Phenidone	40	0.41 ± 0.01*	22.6
EACP	20	0.49 ± 0.01	7.5
EACP	40	0.44 ± 0.01*	17.0

Results are expressed as mean ± S.E. ($n=6$). * $p<0.05$, significant as compared to the control by Dunnett's t -test. EV: edema volume, EI: edema inhibition.

Table 2. Effects of EACP on Cotton Pellet-Induced Granuloma Formation in Rats

Group	Dose (mg/kg)	Transudate weight (mg)	GW/C (mg/mg)	GI (%)	Dry thymus weight (mg/100 g)	BWG (g)
Control	—	288.4 ± 5.8	2.4 ± 0.1	—	62.4 ± 2.0	51.7 ± 1.1
Indomethacin	5	216.3 ± 6.2**	1.7 ± 0.1**	29.2	59.1 ± 2.2	56.3 ± 2.8
Prednisolone	5	209.4 ± 4.6**	1.5 ± 0.1**	37.5	34.9 ± 2.1**	23.2 ± 1.6**
EACP	15	283.4 ± 5.8	2.4 ± 0.1	0	62.6 ± 1.8	52.0 ± 1.8
EACP	30	268.3 ± 5.2*	2.3 ± 0.1	4.2	62.6 ± 1.2	53.5 ± 1.9

Results are expressed as mean ± S.E. ($n=6$). * $p<0.05$, significant as compared to the control by Dunnett's t -test. ** $p<0.001$, significant as compared to the control by Dunnett's t -test. GW/C: granuloma weight/cotton, GI: granuloma inhibition, BWG: Body weight gain.

DISCUSSION

The anti-inflammatory and anti-nociceptive effects of EACP were investigated in *in vivo* animal models. The anti-

Fig. 2. The Effects of EACP on Formalin-Induced Licking Time in Mice
ASA: aspirin, Indo: indomethacin. * $p<0.05$, significant as compared to the control by Dunnett's t -test.

Fig. 3. The Effects of the Hexane, EtOAc, BuOH and H₂O Fraction of *Cynanchum paniculatum* on Acetic Acid-Induced Writhing Response in Mice
* $p<0.05$, significant as compared to the control by Dunnett's t -test.

Fig. 4. The Effects of EACP on Pentobarbital-Induced Sleeping Time in Mice

* $p<0.05$, significant as compared to the control by Dunnett's t -test.

Fig. 5. HPLC Chromatograms of EACP (a) and Paeonol Standard (b)
HPLC indicated that paeonol of EACP was detected at t_R 21.60 min.

inflammatory activities were evaluated by acetic acid-induced vascular permeability, AA-induced paw edema, cotton pellet-induced granuloma formation, and formalin-induced licking time. The vascular permeability was induced by acetic acid, which could cause an increase in peritoneal fluids of prostaglandin E_2 (PGE_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), serotonin, and histamine.¹³ This leads to a dilation of the arterioles and venules and to an increased vascular permeability. As a consequence, fluid and plasma proteins are extravasated, and edema forms. Indomethacin and EACP showed significant inhibition of acetic acid-induced vascular permeability in mice. This result suggested that EACP probably has an anti-inflammatory property like indomethacin (nonselective COX inhibitor), acting through inhibition of the inflammatory mediators of the acute phase of inflammation.

The mediators, including histamine, 5-HT, the kinins and their complements, have become the recent focus of attention as the metabolites of AA. Alone or in appropriate combination, AA products of COX and LOX pathways are capable of producing the characteristic signs of inflammation: vasodilatation, hyperemia, pain, edema, and cellular infiltration.^{14,15} The COX products, particularly prostaglandin E_2 (PGE_2), contribute to increased blood flow through a vasodilatation action, but the lipoxygenase pathway is necessary for vascular leakage and edema consequently on cellular infiltration.¹⁶ EACP, as well as phenidone, a dual blocker of COX and LOX, significantly reduced paw edema. This result suggested that the anti-inflammatory activity of EACP was related to the COX and LOX pathways.

The response to subcutaneously implanted cotton pellet in rat has been divided into three phases: a transudative phase, an exudative phase, and a proliferative phase.⁹ In this test, EACP was effective at inhibiting the transudative formation. Indomethacin and prednisolone elicited significant inhibitory activity on granuloma formation. Swingle and Shideman⁹ showed that corticosteroids effectively inhibit the proliferative phase of inflammation. Moreover, prednisolone significantly reduced the dry thymus weight and body weight gain, whereas EACP did not exert any effect. These results sug-

gested that the test drugs did not possess any steroidal-like activity, revealed a difference in the mechanisms of the anti-inflammatory actions of EACP and prednisolone, and supported the hypothesis of the effect of EACP on the mediators involved in the immediate response of inflammation in rats. This effect also appeared to be related to the stabilization of the lysosomal membrane system,¹⁷ since the activity of serum alkaline phosphatase that was raised in rats in the cotton pellet-induced granuloma model was normalized by EACP.

The formalin test possesses two distinctive phases, possibly reflecting different types of pain. The earlier phase reflects a direct effect of formalin on nociceptors, *i.e.* non-inflammatory pain, whereas the late phase reflects inflammation. It is well known that substance P and bradykinin participate in the early phase, while histamine, serotonin, prostaglandins and bradykinin are involved in the late phase.¹⁸ NSAIDs, such as indomethacin, reduce nociceptive behavior during the late phase, while the early phase seems unaffected.^{10,19} From this study, EACP showed only slight analgesic activity on the early phase but exhibited significant effect on the late phase. The slight inhibition in the early phase may be due to a sedative effect, as was demonstrated by the pentobarbital-induced sleep. The effect on the late phase of EACP indicates that its inhibitory activity on pain arose from inflammation, which reflects the effect on the synthesis and/or release of PGs, *etc.*

The anti-nociceptive and sedative activities were evaluated by acetic acid-induced writhing response and pentobarbital-induced sleep time. The writhing response of the mouse to an intraperitoneal injection of noxious chemical is used to screen for both peripherally and centrally acting, anti-nociceptive activity. Acetic acid causes pain by liberating endogenous substances and many others that excite pain nerve endings.²⁰ NSAIDs can inhibit COX in peripheral tissues, thus interfering with the mechanism of transduction in primary afferent nociceptors. The mechanism of the analgesic action of EACP may be due to the blockade of the effect or the release of endogenous substances that excite pain nerve endings similarly to indomethacin, which is mediated *via* a peripheral mechanism. This inhibition revealed an anti-nociceptive effect on acetic acid-induced writhing response, as well as an anti-inflammatory effect on acetic acid-induced vascular permeability.

EACP was employed in a pentobarbital-induced, sleeping time study in order to test its sedative/hypnotic effects. EACP significantly prolonged the pentobarbital-induced sleeping time in mice. These results raise the possibility that the reduction in acetic acid-induced writhing and the early phase of formalin licking time by EACP are the result of both its sedative property and the blockade of endogenous substances.

In conclusion, we have demonstrated the anti-inflammatory and anti-nociceptive/sedative effects of EACP. Furthermore, since we found paeonol in EACP (Fig. 5), the effects of EACP may be due to paeonol or other principles or interaction among them. The results of this study clearly demonstrated the anti-inflammatory property of EACP in the acute phase of inflammation. It seems that EACP significantly inhibits the acute phase of inflammation *via* blockage of the mediators that cause edema. However, it did not show any ef-

fect on the proliferative phase of inflammation, in cotton pellet-induced granuloma formation. In addition, the anti-nociceptive results suggest that its effects are related to sedative and muscle relaxant mechanisms involved in this pharmacological response. Considering these results, EACP may represent a potential new source of drugs for the treatment of anti-inflammatory pain. Further research is therefore obviously required to purify and identify the structure of the active principle(s) included in this fraction, as well as to determine its anti-inflammatory properties against gastritis, in line with its historical use in Chinese traditional medicine.

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