Antinociceptive Activities of 70% Methanol Extract of Evodiae Fructus (Fruit of *Evodia rutaecarpa* var. *bodinieri*) and Its Alkaloidal Components

Hideaki Matsuda,*,† Jian-xin Wu,*, Toshiyuki Tanaka,† Munekazu Iinuma,‡ and Michinori Kubo

Faculty of Pharmaceutical Sciences, Kinki University,*, 3–4–1 Kowakae, Higashiosaka, Osaka 577, Japan and Department of Pharmacognosy, Gifu Pharmaceutical University,‡ 5–6–1 Mitahora-higashi, Gifu 502, Japan.

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The effects of 70% methanol extract (EA-ext) from Evodiae Fructus (EA) consisting of dried fruits of *Evodia rutaecarpa* var. *bodinieri* (Rutaceae) on nociceptive responses were investigated. Oral administration of 50 or 200 mg/kg EA-ext had the same antinociceptive effect on writhing responses as induced by acetic acid. Its major alkaloidal constituents, evodiamine and rutaecarpine also had the antinociceptive effect. EA-ext significantly decreased the frequency of licking behavior within a unit of time at the late phase without affecting that of the early phase in the formalin test. EA-ext also increased nociceptive threshold of the inflamed paw without increasing that in the non-inflamed paw in the Randall–Selitto test. Although EA-ext inhibited the rise of vascular permeability induced by acetic acid and the increase of paw edema induced by carrageenin, it was ineffective on nociceptive response in the hot plate test and on locomotor activity. These results suggest that EA possesses antinociceptive effects and its mode of action may be mediated by anti-inflammatory action, and that the antinociceptive constituents are only partially attributable to alkaloidal components mentioned above.

**Key words** *Evodia rutaecarpa* var. *bodinieri*; Rutaceae; antinociceptive activity; anti-inflammatory activity; evodiamine; rutaecarpine

Evodiae Fructus (Chinese; Wu Zhu Yu, Japanese; Goshuyu) which originates from a variety of *Evodia* species (Rutaceae) has been used for treatment of headache, thoracic-abdominal pain and vomiting as analgesic agent or cold constitution as improving agent of blood circulation in traditional Chinese medicine. Effects related to the improvement of blood circulation of Evodiae Fructus were reported, but there have been few reports about its antinociceptive effect. Evodiamine and rutaecarpine, isolated from Ashina and Kashiwagi and known as main alkaloidal components in Evodiae Fructus were not examined, although isoevodiamine, an analogous constituent of evodiamine, was reported to have an analgesic effect.

This paper deals with the effects on nociceptive responses of 70% methanol extract (EA-ext) from Evodiae Fructus originating from *E. rutaecarpa* var. *bodinieri* using various kinds of nociceptive response models, and also its mechanism of action. The antinociceptive effects of evodiamine and rutaecarpine are also described.

**MATERIALS AND METHODS**

**Plant Material** Evodiae Fructus (EA, dried fruits of *Evodia rutaecarpa* var. *bodinieri* (DODE) Huang produced in Guizhou province, China) was offered by Nippon Funmatsu Yukuhin Co., Ltd. (Japan) and its origin was identified by Dr. Zhengtao Wang of China Pharmaceutical University.

**Extraction and Separation** The powdered fruits were extracted at about 80°C for 2h (two times) in 70% methanol of decuple of the powder for the pharmacological tests. The extract (EA-ext) was evaporated and then dried in a vacuum (yield: 43.4%).

In order to screen the active component(s), the crushed fruits (50 kg), as shown in Chart 1, were newly extracted with methanol (250 l) at room temperature for 24 h. The extract was evaporated and dried (2.85 kg), then poured into water and partitioned with ethyl acetate (EtOAc). The EtOAc layer was chromatographed on silica gel eluted with an ether-methanol system to get three fractions (frs. 1—3). Fraction 2 was further chromatographed on silica gel with an n-hexane—EtOAc—methanol system to afford six fractions (frs. 2—1—2—6). The mixture of fr. 1 and fr. 2—2 was chromatographed on silica gel column eluted with a n-hexane—EtOAc system and an ODS column with 70% methanol to isolate evodiamine (64.86 g) and rutaecarpine (82.57 g), respectively. Fraction 2—4 was chromatographed on silica gel column eluted with n-hexane—EtOAc to get limonin (2.05 g). The structures of these components were identified by spectroscopic analysis.

**Measurement of Evodiamine and Rutaecarpine Contents** On the basis of the method described by Kano et al., the contents of evodiamine and rutaecarpine in the EA-ext were determined by HPLC method (conditions: column, Symmetry C18 (Waters) (4.6 mm i.d. × 250 mm); detection, UV absorption at 230 nm; mobile phase, (A) 0.5 M l-pentane sulfonic acid 10 mL phosphate-buffered saline (PBS) buffer (pH 3.5) 1000 mL; (B) acetonitrile; (C) tetrahydrofuran; (A):(B):(C)=60:40:6; flow rate, 0.8 mL/min; column temperature, 40°C; injection volume, 10 μL). The contents of evodiamine and rutaecarpine in the extract were 3.46 and 1.84%, respectively.

**Measurement of Limonin Content** The content of limonin in the EA-ext was determined by HPLC method (conditions: column, Cosmosil 5 C18 (4.6 mm i.d. × 150 mm); detection, UV absorption at 210 nm; mobile phase, CH3CN:CH3OH:H2O=28:13:59; flow rate, 1.2 mL/min; column temperature, 40°C; injection volume, 10 μL). The content was determined to be 2.64%.

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Pharmacological Tests Animals: Male Slc:Wistar strain rats (100—120, 160—180 g) and male Slc:ddY strain mice (18—22 g) were used. They were maintained in an air-conditioned room with lighting from 7 a.m. to 7 p.m. The room temperature (about 23 °C) and humidity (about 60%) were controlled automatically. A laboratory pellet chow and water were given freely.

Drugs: The following drugs were used in the study: formalin, indomethacin, acetysalicylic acid (aspirin), acetic acid (Nacalai Tesque), \( \lambda \)-carrageenin, brewer's yeast (Sigma), pontamine sky blue (Tokyo Kasei), antipyrine (Hoei-yakko). Test samples were suspended with 0.5% carboxymethyl cellulose sodium (CMC·Na). The vehicle without sample was administered for control.

Antinociceptive Effects by Acetic Acid-Induced Writhing Test Acetic acid-induced writhing test was performed by the method of Koster et al.11 Samples were orally given 1 h prior to an intraperitoneal injection of 1.0% acetic acid (0.1 ml/10 g mouse). The number of writhings and stretchings was counted for 10 min from 5 min after the injection.

Antinociceptive Effects by Hot Plate Test Hot plate test was performed by the method described by Woolfe and McDonald.12 Briefly, a mouse was placed on a hot plate maintained at 55 ± 1 °C. The latency of nociceptive responses such as licking or jumping was measured. Only the mice that showed the nociceptive responses within 15 s were used for this experiment. One hour after the oral administration of samples, the latency of nociceptive responses was measured.

Antinociceptive Effects by Formalin Test Formalin test was carried out according to the method of Shibata et al.13 One hour after the oral administration of samples, 25\( \mu \)l of 0.5% formalin in saline was subcutaneously injected to the right hind paw of mice. Each animal was then returned to the chamber and pain response was recorded for a period of 30 min. The summation of time (in seconds) spent in licking and biting responses of the injected paw during each 5 min block was measured as an indicator of pain response.

Antinociceptive Effects by Randall–Selitto Paw Pressure Test Randall–Selitto paw pressure test was performed by the modified method of Randall and Selitto.14 Gradi-ent pressure was given to the right hind paw of rats (100—120 g) using an analgesy-meter (Ugo Basile, Italy). Inflammation was caused by subcutaneously injecting 0.1 ml of 1% \( \lambda \)-carrageenin in saline into the paw. After 2 h, the samples were orally administered. The nociceptive threshold was defined as the pressure causing the animals to struggle, turn to bite and/or vocalize, and it was recorded at 1, 2 and 3 h after the administration.

Antipyretic Effect Antipyretic test was performed by the method of Adams et al.15 Rectal temperature of Wistar strain rats (160—180 g) was measured by digital thermometer (MGA-III, Natsume). Rats which had a rectal temperature higher (about 1 °C) than the mean rectal temperature 17 h after the subcutaneous injection of 20% brewer’s yeast (10 ml/kg) were used in this experiment. After oral administration of the sample, the temperature was again measured at 0.5, 1, 3 and 5 h.

Spontaneous Motor Effect To habituate a mouse to new surroundings, the animal was placed in a doughnut-shaped cage (Laboratory Ambulometer, Model SMA-10, OHC O’hara & Co., Ltd.) 30 min after the oral administration of sample. After 30 min, changes in spontaneous motor activity were recorded with a Drinco- order (GI-77115, OHC O’hara & Co., Ltd.) for an 1 h period.

Anti-inflammatory Effect by Acetic Acid-Induced Vascular Permeability Test Acetic acid-induced vascular permeability test was performed by the method of Whittle.16 The mice were dosed orally with samples 1 h before the intravenous injection of 4% pontamine sky blue (10
ml/kg). Fifteen min after injection of the dye, 0.7% acetic acid (10 ml/kg) was injected intraperitoneally. Twenty min later, the mice were killed by dislocation of the neck and the viscera were exposed after a 1 min period to allow blood to drain away from the abdominal wall. The animal was held by a flap of the abdominal wall and the viscera were irrigated with 10 ml of saline over a petri dish. The washing was filtered through glass wool and transferred to a test tube. To each tube was added 0.1 ml of 1 N NaOH in order to clear any turbidity due to protein, and the absorbance was read at 590 nm with a Shimadzu model UV-160 spectrophotometer.

**Anti-inflammatory Effect by Carrageenin Edema Test**

The method was based on that of Nakamura et al. Initial hind paw volume of Wistar strain rats (160—180 g) was volumetrically determined. A 1% solution of δ-carrageenin in saline (0.1 ml/rat) was injected subcutaneously into the right hind paw 1 h after the samples had been administered orally. Paw volume was measured from 1 to 5 h after the injection, and the edema was determined. The results were expressed as percentage of the swelling compared with the initial hind paw volume.

**Statistical Analysis**
The experimental data were tested for statistical differences using Sheffe’s F-test or Bonferroni/Dunn’s Multiple Range Test.

**RESULTS**

**Antinociceptive Effects** 1. Acetic Acid-Induced Writhing Test As shown in Fig. 1, EA-ext at oral doses of 50, 200 mg/kg had an antinociceptive effect on the writhing responses induced by 1% acetic acid solution in mice. A positive control agent, antipyrine (100 mg/kg, p.o.) showed an inhibitory effect.

2. Hot Plate Test Oral administrations of EA-ext (50, 200 mg/kg) or antipyrine (100 mg/kg, p.o.) were ineffective on the reaction to thermal stimuli in mice (data not shown).

3. Formalin Test As shown in Fig. 2, EA-ext (50, 200 mg/kg, p.o.) did not inhibit the duration of licking activity in the early phase, while the extract significantly inhibited the duration in the late phase. A reference antinociceptive agent, aspirin (200 mg/kg, p.o.) had similar effect.

4. Randall–Selitto Paw Pressure Test As shown in Fig. 3, EA-ext (50, 200 mg/kg, p.o.) increased the nociceptive threshold of the inflamed but not the non-inflamed paw in this test. A reference drug, indomethacin (10 mg/kg, p.o.) also significantly increased only the inflamed paw.

**Antipyretic Effect** A reference drug, aspirin (200 mg/kg, p.o.) reversed hyperthermia induced by the yeast in rats, while EA-ext (50, 200 mg/kg, p.o.) did not have this effect (data not shown).

**Spontaneous Motor Effect** EA-ext (50, 200 mg/kg, p.o.) did not change the spontaneous locomotor activity (data not shown).

**Anti-inflammatory Effects** 1. Capillary Permeability Test As shown in Fig. 4, EA-ext at doses of 50, 200 mg/kg (p.o.) inhibited the increase of peritoneal vascular permeability caused by 0.7% acetic acid. A standard drug, indomethacin (10 mg/kg, p.o.) also reduced the leakage.

2. Carrageenin Edema Test EA-ext (50, 200 mg/kg, p.o.) had a significant inhibitory effect on the edema 1—5 h after the injection of carrageenin, as shown in Fig. 5. A standard drug, indomethacin (10 mg/kg, p.o.) showed more potent inhibition than did the extract.

**Screening of Antinociceptive Constituents** As shown in Chart 1, the fractions from Evodiae Fructus were screened for antinociceptive activity. EtOAc phase from methanol extract showed an inhibitory effect, and further screening led to the isolation of evodiamine, rutaecarpine and limonin (Fig. 6).

**DISCUSSION**

The antinociceptive effects of 70% methanol extract (EA-ext) from a Chinese crude drug, Evodiae Fructus originating from Evodia rutaecarpa var. bodinieri were investigated using experimental models on various nociceptive responses.

EA-ext exhibited antinociceptive effects as shown by the inhibition of abdominal writhing responses in mice induced by acetic acid which activates a chemosensitive nociceptor in the abdominal cavity. Antinociceptive drugs are generally classified into central or peripheral at the respective site of action. The results of this writhing test alone did not ascertain whether the antinociceptive effects are central or peripheral.

To learn the mode of the inhibitory effect of EA-ext on the nociceptive responses, the effects of EA-ext on hot plate, formalin and Randall–Selitto paw pressure tests was examined. It is known that formalin is useful to cause neurogenic pain and inflammatory pain and that its subcutaneous injection produces a biphasic pain response in rats. In mice used, the first phase response (first phase) was observed from 0 to 10 min after its injection and the second phase (second dose) from 10 to 30 min. According to Dubuisson and Dennis, centrally acting drugs inhibit the pain response in both phases equally, while the peripherally acting drugs inhibit the second phase only. EA-ext inhibited only the second
Fig. 2. Effects of 70% Methanol Extract (EA-ext) from Evodiae Fructus and Aspirin on Paw Licking in the Formalin Test in Mice

EA-ext and aspirin were administered orally to mice. Formalin (0.5%) was injected subcutaneously to the right hind paw of mice in a volume of 25 µl. Paw-licking time was measured from 0 to 30 min after the injection. (A) Time course of paw-licking after formalin injection; (B) Effects of EA-ext and aspirin on paw-licking in the early phase (0–10 min after formalin injection) and late phase (10–30 min after the injection). Each point or column represents the mean ± S.E. of 10 mice. Significantly different from control group, *p < 0.05, **p < 0.01 (Bonferroni/Dunn's multiple range test). ○, control; ▲, EA-ext 50 mg/kg; ■, EA-ext 200 mg/kg; ●, aspirin 200 mg/kg.

Fig. 3. Antinociceptive Effects of 70% Methanol Extract (EA-ext) from Evodiae Fructus and Indomethacin in Randall-Selitto Test in Rats

One percentage of l-carrageenin was injected subcutaneously to the right hind paw 2 h before the administration of EA-ext or indomethacin, and the nociceptive thresholds of the inflamed (A) and non-inflamed (B) paw were measured at 1 h intervals for 3 h after the administration of samples. Each point represents the mean ± S.E. of 8 rats. Significantly different from control group, *p < 0.05, **p < 0.01 (Bonferroni/Dunn's multiple range test). ○, control; ▲, EA-ext 50 mg/kg; ■, EA-ext 200 mg/kg; ●, indomethacin 10 mg/kg.
Fig. 4. Effects of 70% Methanol Extract (EA-ext) from Evodiae Fructus and Indomethacin (Indo.) on Capillary Permeability Induced by Acetic Acid in Mice

EA-ext and indomethacin were administered orally to mice before the intravenous injection of 4% pontamine sky blue. Fifteen min later, 0.7% acetic acid was injected intraperitoneally, and 20 min thereafter the mice were killed by decapitation and the vascular permeability was measured in terms of dye which leaked into the intraperitoneal cavity. Each column represents the mean ± S.E. of 10–12 mice. Significantly different from the control group, *p < 0.05, **p < 0.01 (Bonferroni/Dunn’s multiple range test).

Fig. 5. Effects of 70% Methanol Extract (EA-ext) from Evodiae Fructus and Indomethacin on Hind Paw Edema Induced by Carrageenin in Rats

EA-ext and indomethacin were administered orally 1 h before the subcutaneous injection of 1% λ-carrageenin, and the paw edema was measured from 1 h to 5 h after the injection of carrageenin. Each point represents the mean ± S.E. of 10–12 rats. Significantly different from control group, *p < 0.05, **p < 0.01 (Bonferroni/Dunn’s multiple range test). ▲, control; ▲, EA-ext 50 mg/kg; □, EA-ext 200 mg/kg; ●, indomethacin 10 mg/kg.

Fig. 6. Antinociceptive Effects of Evodiamine, Rutacarpine and Limonin Isolated from Evodiae Fructus and Antipyrine (Anti.) on Writhing Responses Induced by Acetic Acid in Mice

Evodiamine, rutacarpine, limonin and antipyrene were administered orally to mice 1 h before the intraperitoneal injection of 1.0% acetic acid. Writhing responses were measured by counting for 10 min. Each column represents the mean ± S.E. of 10–12 mice. Significantly different from control group, *p < 0.01 (Sheffe’s F-test).

phase.

In the Randall–Selitto test, EA-ext significantly elevated the pain threshold of the inflamed foot compared with that of the control foot. Aminopyrine and narcotic analgesics reportedly raise the pain threshold of both the normal and the inflamed foot, while salicylate and phenylbutazone affect only the inflamed foot.¹⁴

In the hot plate test, EA-ext did not show the inhibitory effect. Woolfe and MacDonald¹⁵ describe that aminopyrine and antipyrene are effective only in very large doses, but phenobarbital sodium and aspirin had no analgesic action that was detected by this test.

EA-ext significantly inhibited the rise of vascular permeability of the dye caused by acetic acid administered intraperitoneally and the increase of paw edema induced by carrageenin.

These findings proved that the antinociceptive effect of EA-ext is brought about through a peripheral site of action and is related to anti-inflammatory action. In the experimental models used this time, there is a possibility that sedative drugs also showed antinociceptive action. However, the fact that EA-ext showed no effect on locomotor behavior in mice denied this possibility.

The active components in EA-ext were pursued by monitoring antinociceptive activity by the acetic acid writhing method. Two alkaloids, evodiamine and rutacarpine showed the inhibitory effect; a non-alkaloidal component, limonin, was also effective. The contents of evodiamine, rutacarpine and limonin in EA-ext were 3.46, 1.84 and 2.64%, respectively. It was reported that limonin
content in Evodiae Fructus originating from *E. rutaecarpa* var. *bodinieri* was higher than that of Evodiae Fructus originating from *E. rutaecarpa* or *E. rutaecarpa* var. *officinalis*. In the original plant which provides Evodiae Fructus, Japanese Pharmacopoeia XIII describes two species, *E. rutaecarpa* and *E. rutaecarpa* var. *officinalis*, while Chinese Pharmacopoeia gives, in addition, *E. rutaecarpa* var. *bodinieri*. We dealt with the antinociceptive effects (acetic acid writhing test) of the 70% methanol extracts from Evodiae Fructus mentioned above and alkaloid (evodiamine and rutaecarpine) contents, but no relation between the content of alkaloid and the antinociceptive activity was observed. These results indicate that limonin plays an important role in Evodiae Fructus, although the antinociceptive effects of this fruit are generally attributed to alkaloidal components. We believe that it is possible to evaluate the quality of Evodiae Fructus from the market using the antinociceptive effect and the content of evodiamine, rutaecarpine and limonin as indexes. However, all of the antinociceptive constituents and the mechanisms of evodiamine, rutaecarpine and limonin are now under investigation.

The clinical application of Evodiae Fructus in traditional Chinese medicine, that is, the analgesic effect was demonstrated in this report.

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REFERENCES