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Analysis and Pharmacotoxicity of Feruloyltyramine as a New Constituent and p-Coumaroyltyramine in *Cannabis sativa* L.

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YAMAMOTO, I., T. MATSUNAGA, H. KOBAYASHI, K. WATANABE AND H. YOSHIMURA. *Analysis and pharmacotoxicity of feruloyltyramine as a new constituent and p-coumaroyltyramine in* Cannabis sativa *L.* PHARMACOL BIOCHEM BEHAV **40**(3) 465–469, 1991.—Feruloyltyramine (FT), a new amide compound, together with p-coumaroyltyramine (p-CT) was isolated and identified in ethanol extract of cannabis seeds. FT and p-CT were also detected in the roots, leaves and resin of *Cannabis sativa* L. The intracerebroventicular injection of these amides caused hypothermia and motor incoordination in mice, and the maximal effects were caused 160 to 240 min after the injection. Furthermore, p-CT also exhibited cataleptogenic effect in mice, although FT did not show any effect. These results suggest that these amide compounds may be responsible for some pharmacotoxicity of marihuana.

Amide compound p-Coumaroyltyramine Feruloyltyramine Isolation Determination Analysis
Pharmacotoxicity Hypothermia Motor incoordination Cataleptogenic effect

CANNABINOIDS such as tetrahydrocannabinol and cannabinol are known as the major constituents and pharmacologically active compounds in marihuana. In our previous study, the presence of other potentially active classes of compounds in marihuana has been suggested from the comparative experiment in mice of pharmacological effects of cannabinoids and cannabis extract (15). Several nitrogen-containing compounds such as amide, choline, muscarine, betanine and trigonelline have been isolated from *Cannabis sativa* L. (14). The only amide known to exist in cannabis is N-(p-hydroxy-β-phenylethyl)-p-hydroxy-*trans*-cinnamide (p-coumaroyltyramine, p-CT), that has been isolated from the roots (13). N-Cinnamoyl-β-phenethylamine derivatives, including p-CT and feruloyltyramine (FT), have been isolated from several plants (2, 7, 8, 16). However, pharmacological activity of these amides was not adequately understood. In order to elucidate the pharmacotoxicological contribution of the amide, we analyzed p-CT and a related compound in marihuana and evaluated their pharmacological activities (hypothermia, catalepsy and motor incoordination) in mice.

METHOD

Plant Material

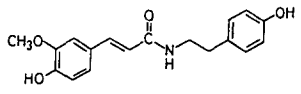
The leaves, roots and seeds were harvested from *Cannabis sativa* L. of Mexican origin grown in the botanical garden of Hokuriku University.

Extraction and Isolation

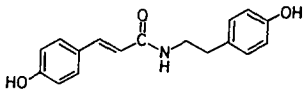
The crushed seeds (200 g) were extracted with n-hexane (2 × 1.0 l) followed by ethanol (4 × 1.0 l). The ethanol extract was evaporated in vacuo. The residue (1.0 g) was chromatographed over silica gel (150 g). The elution with 10% methanol-chloroform was fractionated into A and B. Each fraction was rechromatographed over silica gel. Extracts A and B were isolated from rechromatographed fractions A and B, respectively, by high performance liquid chromatography (HPLC). Extract A (feruloyltyramine) 0.5 mg. Mass spectrum (MS) m/z (rel. int. %): 313(M^+, 21), 194(34), 193(58), 192(56), 177(100), 145(39), 120(39), 107(23), 89(14), 77(18). Extract B (p-coumaroyltyramine) 0.1 mg. MS m/z (rel. int. %): 283(M^+, 8), 164(71), 147(100), 120(59), 107(12), 91(15), 77(7), 65(12).

Analyses of Amides

Ethanol extracts of the leaves, seeds, roots and resin were evaporated in vacuo. The residues were dissolved in ether, and



Feruloyltyramine(FT)



p-Coumaroyltyramine(p-CT)

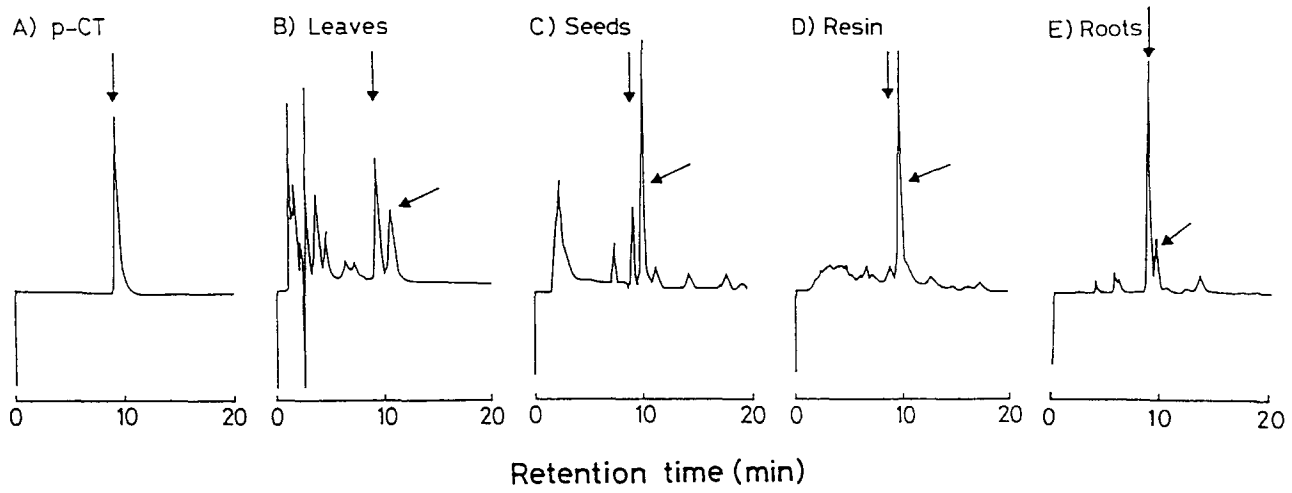


FIG. 1. HPLC chromatograms of p-CT and ethanol extracts of leaves, seeds, resin and roots. Mobile phase; acetonitrile:methanol:water (5:40:55, v/v/v).

shaken with 5% sodium bicarbonate and 0.1 N NaOH. Sodium hydroxide layers were acidified with 1 N HCl and then shaken with ether. The ether layers were evaporated in vacuo. The residues were cleaned up with Bond Elut C18 column, and analyzed by HPLC (Hitachi 655 type). HPLC conditions were as follows:

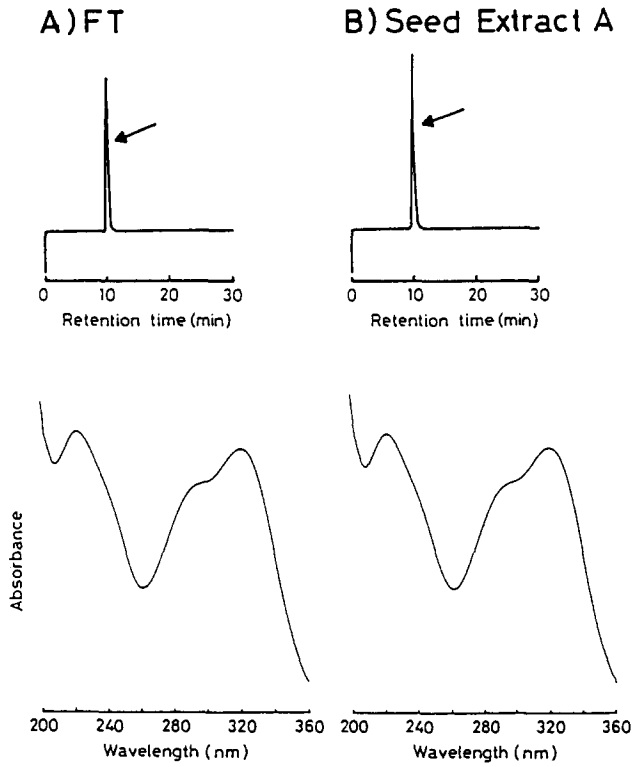


FIG. 2. HPLC chromatograms and UV spectra of FT and seed Extract A. The UV spectra of the peaks (the arrow at the top) of the FT and seed Extract A are presented to the right and left of the bottom, respectively. Mobile phase; acetonitrile:methanol:water (5:40:55, v/v/v).

column; DuPont ZORBAX ODS (4.6 mm i.d. × 15 cm), absorption of UV; 310 nm (with a variable wavelength UV detector, 655A-21 type) and 200 to 360 nm (with a photo diode array detector, Hitachi L-3000), mobile phase; acetonitrile:methanol: water (5:40:55, v/v/v) or 0.1% acetic acid:methanol (58:42, v/v), and flow rate; 1 ml/min. MS conditions were as follows: apparatus; JEOL JMS-DX 300 mass spectrometer and JEOL JMS-DA 5000 mass data system, ionizing energy; 70 eV and ionizing current; 300 μA.

Syntheses of FT and p-CT

The synthesis of FT was carried out as follows. Pyridine solution of tyramine (4.2 g) was added dropwise to chloride of ferulic acid (3.0 g) obtained by reaction with oxalyl chloride in benzene. After 2 h reaction mixture was evaporated in vacuo. The residue suspended with ethanol (60 ml) was added 3% NaOH (90 ml) and heated for 2 h at 70°C to hydrolyze ester bond of by-product, di-feruloyltyramine. The solution was acidified with 5 N HCl and then extracted with ethylacetate. The ethylacetate layer was evaporated in vacuo and the residue was chromatographed over silica gel (450 g), and eluted with 4 and 10% methanol-chloroform. FT (2.03 g) was obtained as a colorless needle (m.p. 144–145°C) from chloroform and acetone. MS: $C_{18}H_{19}NO_4$ m/z (rel. int. %): 313(M^+, 25), 194(33), 193(54), 192(54), 177(100), 145(24), 120(35), 107(13), 89(11), 77(11). UV_{max}^{EtOH}(nm) (log ε): 295(4.11), 322(4.20). ^{1}H-NMR ($CDCl_3$-d_1) δ: 2.68(2H, m), 3.38(2H, m), 3.83(3H, s), 6.47(1H, d, J = 15.76 Hz), 6.70(2H, d), 6.83(1H, d), 7.03(3H, m), 7.15(1H, s), 7.34(1H, d, J = 15.76 Hz), 8.01(1H, m), 9.20(1H, s), 9.44(1H, s). The synthesis of p-CT was carried out as described (12) from p-coumaric acid (0.9 g) and tyramine (0.67 g) in the presence of dicyclohexylcarbodiimide (1.06 g). The crystalline dicyclohexylurea was filtered from the reaction mixture, and the filtrate was chromatographed over silica gel (400 g) and eluted with 4 and 10% methanol-chloroform. p-CT (0.17 g) was obtained as a colorless needle (m.p. 253–254°C) from chloroform and methanol. MS: $C_{17}H_{17}NO_3$ m/z (rel. int. %): 283(M^+, 9), 164(67), 147(100), 120(64), 107(14), 91(29), 77(9), 65(17). UV_{max}^{EtOH} (nm) (log ε): 293(4.46), 300(4.45), 309(4.45). ^{1}H-NMR(DMSO-d_6) δ: 2.64(2H, m), 3.32(2H, m), 6.39(1H, d, J = 15.76 Hz), 6.68(2H,

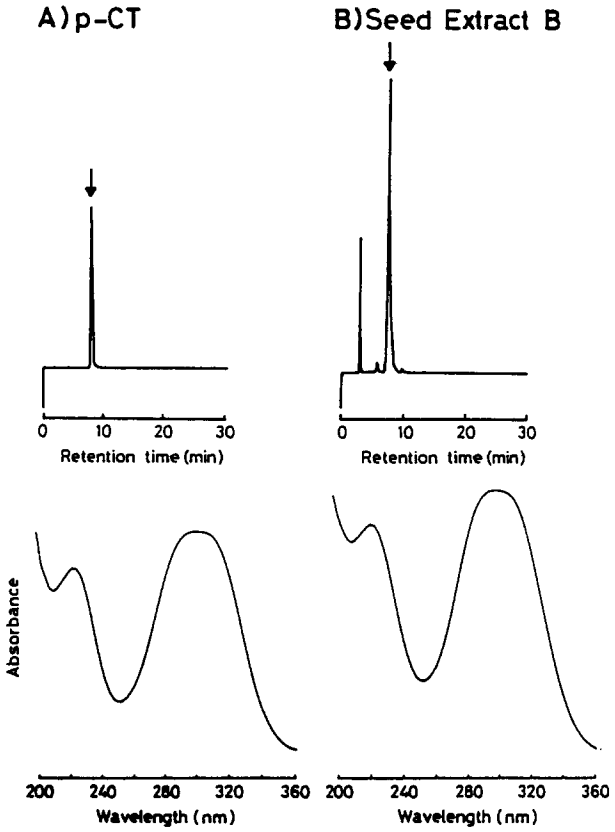


FIG. 3. HPLC chromatograms and UV spectra of p-CT and seed Extract B. The UV spectra of the peaks (the arrow at the top) of the p-CT and seed Extract B are presented to the right and left at the bottom, respectively. Mobile phase; acetonitrile:methanol:water (5:40:55, v/v/v).

d), 6.78(2H, d), 7.00(2H, d), 7.30(1H, d, J = 15.76 Hz), 7.37(2H, d), 7.99(1H, m), 9.15(1H, s), 9.80(1H, s).

Pharmacological Experiments

Male ddN mice (20–25 g) were used in all pharmacological experiments. FT (133 and 200 μg/mouse) and p-CT (125, 190 and 500 μg/mouse) were suspended in saline containing 1% (v/v) Tween 80 and injected intracerebroventricularly (ICV). The hypothermic effect of the amides was evaluated at 40, 80, 120, 160, 200, 240 and 280 min after the ICV administration by measuring rectal temperature of mice. The cataleptogenic effect was assessed by the method described previously (17) 40 to 240 min after the administration of the amides. Motor incoordination was measured by the method described by Flint and Ho (4). The control groups were treated with vehicle.

TABLE 1

THE CONTENTS OF p-CT AND FT IN THE LEAVES, SEEDS AND ROOTS

	p-CT mg/100 g	FT mg/100 g
Leaves	0.2 (2)	0.4 (2)
Seeds	0.75 ± 0.05 (4)	12.4 ± 0.6 (4)
Roots	0.51 ± 0.04 (3)	0.26 ± 0.02 (3)

Mobile phase, 0.1% acetic acid:methanol (58:42, v/v).

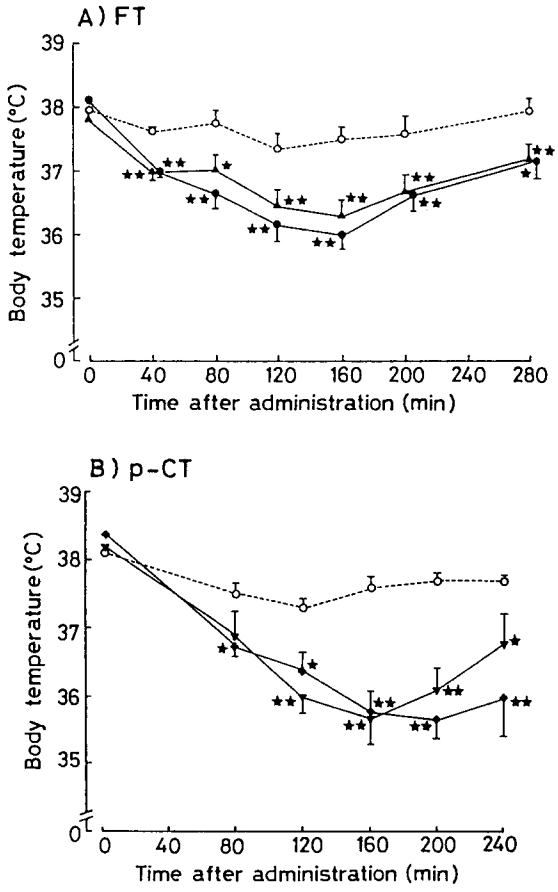


FIG. 4. Time course of change in body temperature produced by FT and p-CT. (A) ○: control, n = 8; ▲: FT 133 μg/mouse, n = 8; ●: FT 200 μg/mouse, n = 8. (B) ○: control, n = 7; ▼: p-CT 125 μg/mouse, n = 7; ◆; p-CT 190 μg/mouse, n = 8. ★Significantly different from control ($p<0.05$). ★★Significantly different from control ($p<0.01$).

Statistics

Statistical significance of difference was tested using the Student's *t*-test.

RESULTS

Figure 1 shows chromatograms of p-CT, and ethanol extracts of the leaves, seeds, resin and roots. A peak corresponding to p-CT (retention time 9 min) appeared on the chromatograms of all samples, and another peak with a retention time of 10.5 min appeared subsequently to a peak of p-CT. In preliminary experiment, a UV spectrum of the peak which was recorded by HPLC with a photo diode array detector was similar to that of ferulic acid. This result suggested that it was ferulic acid derivatives. Furthermore, Negrel and Jeandet reported that feruloyltyramine was eluted subsequently to p-CT from Waters Radialpack C18 column (9). Figures 2 and 3 show chromatograms and UV spectra of extracts A and B isolated from the seeds, respectively.

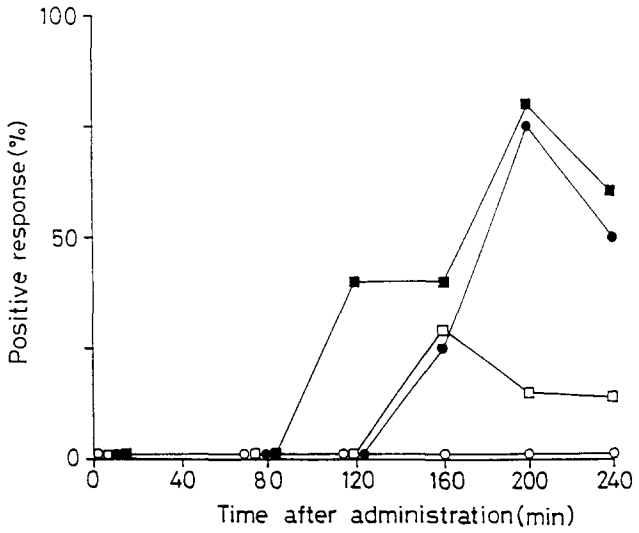


FIG. 5. Time course of cataleptogenic effect of p-CT. Each point represents a percentage of positive response in mice. ○: control, n = 10; □: 125 μg/mouse, n = 7; ●: 190 μg/mouse, n = 8; ■: 500 μg/mouse, n = 5.

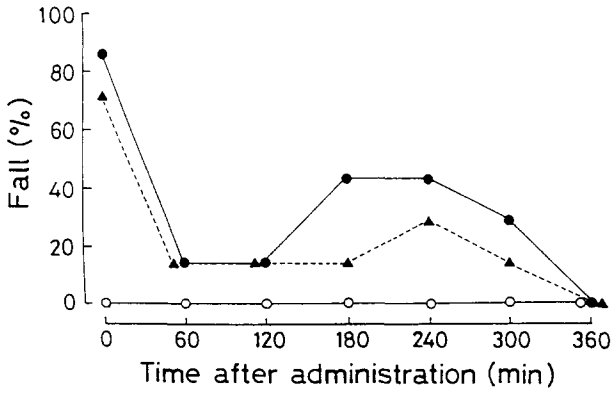


FIG. 6. Motor incoordination effect of p-CT and FT. Each point represents a percentage of mice falled rods within 30 seconds. ○: control, n = 7; ●: FT 200 μg/mouse, n = 7; ▲: p-CT 190 μg/mouse, n = 7.

The retention times and UV spectra of extracts A and B were identical with those of the synthetic FT and p-CT, respectively. MS spectra of extracts A and B were also identical with those of synthetic standards (see the Method section). These results supported that extracts A and B are FT and p-CT, respectively. The contents of FT and p-CT determined by HPLC are summarized in Table 1. FT was preferentially contained in the seeds, and its content of the seeds was 12.4 mg/100 g. The leaves and roots contained both amides to almost similar extent.

The hypothermic effect of FT and p-CT is shown in Fig. 4. A significant hypothermia was observed after 40 and 80 min, and the maximal effects were caused 160 min and 160 or 200 min after the administration of FT and p-CT, respectively. Approximately 80% of mice showed catalepsy at 200 min after administration of p-CT 190 and 500 μg/mouse (Fig. 5). On the other hand, administration of FT did not cause any cataleptogenic effect. Motor incoordination was observed by FT and p-CT administration, and the maximal effects occurred 180 and 240 min after injection (Fig. 6). The effect of FT was stronger than that of p-CT.

DISCUSSION

In the present study, FT was isolated and identified as a new constituent of *Cannabis sativa* L. from the seeds. FT and p-CT were detected not only in the seeds and roots but also in the leaves and resin. Slatkin et al. reported that a mild analgesic activity has been observed in mice by subcutaneous administration of p-CT (13). We could not detect any significant analgesic activity of p-CT in mice (data not shown). However, the present study demonstrated that p-CT and FT have some pharmacological activity (hypothermia, catalepsy and motor incoordination). The effects of these amides in spite of ICV administration appeared later than those of THC (18). The mechanism by which the amides cause the pharmacological actions on mice is not clear. FT and p-CT have been isolated from medicinal plants used to treat stenocardia, heart asthma and so-called stagnant blood, and those amides inhibit in vitro prostaglandin biosynthesis (5,6). Prostaglandins have been implicated in the pharmacological actions of cannabinoids. There is indirect evidence supporting the view that prostaglandin formation is involved in mediating some of the central actions of THC (1,3). Conversely, inhibition of prostaglandin formation in vitro by THC has also been observed in synaptosomes (10) and in brain (11). Pharmacological effects of the amides may be mediated with disturbance of prostaglandin biosynthesis. Even though only small concentrations of these compounds are present in the plants their significance cannot be dismissed in light of the possibility of cooperative effects with cannabinoid and cumulative effects of the chronic use of cannabis preparations. These results suggest that FT and p-CT have some action on the central nervous system and may contribute in part to the pharmacological effects of marihuana.

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