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Cannabis sativa L. (Marijuana) V: Pharmacological Evaluation of Marijuana Aqueous Extract and Volatile Oil

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Abstract □ The aqueous extract (marijuana tea) and volatile oil prepared from marijuana were compared with (−)-*trans*-Δ^9-tetrahydrocannabinol for their effect on hexobarbital sleeping time and analgesic action in mice. All three substances prolonged hexobarbital sleeping time with an order of potency of (−)-*trans*-Δ^9-tetrahydrocannabinol > aqueous extract > volatile oil. Each agent produced significant analgesic activity. However, the potencies of the aqueous extract and the volatile oil were similar to each other but only 1/200 that of (−)-*trans*-Δ^9-tetrahydrocannabinol.

Keyphrases □ *Cannabis sativa* L.—pharmacological evaluation of marijuana aqueous extract and volatile oil, hexobarbital sleeping time and analgesia, mice □ Marijuana—pharmacological evaluation of aqueous extract and volatile oil, hexobarbital sleeping time and analgesia, mice

A recent report (1) described the preparation of marijuana[1] tea, a simple aqueous extract that was obtained by continuously heating *Cannabis* plant material in water under reflux for 6 hr. In addition, a volatile oil was removed and retained. Since the use of marijuana tea has been stated to prolong and intensify significantly the effects resulting from smoking marijuana (1), the effects of marijuana tea were compared to (−)-*trans*-Δ^9-tetrahydrocannabinol (I), the major psychoactive constituent of marijuana (2, 3). Two pharmacological parameters known to be affected by I (4, 5) were used, *i.e.*, hexobarbital sleeping time and alteration of painful stimuli. Moreover, since no pharmacological data have been reported for the volatile oil, it was also included.

EXPERIMENTAL

Test Animals—Male, albino mice[2] (CD-1 strain) weighing 20–28 g at the time of testing were used. The animals, housed in groups of eight each, were allowed at least 5 days of acclimation to laboratory housing conditions of 12 hr of light and 12 hr of dark at a temperature of 21–23°, with food and water continuously available up until the time of testing.

[1] The term marijuana as used here refers generally to the crushed and broken tops, including leaves and small stems, derived from flowering or nonflowering *Cannabis sativa* L. plants of either sex.

[2] Charles River Breeding Laboratories, Wilmington, Mass.

Table I—Effects of I, III, and IV on Hexobarbital Sleeping Time in Mice

Test Drug	Dose, mg/kg ip	Mice	Sleeping Time, Minutes (Mean ± *SE*)	Increase, %
Propylene glycol vehicle	—	16	49 ± 4	—
I	5.0	8	49 ± 2	0
	10.0	8	100 ± 7[a]	104
	20.0	8	105 ± 12[a]	114
IV	40.0	8	53 ± 5	8
	80.0	8	75 ± 6[a]	53
	160.0	8	87 ± 6[a]	78
	320.0	8	140 ± 11[a]	186
Distilled water vehicle	—	16	41 ± 2	—
III	12.5	8	44 ± 4	7
	25.0	8	58 ± 5[a]	41
	50.0	8	59 ± 3[a]	44
	100.0	8	81 ± 8[a]	98

[a] When compared with its respective vehicle-treated control group, $p \leq 0.05$.

Preparation of Aqueous Extract from Marijuana Tea—Marijuana tea was prepared from 100 g of marijuana[3] and lyophilized as described previously (1) to give 30 g of aqueous extract (II). Then II was purified by stirring with petroleum ether (bp 30–60°) to remove trace amounts of I and other petroleum ether-soluble fast blue B reactive substances, which were detected by TLC (6). The petroleum ether-extracted II was dried *in vacuo* at 40° to give a cannabinoid-free aqueous extract (III)[4].

Preparation of Volatile Oil—Marijuana plant material[3] was subjected to direct steam distillation. The water-insoluble volatile constituents were recovered from the aqueous distillate using an oil separator and dried (anhydrous sodium sulfate) to give a pale yellow aromatic oil, yielding 0.18% (w/w) (n_D^{20} 1.494 and d_{20}^{20} 0.8788) (IV). This IV was found to contain no detectable amounts of cannabinoids[5] as indicated by TLC (6).

Dosage Forms—All doses of I[6], III, and IV were prepared in a concentration to permit a constant volume of 0.1 ml/10 g body

[3] Female plant material representing a Mexican strain of *C. sativa* (1).

[4] Extract III was used for all pharmacological studies, although preliminary pharmacological experiments showed no significant differences in results when II and III were compared.

[5] The volatile oil obtained by water distillation during the preparation of marijuana tea (1) was shown by TLC (6) to contain significant amounts of I and thus was not used in the present pharmacological study.

[6] Obtained from the National Institute of Mental Health, Rockville, Md.

Table II—Effect of I, III, and IV in the Hot-Plate Analgesia Test in Mice

Test Drug	Dose, mg/kg ip	Control[a] Reaction Time, sec	Drug[a] Reaction Time, sec	Increase in Mean Reaction Time, %	Number Analgesic[b] / Number Tested	ED_{50}, mg/kg[b] (95% Confidence Limits)
Propylene glycol vehicle	—	7.8 ± 0.7	7.7 ± 0.6	0.0	0/8	—
I	0.3	8.3 ± 0.6	10.0 ± 1.5	20.7	3/8	1.3
	0.625	7.4 ± 0.5	11.6 ± 1.5[c]	57.3	4/8	(0.5–3.1)
	1.25	7.6 ± 0.4	11.0 ± 1.0[c]	45.2	5/8	
	2.5	7.5 ± 0.6	11.7 ± 1.1[c]	56.2	5/8	
	5.0	7.6 ± 0.7	12.7 ± 2.1[c]	68.7	5/8	
	10.0	7.4 ± 0.6	16.8 ± 1.1[c]	126.2	7/8	
IV	100.0	8.0 ± 0.7	9.1 ± 1.0	14.1	2/8	260.0
	200.0	7.4 ± 0.7	11.6 ± 1.0[c]	56.2	4/8	(138.0–489.0)
	400.0	8.6 ± 0.6	13.3 ± 1.6[c]	55.0	4/8	
	800.0	7.0 ± 0.6	19.5 ± 0.5[c]	178.4	8/8	
Distilled water vehicle	—	7.9 ± 0.2	7.9 ± 0.5	0.0	0/8	—
III	100.0	8.1 ± 0.7	8.9 ± 0.9	11.0	2/8	216.0
	200.0	7.0 ± 0.5	9.6 ± 0.5[c]	37.0	4/8	(135.0–346.0)
	300.0	8.0 ± 0.6	12.4 ± 1.7[c]	54.7	6/8	
	400.0	7.5 ± 0.6	12.0 ± 1.0[c]	60.6	7/8	

[a] These data represent the mean ± *SE* for eight mice in a group. [b] The number of mice per group showing a 40% or greater increase in their predrug reaction time. These data are used to calculate the ED_{50} value. [c] $p \leq 0.05$ when compared with its respective predrug control reaction time.

weight to be given each animal. The vehicle used for I and IV was 10% propylene glycol–1% polysorbate 80[7]–0.9% saline (7), while III was dissolved in glass-distilled water. All injections were by the intraperitoneal route.

Hexobarbital Sleeping Time—Thirty minutes following administration of the test drugs or their vehicles, each mouse was given an injection of sodium hexobarbital (125 mg/kg ip). Sleeping time for each animal was measured by the time in minutes from the loss to the regaining of the righting reflex observed for at least 10 sec after the animal was placed on its back. The mean sleeping time for each group was calculated, and a statistical comparison between groups was evaluated by the Student *t* test.

Hot-Plate Test—This method for assessing analgesic activity was based on the reaction time of mice to lick their forepaws and/or jump after exposure to a copper surface hot plate heated and maintained at 54–56° (8). A control reaction time (measured to the nearest 0.1 sec) was obtained 24 hr prior to any test for drug effect. Only mice with a control reaction time of 10 sec or less were used. On the test day, mice were administered the test drugs or their vehicles; 30 min later, each mouse in a group was re-exposed to the hot-plate surface and reaction time once again was recorded. A cutoff time of 30 sec was used. The percent change in mean reaction time was obtained and statistically evaluated by comparing postdrug and predrug values. In addition, the number of mice in each group that showed a 40% or greater increase in reaction time from their respective predrug control value was noted and ED_{50} values were determined (9).

RESULTS

Hexobarbital Sleeping Time—Table I shows the results of each treatment on hexobarbital-induced anesthesia in mice. A significant prolongation of sleeping time was observed in mice treated with either 10.0 or 20.0 mg/kg of I. However, this effect with the data of this study appeared not to be dose related since a similar degree of activity was obtained with both doses, 104 or 114%, respectively.

Varying the pretreatment dose of IV produced a significant prolongation of hexobarbital sleeping time after the three highest doses. The 80.0- and 160.0-mg/kg doses produced similar degrees of enhancement (53 and 78%, respectively), while 320.0 mg/kg drastically increased hexobarbital sleeping time by 186%. Finally, a pattern of activity similar to that obtained with IV was noted when the pretreatment regimen was III. The low dose of 12.5 mg/kg was ineffective, while doses of 25.0 and 50.0 mg/kg produced 41 and 44% increases in hexobarbital anesthesia, respectively. When the dose of III was increased to 100.0 mg/kg, sleeping time was increased by 98%. Based on the data presented in Table I, the order of potency (on a milligram per kilogram basis) to prolong hexobarbital sleeping time was I > III > IV.

Hot-Plate Test—Table II summarizes the data obtained in this test system with each treatment group. All doses of I, except 0.3 mg/kg, produced a significant increase in mean reaction time to the noxious stimulus (heat). The intermediate doses of 0.625–5.0 mg/kg produced similar elevations in pain threshold (45.2–68.7%), while the highest dose of I (10.0 mg/kg) caused a 126.2% increase in mean reaction time. Both III and IV showed equal analgesic effectiveness when compared with each other but were much less potent than I (ED_{50} values of each were approximately 1/200 that of I). Neither the 10% propylene glycol–1% polysorbate 80–0.9% saline nor the distilled water vehicles produced analgesic activity.

DISCUSSION

These results demonstrate that III and IV prolong hexobarbital sleeping time and possess analgesic activity in mice similar to, but less potent than, I. Since no measurable amounts of I or any other cannabinoids could be detected in either III or IV, their pharmacological activity must be due to one or more chemical entities as yet unidentified. Further work is underway in these laboratories to isolate and identify the constituents in III and IV responsible for the observed activities.

Moreover, since it has been claimed that the effects of smoked marijuana in humans can be augmented by the ingestion of marijuana teas (1) and because it is reasonable to expect that some *Cannabis* volatile oil constituents cooccur with I in the mainstream smoke resulting from smoking marijuana, animal experiments have been initiated to determine the extent of any interaction of either III or IV or both with I.

REFERENCES

(1) A. B. Segelman and R. D. Sofia, *J. Pharm. Sci.*, **62**, 2045(1973).

(2) H. Isbell, C. W. Gorodetzsky, D. Jasinski, U. Claussen, F. von Spulak, and F. Korte, *Psychopharmacologia*, **11**, 184(1967).

(3) R. Mechoulam, *Science*, **168**, 1159(1970).

(4) J. C. Garriott, R. B. Forney, F. W. Hughes, and A. B. Richards, *Arch. Int. Pharmacodyn. Ther.*, **171**, 425(1968).

(5) R. D. Sofia and H. Barry, III, *Fed. Proc.*, **31**, 506(1972).

[7] Tween 80.

(6) A. B. Segelman, *J. Chromatogr.*, **82**, 151(1973).
(7) R. D. Sofia, R. K. Kubena, and H. Barry, III, *J. Pharm. Pharmacol.*, **23**, 889(1971).
(8) N. B. Eddy and D. Leimbach, *J. Pharmacol. Exp. Ther.*, **107**, 385(1953).
(9) J. T. Litchfield, Jr., and F. Wilcoxon, *ibid.*, **96**, 99(1949).

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Effect of γ-Radiation on Yield of Insulin from Beef Pancreas Glands

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Abstract □ Beef pancreas glands were subjected to varying doses of γ-radiation to inactivate proteolytic enzymes and thus allow greater insulin yields *via* normal extraction procedures. An increase in yield was seen with doses greater than 10,000 rads, with the maximum yield obtained from a 20,000-rad dose.

Keyphrases □ Radiation, gamma—effect on yield of insulin from beef pancreas glands □ Insulin—increased yields by γ-irradiation of beef pancreas glands

Recent beef shortages as well as recommended restrictions by the Food and Drug Administration on the use of oral hypoglycemics (1) have placed a serious strain on the supply of insulin from cattle and hogs. Although an increase in animal slaughter will somewhat alleviate this shortage, an increasing population carries with it an increasing demand for insulin. Thus, there is a need for methods of increasing the yield of insulin extracted from the pancreas glands of these animals.

The commercial insulin procedure is a combination of acid-alcohol extractions and isoelectric precipitations to achieve a purified insulin (2). Frozen glands in 45.9–68.1-kg (100–150-lb) lots are ground and placed in large extraction tanks filled with cold acidified alcohol. The acid–alcohol extracts the insulin from the glands and inactivates enzymes that could destroy the insulin in the extract. This step is most important, and it is felt that a loss of insulin in the grinding–extraction process could still be due to some action of the proteolytic enzymes on the insulin. Thus, if enzymes could be further inactivated, less degradation of insulin would occur and the yield would be improved.

One such means of inactivating the enzymes would be through the use of ionizing radiation (3). Irradiation of frozen glands prior to the grinding step could inactivate the enzymes that may be degrading the insulin during the subsequent steps.

Table I—Results of Insulin Assays of Irradiated Pancreas Glands

Sample Dose, rads	Yield, Units/ 0.454 kg	Change from Control, %	*p*
0 (Control)	1456 ± 54	—	—
4,000	1469 ± 45	+0.9	—
6,000	1393 ± 48	−4.3	<0.02
8,000	1278 ± 55	−12.2	<0.001
10,000	1457 ± 102	0	—
12,000	1742 ± 104	+19.6	<0.001
14,000	1702 ± 123	+16.9	<0.001
16,000	1657 ± 126	+13.8	<0.001
18,000	1831 ± 155	+25.8	<0.001
20,000	1954 ± 160	+34.2	<0.001
50,000	1820 ± 80	+25	<0.001
100,000	1490 ± 174	+2.03	—

EXPERIMENTAL

Approximately 11.6 kg (25 lb) of beef pancreas glands was obtained from a commercial slaughterhouse. The glands had been collected during the previous day's slaughter and were stored at 5°. These glands were transported in a refrigerated container to the laboratory and then placed in a −10° freezer for 24 hr. The frozen glands were chopped into pieces approximately 2.54–5.08 cm (1–2 in.) square, and all pieces were uniformly mixed. This procedure was done to approximate a uniform sample and avoid individual gland variation. Lots (0.2 kg, 0.5 lb) of the mixed frozen glands were then placed in polyethylene bags for irradiation.

Samples (0.9 kg, 2 lb) of the glands were then irradiated in 0.2-kg (0.5-lb) lots in a cobalt-60 (^{60}Co) pool-type irradiator[1] (7 cm i.d.; 1.85×10^2 rads/sec) for varying doses. All samples remained frozen during the irradiation. There was no induced radioactivity in the sample as determined by using a NaI (Tl) detector. Following irradiation, the glands were ground, weighed, and extracted with cold (1–4°) 95% ethanol acidified to pH 2.5 with concentrated phosphoric acid. The extraction time was 30 min and the temperature of the final extraction mixture was measured; then this mixture was filtered to collect the crude alcoholic extract. Ten aliquots of the alcoholic filtrate were collected for assay.

The radioimmunoassay technique of Herbert *et al.* (4) was uti-

[1] Lockhead-Georgia Co., Marietta, Ga.