

ISOLATION OF AN ANTI-INFLAMMATORY PRINCIPLE FROM THE
FRUIT JUICE OF *ECBALLIUM ELATERIUM*ERDEM YESILADA,¹ SHIGEO TANAKA, EKREM SEZIK,² and MAMORU TABATA*

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ABSTRACT.—The fruit juice of *Ecballium elaterium*, used as a folk medicine in Turkey for the treatment of sinusitis, was investigated for its anti-inflammatory activity in mice. Fractions obtained from the juice were tested in mice for their effects on increased vascular permeability, as induced by HOAc ip. The active principle was isolated from the CHCl₃ extract as well as the H₂O-insoluble part of the fruit juice and identified as cucurbitacin B. This is the first report that cucurbitacin B has a significant anti-inflammatory activity.

Ecballium elaterium (L.) A. Rich. (Cucurbitaceae), squirting cucumber, grows naturally in the Mediterranean region. The juice of its fruit is a well-known powerful hydragogue cathartic in folk medicine and is also used for its diuretic activity, especially in edema caused by kidney trouble. The determination of antitumor activity of cucurbitacins stimulated investigations on this plant (1).

In Turkey, the fresh juice of this fruit has been used externally for the treatment of sinusitis. Dioscorides (2) described that the application of powdered elaterium (precipitate of the fruit juice) mixed with milk into nostrils cleared away icterus and cured a headache of long continuance. Such a use for elaterium, however, is not commonly found in any other country except Turkey, where the fresh fruit juice is applied directly into the nostrils of the patient. This practice often causes severe congestion of the upper respiratory tract because of the high cytotoxicity of cucurbitacins contained in the fruit juice. To solve this problem, Sezik *et al.* (3) performed clinical tests to determine a proper and nontoxic dose of the drug for the treatment of sinusitis, and through the application of properly diluted juice to voluntary patients, they observed a good recovery in 87% of the treated patients. However, the active principle of this juice remains unknown.

In this report, we have separated various fractions of *E. elaterium* and identified one compound, cucurbitacin B, which possesses potent *in vivo* effects using the inhibition of vascular permeability in mice as an index of anti-inflammatory activity.

RESULTS AND DISCUSSION

The anti-inflammatory activity of *E. elaterium* fruit juice was investigated by the Whittle method (4) with some slight modifications. The freeze-dried fruit juice was administered orally to the mice at four different dosage levels. As shown in Table 1, a significant activity was observed at a dose of 50 mg/kg body weight, and the activity increased with increasing dosage. Although the highest inhibition of dye leakage was observed at 400 mg/kg, this dosage was highly toxic to mice. At a dosage of 200 mg/kg, however, no apparent toxic effect was observed, in spite of high anti-inflammatory activity.

To isolate the active principle, the freeze-dried fruit juice was dissolved in H₂O and subjected to a series of successive fractionations with CHCl₃, EtOAc, and *n*-BuOH. Each extract was administered orally to mice in an amount equivalent to 400 mg of freeze-dried juice. As shown in Table 2, the CHCl₃ extract significantly inhibited the

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TABLE 1. Effect of the Oral Administration of *Ecballium elaterium* Fruit Juice on Dye Leakage in Mice.

	Dose (mg/kg)	Dye Leakage (µg/mouse) Mean ± SE	Inhibition (%)
Control	0	238.3 ± 33.8	—
Fruit juice (freeze-dried)	50	163.3 ± 13.8 ^a	31.3
	100	151.4 ± 15.2 ^a	36.3
	200	70.0 ± 9.2 ^b	70.3
	400	64.2 ± 4.6 ^b	72.7
Control	0	101.2 ± 4.1	—
Acetylsalicylic acid	200	53.5 ± 3.3 ^b	46.6

^a*p* < 0.05.

^b*p* < 0.001.

vascular permeability induced by HOAc at a dose of 20 mg/kg. The H₂O-insoluble fraction of freeze-dried juice also showed an inhibitory effect.

The CHCl₃ extract was separated into three equal fractions by preparative tlc, and each fraction extracted from the Si gel layer with CHCl₃-MeOH (1:1) was given to mice in an amount equivalent to 40 mg of the CHCl₃ extract. It was observed that only frac-

TABLE 2. Effect of Various Extracts of *Ecballium elaterium* Fruit Juice on Dye Leakage in Mice.

	Dose (mg/kg) po	Dye Leakage (µg/mouse) Mean ± SE	Inhibition (%)
Control	0	170.1 ± 20.0	—
CHCl ₃ extract	20	43.9 ± 5.6 ^a	73.3
EtOAc extract	10	145.0 ± 13.6	14.6
<i>n</i> -BuOH extract	20	156.2 ± 11.3	8.1
Remaining aqueous extract	330	166.6 ± 9.9	3.4
Control	0	132.5 ± 9.4	—
H ₂ O-insoluble fraction	100	82.7 ± 8.0 ^b	43.6

^a*p* < 0.001.

^b*p* < 0.01.

tion III showed a significant anti-inflammatory effect (Table 3). Fraction III was subjected to preparative tlc with the solvent system CHCl₃-MeOH (11:0.4) to be separated into four fractions, which were separately eluted from the Si gel with CHCl₃-MeOH (1:1). Of these fractions, only fraction III-2 showed a significant anti-inflammatory activity (Table 4). Tlc analysis of fraction III-2 revealed only one distinct spot under uv light or spraying the plate with the vanillin-phosphoric acid reagent (VAP reagent). The compound corresponding to this spot was isolated from a larger sample of the original CHCl₃ extract by cc and by preparative tlc. An anti-inflammatory compound was also isolated from the H₂O-insoluble fraction and proved to be identical with the compound from fraction III-2 by comparison of mp and uv absorption spectra and also by mmp and co-tlc.

This compound gave a positive Liebermann-Burchard's reaction, suggesting a steroidal or triterpenoidal structure. It has been reported that cucurbitacins, lanostane-

TABLE 3. Effect of Fractions of the CHCl₃ Extract on Dye Leakage in Mice.

	Dose (mg/kg) po	Dye Leakage (μg/mouse) Mean ± SE	Inhibition (%)
Control	0	151.5 ± 8.2	—
Fraction I	4.3	157.9 ± 12.8	-4.2
Fraction II	26.4	124.6 ± 16.0	17.6
Fraction III	8.3	55.5 ± 6.7 ^a	62.8

^a*p* < 0.001.

type triterpenoids, were the main triterpenoids of *E. elaterium* (5,6), and cucurbitacin E, B, D, and I were isolated from the CHCl₃ extract of the fruit juice (7).

The compound isolated in the present study gave negative response to 5% FeCl₃ solution in EtOH, a specific reagent for the diosphenol structure in ring A of cucurbitacins (7), but when treated with the VAP reagent, it showed red fluorescence, characteristic of Δ²³ cucurbitacins, under uv light (7). Its uv spectrum, showing a maximum peak at 228 nm, was identical with that reported for cucurbitacin B (8). This compound was finally identified as cucurbitacin B by measurements of mp, optical rotation, and ir, ¹H-nmr and ms spectra in comparison with previous data (9, 10). On the basis of the pharmacological dose-response curves, the ED₅₀ and LD₅₀ of cucurbitacin B have been estimated as 6.1 and 10.9 mg/kg, respectively. Cucurbitacin B is known to exhibit various physiological activities such as purgative action, antitumor activity, and insect attractant property (6). Some cucurbitaceous plants such as *Bryonia alba* and *Cayaponia tayuya* containing cucurbitacin B have been used for the treatment of rheumatism and arthritis in folk medicine (7), but this is the first report demonstrating the potent anti-inflammatory activity of cucurbitacin B.

TABLE 4. Effect of Four Subfractions of the CHCl₃ Fraction III on Dye Leakage in Mice.

	Dose (mg/kg) po	Dye Leakage (μg/mouse) Mean ± SE	Inhibition (%)
Control	0	191.8 ± 15.3	—
Fraction III-1	22.6	195.7 ± 20.5	-2.0
Fraction III-2	5.9	57.5 ± 11.3 ^a	69.6
Fraction III-3	4.3	198.7 ± 17.7	-3.5
Fraction III-4	22.3	226.4 ± 14.6	-17.9

^a*p* < 0.001.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Melting points (uncorrected) were determined with a Yanagimoto Micro Melting Point Apparatus; ir and uv spectra were recorded with Shimadzu IR-435 and Hitachi 124 spectrometers, respectively. Ms and ¹H-nmr spectra were obtained with JEOL JMS-01SG-2 (75 eV) and JEOL FX-200 spectrometers (200 MHz), respectively. Optical rotations were determined using a JASCO DIP 360 digital polarimeter. Si gel 60F₂₅₄ and Si gel PF₂₅₄ (Merck) were used for tlc and preparative tlc, respectively. For the detection of spots, tlc plates sprayed with VAP reagent (2% EtOH solution of vanillin, 85% *o*-phosphoric acid, and EtOH, 1:2:8) were heated for 10 min at 120° and examined under a uv (365 nm) lamp.

PLANT MATERIAL.—Green, ripe fruits (10 kg) of *E. elaterium* were collected in August 1986, in Nar-

lidere, Izmir, Turkey, and a voucher specimen (No. 86002) is preserved in the herbarium of HUEF. The juice was squeezed by hand from cut fruits, filtered through a double layer of muslin (1.6 liters), and freeze-dried to give 77 g of powder.

FRACTIONATION OF FRUIT JUICE.—A sample of freeze-dried fruit juice (33.8 g) was dissolved in distilled H₂O (850 ml), and the H₂O-insoluble fraction was filtered under reduced pressure (9.1 g). The H₂O-soluble fraction was extracted with CHCl₃ (200 ml × 4), and the CHCl₃ extract washed with distilled H₂O was dried over anhydrous MgSO₄ and then concentrated to dryness in vacuo at 35° to give 1.37 g of extract. The aqueous phase was extracted with EtOAc (200 ml × 4) and H₂O-saturated *n*-BuOH (200 ml × 5), successively, and each extract washed with H₂O was concentrated to dryness in vacuo at 35° to yield 0.51 g and 1.07 g of extracts, respectively. The remaining aqueous solution was dried by freeze-drying.

FRACTIONATION OF CHCl₃ EXTRACT.—The CHCl₃ extract (40 mg) of the fruit juice was subjected to preparative tlc on Si gel (solvent system: EtOAc-MeOH-H₂O, 10:1:3, upper layer), and 15 spots detected by uv (254 nm) and VAP reagent were separated into three zones: I (lower 8 weak spots), II (middle 4 spots), and III (upper 3 spots). Each zone was scraped off from the plate and extracted with CHCl₃-MeOH (1:1) several times. Combined extracts were evaporated to dryness in vacuo to yield fractions I (4.3 mg), II (26.4 mg), and III (8.3 mg). Fraction III, which gave 10 spots in tlc using the solvent system CHCl₃-MeOH (8:0.4), was subjected to further fractionation by preparative tlc (CHCl₃-MeOH, 11:0.4) to give four fractions: III-1 (6.8 mg), III-2 (1.8 mg), III-3 (1.3 mg), and III-4 (6.7 mg) from 80 mg of the original CHCl₃ extract.

FRACTIONATION OF H₂O-INSOLUBLE FRACTION (WIS).—The H₂O-insoluble fraction of the freeze-dried fruit juice (420 mg) was extracted with CHCl₃-MeOH (1:1), and the organic phase was evaporated to dryness in vacuo at 35°. This extract (115 mg) was separated into three fractions by preparative tlc (solvent system: CHCl₃-MeOH, 95:5): WIS-I (8.1 mg), WIS-II (85.0 mg), and WIS-III (10.1 mg), which correspond to the lower, middle, and upper zones of the plate, respectively. WIS-II was fractionated into two fractions, each containing a different compound by preparative tlc (solvent system: CHCl₃-MeOH, 11:0.4): WIS-II-1 (14.0 mg) (lower zone) and WIS-II-2 (28.3 mg) (upper zone).

ISOLATION OF ACTIVE COMPOUND CUCURBITACIN B.—Fraction III and WIS-II were separately applied to Si gel cc and eluted with CHCl₃ and CHCl₃-MeOH (100:1), respectively. Active fractions III-2 and WIS-II-1 were subjected to preparative tlc (solvent system: Et₂O-C₆H₆, 8:2) and crystallized from MeOH to yield colorless needles. The physical data (ir, uv, ms, ¹H nmr) of this compound were identical with previously published values (7-11) for cucurbitacin B: mp 182-184°; [α]^D₂₀ +75.9° (c = 0.98, EtOH).

ASSAY FOR ANTI-INFLAMMATORY ACTIVITY.—Effect of the test samples on the increased vascular permeability induced by HOAc in male mice (dd strain) was determined according to the Whittle method (4) with slight modifications. Each test substance was administered orally to a group of 10 mice in 0.2 ml per 20 ± 1 g of body weight; 30 min after drug administration, each animal was injected with 0.1 ml of 4% pontamine sky blue in saline solution iv at the tail. Then, 10 min after the iv injection of the dye solution, 0.4 ml of 0.5% (v/v) HOAc was injected ip. After 20 min, the mice were killed by dislocation of the neck, and the viscera were exposed and irrigated with distilled H₂O, which was then poured into 10-ml volumetric flasks through glass wool. Each flask was made up to 10 ml with distilled H₂O, 0.1 ml of 0.1 N NaOH solution was added to the flask, and the absorption of the final solution was measured at 590 nm. In control animals, a mixture of distilled H₂O and 0.5% sodium carboxymethyl cellulose (CMC) was given orally, and they were treated in the same manner as described earlier. LD₅₀ value was calculated according to the Weil method (12).

PREPARATION OF SAMPLES FOR BIOASSAY.—Freeze-dried fruit juice was homogenized in distilled H₂O, and the homogenate including the insoluble fraction was administered to mice.

Each of CHCl₃, EtOAc, *n*-BuOH extracts, the H₂O-insoluble fraction, and their fractionated samples was given to mice after suspending in a mixture of distilled H₂O and 0.5% CMC by the use of a homogenizer.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Kazuo Iguchi, Tokyo College of Pharmacy, for providing us with the copies of ¹H-nmr and ir spectra of cucurbitacin B and its related compounds.

This study was supported in part by grant-in-aid No. 62043045 from the Ministry of Education, Science, and Culture, Japan. The first author was also supported by the JSPS fellowship for research in Japan from the Japan Society for the Promotion of Science.

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Received 1 October 1987