[Chem. Pharm. Bull. 34(6)2614—2617(1986)]

Anti-inflammatory Constituents of Topically Applied Crude Drugs. I. Constituents and Anti-inflammatory Effect of *Eriobotrya japonica* LINDL.[1)]

MINEO SHIMIZU,* HIDEKI FUKUMURA, HIDEKI TSUJI, SEIICHI TANAAMI, TOSHIMITSU HAYASHI and NAOKATA MORITA

Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-01, Japan

(Received October 21, 1985)

The ether-soluble fraction of EtOH extract from the leaves of *Eriobotrya japonica* LINDL. showed an anti-inflammatory effect when topically applied to rats. Ursolic acid, maslinic acid, methyl maslinate and euscaphic acid were isolated from the ether-soluble fraction. Maslinic acid was found to show an anti-inflammatory effect on carrageenan-induced edema and an inhibitory effect on histamine-induced ileum contraction.

Keywords——*Eriobotrya japonica*; Rosaceae; ursolic acid; methyl maslinate; maslinic acid; euscaphic acid; anti-inflammatory effect; carrageenan edema; histamine-induced contraction

Hot water extract of the leaves of *Eriobotrya japonica* LINDL. has traditionally been used as a diuretic, a stomachic and a remedy for heatstroke, and topical application of the alcohol extract has been used for the treatment of prickly heat, eczema and skin disease. Amygdalin, sugars, organic acids, tannin, *etc.*[2)] have been isolated from this plant but no studies in relation to the anti-inflammatory effect have been done.

In the course of chemical and pharmacological studies on the anti-inflammatory active constituents of topically applied crude drugs, the effects of hot and cold water and alcoholic extracts of *Eriobotrya japonica* were examined by using the carrageenan-induced foot edema method[3)] and only the alcoholic extract was found to have activity. In the present paper, we report the identification of some chemical constituents of the leaves, and their anti-inflammatory activity toward carrageenan-induced edema and inhibitory effect on histamine-induced contraction of the ileum isolated from guinea pigs.

The EtOH extract (fr. 1) was suspended in distilled water and extracted with Et_2O (fr. 2), AcOEt (fr. 3) and *n*-BuOH (fr. 4) successively (Chart 1).

In the carrageenan edema test with topical application of frs. 1—5, fr. 2 showed a stronger effect (5 mg × 4 spread as 5% EtOH solution) than fr. 1 (Table I).

Fraction 2 showed four main spots colored red to orange (designated **1**, **2**, **3**, and **4** from the top downward), excluding the upper spots (chlorophyll) on thin layer chromatography (TLC) (Kieselgel $60F_{254}$) using $CHCl_3$–MeOH (20 : 1) as the eluent. The spots were detected by spraying the plates with 10% H_2SO_4 followed by heating.

Fraction 2 was applied to a column of silica gel, and eluted with petroleum benzin (P.B.)–AcOEt (10 : 3) followed with P.B.–AcOEt (2 : 1) to yield **1**, **2** and a mixture of **3** and **4**. Compounds **3** and **4** could not be separated on a silica gel column because of the similarity of their *Rf* values. The acetate of the mixture was further applied to a column of silica gel and eluted with P.B.–AcOEt (5 : 1) to yield the **3**- and **4**-acetates (**3a** and **4a**), which gave **3** and **4** after saponification with 5% KOH in MeOH.

Compound **1** was identified as ursolic acid by comparison of the physical and spectral

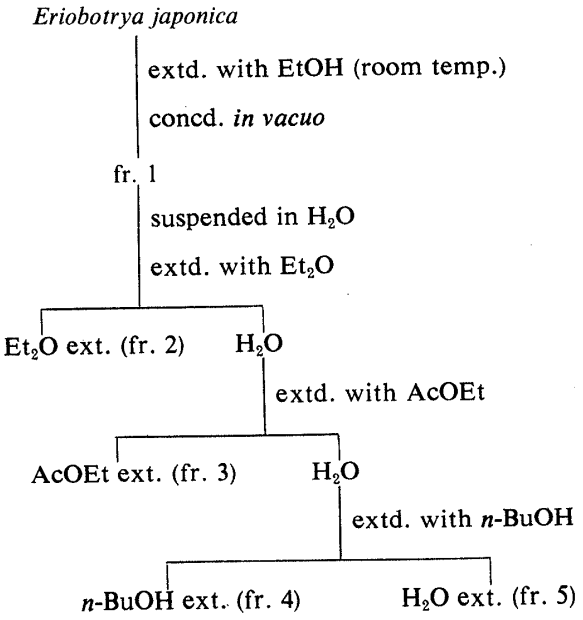

Chart 1

TABLE I. Inhibitory Effects of Frs. 1—5 on Carrageenan Edema

Fraction	Dose (mg/site × 4)	Carrageenan edema (%)[a] Time after injection (h)			
		1	2	3	4
1	5	—	25.6	27.6[b]	—
2	5	18.4	27.3[c]	28.1[b]	7.8
3	5	−15.9	0	0	12.5
4	5	17.8	9.1	4.7	10.9
5	5	0	16.9	16.4	13.2

a) Inhibition %, $n=5$. *b*) $p<0.05$, *c*) $p<0.01$.

data with those of an authentic sample.

Compound **2** was concluded to be methyl maslinate[4] from the physical and spectral data (given in the experimental section), and was confirmed to be identical with an authentic sample.

Compound **3a** gave a methyl ester (**3am**) on methylation with diazomethane; **3am** still showed a hydroxyl absorption band in the infrared (IR) spectrum, indicating the existence of a sterically hindered hydroxyl group. The mass spectrum (MS) showed the characteristic fragmentation pattern of retro-Diels–Alder cleavage, as is seen generally with pentacyclic triterpenes having Δ^{12}-unsaturation. The fragment peaks at m/z 308 and 264 suggested the presence of two acetoxyl groups in ring A or B and one hydroxyl (hindered) and one carboxyl group in ring C or D. From the above results and a comparison of the chemical and physical properties with those given in the literature, **3a** was presumed to be 2α,3α-diacetoxy-19α-hydroxyurs-12-en-28-oic acid.[5]

Compound **3** was obtained by saponification of **3a** with 5% KOH in MeOH and shown to be identical with euscaphic acid[5] isolated from *Euscaphis japonica* PAX (IR, MS and mp).

Compounds **4** and **4a** were identical with authentic maslinic acid [4] and its diacetate, respectively (IR, nuclear magnetic resonance (NMR) and mp).

TABLE II. Inhibitory Effects of Compounds **1**—**4** and Indomethacin on Carrageenan Edema

Compound	Dose (mg/site × 4)	Carrageenan edema (%)[a] Time after injection (h)			
		1	2	3	4
1	5	0	−2.8	−4.3	−5.5
2	5	12.5	13.9	12.8	12.7
3	5	11.7	0	8.8	15.0
4	4	47.1[b]	25.0	0	8.0
Indomethacin	0.5	12.5	19.4	21.3[b]	16.4

a) Inhibition %, $n=5$. *b*) $p<0.05$.

Biological Activities of Compounds 1—4

Compounds **1**—**4** isolated from *E. japonica* in the present study were tested for anti-inflammatory activity after topical application by using the carrageenan-induced foot edema method in rats[3] as described in Experimental (Table II). Among these four compounds, only maslinic acid (**4**) showed activity (16 mg spread per rat, 1 h after carrageenan injection). In the carrageenan-induced foot edema test, histamine, kinins and prostaglandins are known to be involved as chemical mediators at about 1, 2 and 3 h after carrageenan injection, respectively.

Compound **4** also showed a 35% inhibitory effect (2.4×10^{-5} g/ml dose) on histamine-induced (10^{-7} g/ml) contraction in ileum isolated from guinea pig, supporting the result of the carrageenan edema test.

The anti-inflammatory effect of fr. 2 was found 2 and 3 h after carrageenan injection, while the active component (**4**) exhibited its effect at a shorter time. Generally the effect of a crude drug is complex, and is sometimes lost after the separation of each constituent. We concluded that maslinic acid is at least partly responsible for the pharmacological activity of this plant, and its anti-inflammatory effect on topical application may be enhanced in combination with other constituents of the plant.

Experimental

Melting points were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. IR spectra were obtained with a Hitachi 260-0611 spectrometer. NMR spectra were taken with Hitachi R-24B (60 MHz) and JEOL XL-200 (200 MHz) spectrometers with tetramethylsilane as an internal standard, and chemical shifts are given in δ(ppm). The following abbreviations are used: s=singlet, d=doublet, t=triplet, dd=double doublet, m=multiplet, and br=broad. MS were obtained on a JEOL-JMS-D 200 instrument. TLC was performed with Kieselgel $60F_{254}$ plates (Merck) and spots were detected by heating after spraying 10% H_2SO_4.

Bioassay——Anti-inflammatory activity was assessed in male rats weighing 130 to 160 g, in which edema was induced by injecting 0.1 ml of 1% carrageenan. Test material dissolved or suspended in 99.5% EtOH was applied to the hind paw 1 h before and 0, 1 and 2 h after injection of carrageenan, and the paw volumes (in ml) were measured at each time. In the reference group, only 99.5% EtOH was applied.

Inhibitory effect on histamine-induced contraction was assessed in ileum isolated from male guinea pigs weighing 300 to 350 g. Contraction of the ileum was induced by histamine at a concentration of 10^{-7} g/ml in a Tyrode solution aerated with a mixture of 95% O_2 and 5% CO_2 at 32 °C. Test material was applied 3 min before addition of histamine.

Extraction and Fractionation——Fresh leaves of *Eriobotrya japonica* LINDL. (1 kg) were extracted with EtOH (3d at room temperature × 2). The EtOH solution was concentrated *in vacuo* (below 40 °C) to give the EtOH extract (98 g) (fr.1). Fraction 1 was suspended in water and extracted with Et_2O, AcOEt and *n*-BuOH, successively. Each solution were evaporated to dryness under the same conditions as used for fr. 1 to afford fr. 2 (39 g), fr. 3 (10 g), fr. 4 (10 g) and water extract (fr. 5) (34 g) (Chart 1).

Constituents of Active Fraction (Fr.2)——Fraction 2 showed four main spots on TLC (designated as **1**, **2**, **3** and **4** from the top downward) with $CHCl_3$–MeOH (20:1). They were colored red, orange, dark brown and dark brown,

respectively, by spraying with 10% H_2SO_4 followed by heating. Fraction 2 was chromatographed on silica gel to give **1** (300 mg) and **2** (100 mg) from the eluate with P.B.–AcOEt (10 : 3) and a mixture of **3** and **4** from the eluate with P.B.–AcOEt (2 : 1). The mixture of **3** and **4** was acetylated with Ac_2O and pyridine and chromatographed on silica gel using P.B.–AcOEt (5 : 1) to afford **3**-acetate (**3a**) and **4**-acetate (**4a**). **3a** and **4a** were saponified with 5%KOH in MeOH to afford **3** (88 mg) and **4** (90 mg).

Ursolic Acid (1)——White powder, mp 285—287 °C (MeOH). MS *m/z*: 456 (M^+). *Anal.* Calcd for $C_{30}H_{48}O_3 \cdot 1/2H_2O$: C, 77.37; H, 10.61. Found: C, 77.87; H, 10.31. IR ν_{max}^{KBr} cm^{-1}: 3440, 1690.

Methyl Maslinate (2)——Colorless needles, mp 231—233 °C (P.B.–AcOEt). MS *m/z*: 486 (M^+). *Anal.* Calcd for $C_{31}H_{50}O_4 \cdot 1/2H_2O$: C, 75.01; H, 10.37. Found: C, 74.80; H, 10.15. IR ν_{max}^{KBr} cm^{-1}: 3440, 1725, 1620. Liebermann–Burchard reaction: red to violet.

Methyl Maslinate Diacetate (2a)——**2** (10 mg) was acetylated with Ac_2O (0.5 ml) and pyridine (0.5 ml) at room temperature (15 h) and chromatographed on silica gel (P.B.–AcOEt (5 : 1)) to yield **2a** (7 mg), mp 157—159 °C (P.B.–AcOEt). IR ν_{max}^{KBr} cm^{-1}: 1745, 1725, 1620. MS *m/z*: 570 (M^+). NMR ($CDCl_3$) δ: 0.72, 1.08, 1.13 (each 3H, s, CH_3), 0.91 (12H, $CH_3 \times 4$), 2.00, 2.08 (each 3H, s, $OCOCH_3 \times 2$), 2.88 (1H, dd, $J=14$, 4 Hz, C_{18}-H), 3.63 (3H, s, $COOCH_3$), 4.78 (1H, d, $J=10$ Hz, C_3-H), 5.13 (1H, m, C_2-H), 5.29 (1H, m, C_{12}-H).

Euscaphic Acid Diacetate (3a)——Colorless needles, mp 186—188 °C (MeOH). MS *m/z*: 572 (M^+). IR ν_{max}^{KBr} cm^{-1}: 3500, 1745, 1740, 1690, 1640. NMR ($CDCl_3$) δ: 0.74, 0.89, 1.05, 1.22, 1.31 (each 3H, CH_3), 0.95 (6H, $CH_3 \times 2$), 1.98, 2.14 (each 3H, $OCOCH_3$), 2.56 (1H, s, C_{18}-H), 5.00 (1H, d, $J=2.5$ Hz, C_3-H), 5.27 (1H, m, C_2-H), 5.38 (1H, br, C_{12}-H).

Alkaline Hydrolysis of 3a (3)——A solution of **3a** (98 mg) in methanolic 5% KOH was allowed to stand for 5 h at room temperature. After dilution with water, the reaction mixture was neutralized with 1 N HCl and extracted with Et_2O. The Et_2O solution was concentrated to give **3** (88 mg), white crystalline powder, mp 269—270 °C (MeOH). MS *m/z*: 488 (M^+). *Anal.* Calcd for $C_{30}H_{48}O_5 \cdot H_2O$: C, 71.11; H, 9.95. Found: C, 70.73; H, 9.65.

Maslinic Acid Diacetate (4a)——White needles, mp 235—238 °C (MeOH). MS *m/z*: 556 (M^+). IR ν_{max}^{KBr} cm^{-1}: 3440, 1745, 1740, 1695, 1650. NMR ($CDCl_3$) δ: 0.75, 0.91, 1.07, 1.13 (each 3H, CH_3), 0.90 (9H, $CH_3 \times 3$), 2.00, 2.08 (each 3H, $OCOCH_3$), 2.85 (1H, dd, $J=14$, 4 Hz, C_{18}-H), 4.79 (1H, d, $J=10$ Hz, C_3-H), 5.14 (1H, sextet, $J=10$, 10, 4 Hz, C_2-H), 5.30 (1H, m, C_{12}-H).

Alkaline Hydrolysis of 4a (4)——A solution of **4a** (110 mg) in methanolic 5% KOH was treated as in the case of **3a** to give **4** (90 mg), white powder, mp 252—254 °C (MeOH). MS *m/z*: 472 (M^+). *Anal.* Calcd for $C_{30}H_{48}O_4 \cdot H_2O$: C, 73.43; H, 10.27. Found: C, 73.32; H, 10.05. IR ν_{max}^{KBr} cm^{-1}: 3440, 1690.

Acknowledgement The authors wish to thank Prof. J. Sakakibara of Nagoya City University, Dr. M. Takani, Faculty of Pharmaceutical Science, Kanazawa University, and Prof. T. Tomimori of Hokuriku University for providing authentic samples of methyl maslinate, euscaphic acid and ursolic acid. Thanks are also due to Dr. T. Tsuyuki, Faculty of Science, Tokyo University, and Dr. M. Toori of Tokushima-Bunri University for their valuable advice on NMR spectral analysis, and to Kaken Pharmaceutical Co., Ltd. for bioassay.

References and Notes

1) M. Shimizu, H. Fukumura, S. Tanaami and N. Morita, presented at the 105th Annual Meeting of the Pharmaceutical Society of Japan, Kanazawa, April 1985.
2) D. O. Gray, *Phytochemistry*, **11**, 745, 751 (1972).
3) C. A. Winter, E. A. Risley and G. W. Nuss, *Proc. Soc. Exp. Biol.*, **111**, 544 (1962).
4) A. Yagi, N. Okamura, Y. Haraguchi, K. Noda and I. Nishioka, *Chem. Pharm. Bull.*, **26**, 3075 (1978).
5) K. Takahashi, S. Kawaguchi, K. Nishimura, K. Kubota, Y. Tanabe and M. Takani, *Chem. Pharm. Bull.*, **22**, 650 (1974).