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Studies on Bioactive Substances in Crude Drugs Used for Arthritic Diseases in Traditional Chinese Medicine. II. Isolation and Identification of an Anti-inflammatory and Analgesic Principle from the Root of *Angelica pubescens* MAXIM.

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The methanol extract of the root of *Angelica pubescens* MAXIM. was fractionated, and, by following the inhibitory activities on rat hind paw edema induced by carrageenan and on writhing induced by acetic acid in mouse, the active principle was isolated and identified as osthol.

Keywords—anti-inflammatory activity; analgesic activity; *Angelica pubescens*; osthol; Chinese crude drug; anti-arthritic principle

We have previously reported that a group of Chinese crude drugs called Qu-feng-shi-yao (祛風湿薬), which has been used in traditional Chinese medicine as a remedy mostly for arthritic diseases, showed high anti-inflammatory and analgesic activities.[1]

Radix Angelicae Pubescentis ("Du-huo" in Chinese, "Dokkatsu" in Japanese), the root of *Angelica pubescens* MAXIM., has been used in traditional Chinese medicine as a remedy for arthritic diseases, the common cold and so on.[2] Various coumarins have been isolated from this herb.[3,4] This report describes a study on the anti-inflammatory and analgesic principle in the root of *Angelica pubescens* MAXIM.

Anti-inflammatory and analgesic tests were carried out using the carrageenan-induced paw edema method[5] and the acetic acid-induced writhing method,[6] respectively. The methanol extract of the root was fractionated as shown in Chart 1. Table I shows the biological activities of each fraction.

The roots of *Angelica pubescens* MAXIM. were ground and extracted with methanol under reflux. The extract was distributed between the lower layer and the upper layer of a solution of chloroform : methanol : water = 19 : 19 : 12 (v/v). The lower layer showed significant inhibitory activities on both carrageenan-induced edema (50.8% inhibition at a dose of 100 mg/kg) and acetic acid-induced writhing (67.1% inhibition at a dose of 500 mg/kg), but the upper layer showed no inhibitory activities in the bioassays. The active fraction was subjected to column chromatography on alumina with ethyl acetate and methanol as eluents. Anti-inflammatory and analgesic activities were only detected in the ethyl acetate eluate. Finally, the active fraction was further separated into three fractions on silica gel column chromatography with *n*-hexane : ethyl acetate = 5 : 1 (v/v). The active principle was obtained in a crystalline form from fraction No. 2 (fr. 2). The crystalline material was recrystallized from ether to give colorless needles (mp 82—83 °C) in 0.89% yield.

This active principle has the following properties. mp 82—83 °C. Mass spectrum (MS) *m/z*: 244 (M^+), 229, 213, 201, 189, 175, 159, 131, 115. Infrared (IR) ν_{max}^{KBr} cm^{-1}: 1718, 1605,

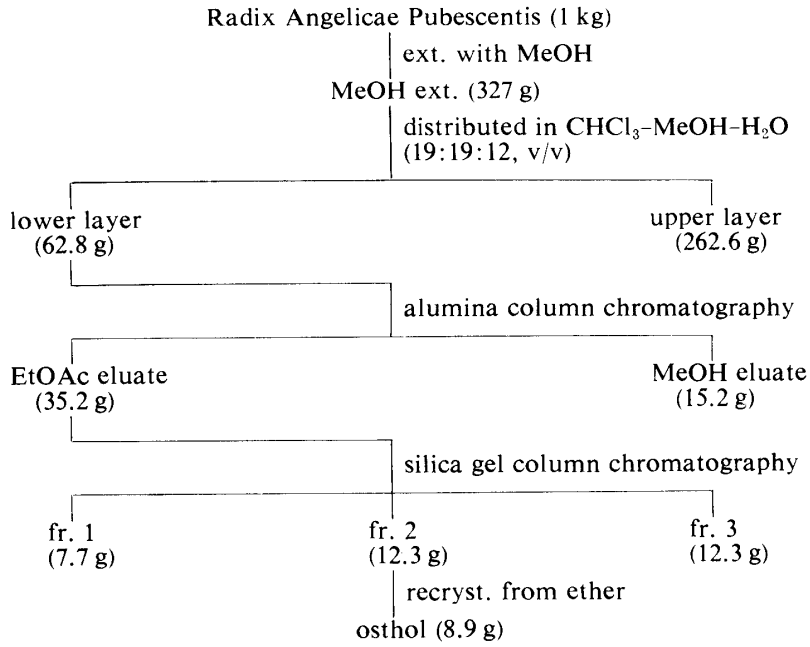

Chart 1. Isolation of Osthol from Radix Angelicae Pubescentis

( ) indicates yield.

TABLE I. Biological Activities of the Active Fractions

Active fractions and compounds	Anti-inflammatory activity[a] inhibition %		Analgesic activity[b] inhibition %	
	Dose (mg/kg) (*i.p.*)	Mean ± S.E.	Dose (mg/kg) (*s.c.*)	Mean ± S.E.
MeOH ext.	400	50.4 ± 2.41[c]	2000	67.8 ± 5.34[c]
Lower layer	100	50.8 ± 4.07[c]	500	67.1 ± 3.48[c]
EtOAc eluate	75	41.8 ± 2.97[c]	250	65.8 ± 3.91[c]
Fraction 2	50	55.7 ± 3.69[c]	75	69.2 ± 4.74[c]
Osthol	50	65.3 ± 3.62[c]	50	61.2 ± 5.14[c]
Indomethacin	10	47.2 ± 2.54[c]		NT
Aminopyrine		NT	50	93.4 ± 3.00[c]
Aspirin		NT	100	69.7 ± 3.00[c]

a) Inhibition % at 3 h after injection of carrageenan. *b*) The number of writhings was counted for 5 min beginning from 10 min after acetic acid *i.p.* injection. NT: not tested. *c*) Significant at $p<0.01$.

1260, 1100. Ultraviolet (UV) λ_{max}^{EtOH} nm (ε): 247 (6431), 258 (7074), 322 (18221). Proton nuclear magnetic resonance (^{1}H-NMR) ($CDCl_3$) δ: 1.65 (3H, s), 1.84 (3H, s), 3.48 (2H, d, $J=7.0$ Hz), 3.88 (3H, s), 5.23 (1H, t, $J=7.0$ Hz), 6.14 (1H, d, $J=9.4$ Hz), 6.79 (1H, d, $J=8.6$ Hz), 7.24 (1H, d, $J=8.6$ Hz), 7.56 (1H, d, $J=9.4$ Hz). Carbon-13 nuclear magnetic resonance (^{13}C-NMR)[7] ($CDCl_3$) δ: 17.8 (q), 22.0 (t), 25.7 (q), 56.0 (q), 107.5 (d), 112.7 (d), 113.0 (s), 117.7 (s), 121.4 (d), 126.4 (d), 132.2 (s), 143.7 (d), 152.8 (s), 160.3 (s), 160.9 (s). *Anal.* Calcd for $C_{15}H_{16}O_3$: C, 73.75; H, 6.60. Found: C, 73.46; H, 6.57.

From the physicochemical data mentioned above, it was concluded that the active principle is osthol. The IR, ^{1}H-NMR and ^{13}C-NMR spectra were identical with those of an authentic sample.

The anti-inflammatory and analgesic activities of osthol are shown in Table I. Osthol

showed significant inhibitory activities on both carrageenan-induced edema (61.8% inhibition at a dose of 500 mg/kg) and acetic acid-induced writhing (53.9% inhibition at a dose of 250 mg/kg) even when given by oral administration.

Osthol has been isolated from many plants, *e.g.*, *Angelica pubscens* MAXIM.,[4] *Cnidium monnieri* (L.) CUSSON,[8] *Murraya paniculata* (L.) JACK.,[9] *Angelica* spp.,[10] *Angelica polymorpha* MAXIM.,[11] *Angelica japonica* A. GRAY[12] and *Angelica kiusiana* MAXIM.[13] and so on. Most of these plants have been used as remedies for arthritic diseases.[2] Therefore, our results are considered to indicate that osthol probably plays an important role in the anti-arthritic action of these plants.

Experimental

The melting point was determined on a Yanaco MP-S3 melting point apparatus and is uncorrected. The low-resolution MS was taken with a JEOL JMS-D 100 instrument. The IR spectrum was recorded on a JASCO A-220 IR spectrophotometer. The UV spectrum was recorded on a Shimadzu UV-220 spectrophotometer. ^{1}H-NMR and ^{13}C-NMR spectra were measured with a JEOL FX-90 NMR spectrometer. Chemical shifts are reported in δ-values downfield from internal tetramethylsilane, and the following abbreviations are used: s = singlet, d = doublet, t = triplet and q = quartet. The coupling constants are quoted in Hz. Elemental analysis was done with a Perkin Elmer 240C elemental analyzer.

Assay for Anti-inflammatory Activity——A carrageenan-induced inflammatory edema described by Winter *et al.*[5] was used in these experiments. Ten male Wistar strain rats weighing 130—150 g were used at each dose level. The samples to be tested were administered *i.p.* or *p.o.* as a 1% Tween 80 suspension, 30 min before the carrageenan treatment. The subplantar injection of 0.1 ml of 1% carrageenan (lambda carrageenan type IV, Sigma Chemical Co.) in saline solution was performed, then the volume of the foot was measured with a Ugo Basile plethysmometer every 1 h for 5 h, and the percent swelling (foot edema) was calculated. The swelling of the paw reached a peak 3 h after the injection of 0.1 ml of carrageenan. Results were expressed as inhibition of swelling at 3 h, relative to the control group given the vehicle.

Assay for Analgesic Activity——An acetic acid-induced writhing method reported by Whittle[6] was used in these experiments. Ten male ddY strain mice weighing 20—22 g were used at each dose level. The samples to be tested were administered *s.c.* or *p.o.* as a 1% Tween 80 suspension 30min before the acetic acid treatment. Writhing was induced in mice by intraperitoneal injection of 0.7% acetic acid (10 ml/kg), and the number of writhings was counted in each animal for 5 min beginning from 10 min after the acetic acid challenge.

Extraction and Partition——The dried roots of *Angelica pubescens* MAXIM. (1 kg), obtained from the Institute of Chinese Materia Medica, Academy of Chinese Traditional Medicine, China, were extracted three times with 5 l each of methanol under reflux. The mixture was filtered and the filtrate was concentrated *in vacuo*, keeping the temperature below 40 °C, to give a methanol extract (327 g). The procedure for isolation of the biologically active principle from the extract is shown in Chart 1. The biological activities of each fraction are shown in Table I. The methanol extract (327 g) was suspended in the upper layer of a solution of chloroform : methanol : water = 19 : 19 : 12 (v/v) and extracted twice with the lower layer of the mixed solvent. The combined extracts were concentrated *in vacuo*, affording the lower layer (62.8 g).

Alumina Column Chromatography——A part of the lower layer (20.0 g) was subjected to column chromatography on alumina (5.0 × 20 cm; grade I; Aluminium oxide 90 active, basic for column chromatography, Merck) with ethyl acetate and methanol as eluents, giving two fractions, the ethyl acetate eluate (11.2 g) and the methanol eluate (4.8 g). In total, 35.2 g of the ethyl acetate eluate was obtained by repeated chromatography.

Silica Gel Column Chromatography——A part of the ethyl acetate eluate (10.0 g) was subjected to column chromatography on silica gel (5.0 × 50 cm, Kieselgel 60, Merk) with *n*-hexane : ethyl acetate = 5 : 1 (v/v) as the eluent, giving three fractions, fr. 1 (2.2 g), fr. 2 (3.5 g) and fr. 3 (3.5 g) based on monitoring by thin layer chromatography (solvent system, *n*-hexane : ethyl acetate = 2 : 1 (v/v), Kieselgel 60 F_{254}, Merck). Anti-inflammatory and analgesic activities were only detected in fr. 2. In total, 12.3 g of the active fraction (fr. 2) was obtained by repeated chromatography. The crystalline material (12.3 g) was recrystallized from ether to afford colorless plates of osthol (8.9 g).

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