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Studies on Antihemorrhagic Substances in Herbs Classified as Hemostatics in Chinese Medicine. IV. On Antihemorrhagic Principles in *Hypericum erectum* THUNB.

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Antihemorrhagic principles were isolated from *Hypericum erectum* THUNB. (Hypericaceae) by a combination of countercurrent distribution and Sephadex LH-20 column chromatography, and identified as wedelolactone[5′,5,6-trihydroxy-7′-methoxycumarino-(3′,4′ : 3,2)-coumarone] (1,8,9-trihydroxy-3-methoxy-6-oxo-6*H*-[*z*]benzofuro[3,2-*c*][*z*]benzopyran) and desmethylwedelolactone [5′,7′,5,6-tetrahydroxyl-cumarino-(3′,4′ : 3,2)-coumarone](1,3,8,9-tetrahydroxy-6-oxo-6*H*-[*z*]-benzofuro[3,2-*c*][*z*]benzopyran).

Keywords——antihemorrhagic principle; hemostatic; countercurrent distribution; gel filtration; *Hypericum erectum*; wedelolactone; desmethylwedelolactone

In the course of studies on the isolation of antihemorrhagic substances in herbs which are commonly used as hemostatic agents in Chinese medicine, we previously isolated 3,3′,4-tri-*O*-methylellagic acid as an antihemorrhagic principle in *Sanguisorba officinallis* L. (Rosaceae).[1] The present paper describes the isolation of the antihemorrhagic principles in *Hypericum erectum* THUNB. (Hypericaceae).

Hypericum erectum THUNB. is an important herb in Chinese medicine as an antihemorrhagic agent, astringent and uretic.[2] Previous studies on the herb focused only on the tannin,[2] coloring matter (hypericin)[3] and essential oils as chemical components in the herb, and no pharmacological study on the antihemorrhagic principle has been reported. We wish to describe here the isolation and identification of the hemostatically active principles in the herb.

During the isolation process, Tajima's method (in mice) was used for the pharmacological measurement of antihemorrhagic activity, as reported previously.[4] Isolation of the active principles was achieved by a combination of countercurrent distribution and Sephadex LH-20 column chromatography. The procedures are summarized in Chart 1.

As shown in Chart 1, the ground herb of *Hypericum erectum* THUNB. was extracted with water under reflux. The extract was separated by countercurrent distribution, using the solvent system of *n*-butanol and water. The distribution pattern and activities are shown in Fig. 1.

As the activity was located in the $r=0$ tube, this fraction was subjected to Sephadex LH-20 column chromatography with methanol. The elution pattern and activities are shown in Fig. 2.

The highest activity was obtained in fraction 5. Final purification of the active fraction II (fraction 5) was achieved by gel filtration through Sephadex LH-20. Elution with methanol afforded two active fractions, III and III′, in that orders. The active fractions, III and III′, were recrystallized from methanol to give colorless plates (37 mg) and green needles (3.2 mg), respectively. A comparison of the spectral data (nuclear magnetic resonance (NMR), infrared

ground herb of *Hypericum erectum* THUNB. (500 g)

↓ extracted with H_2O

extract (93.2 g) [1 g/kg---4.3 min]

↓ countercurrent distribution with *n*-BuOH–H_2O (1 : 1) ($n=2$)

active fraction I ($r=2$) (3.243 g) [35 mg/kg---3.9 min]

↓ gel filtration through Sephadex LH-20 with MeOH

active fraction II (127 mg) [5 mg/kg---4.1 min]

↓ column chromatography on Sephadex LH-20 with MeOH

active fraction III (51 mg)	active fraction III′ (7 mg)
↓ [0.5 mg/kg---4.1 min]	↓ [0.5 mg/kg---4.2 min]
↓ recrystallized from MeOH	↓ recrystallized from MeOH
colorless plates (37 mg) (**1**)	green needles (3.2 mg) (**2**)
[0.5 mg/kg---3.9 min]	[0.5 mg/kg---4.2 min]

( ) indicates yields. [ ] indicates dose and activity (shortening of bleeding time).

Chart 1. Isolation Procedures

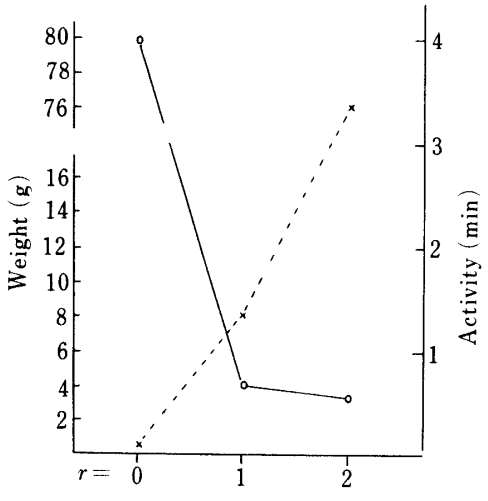


Fig. 1. Countercurrent Distribution Pattern of the H_2O-Extract (93.2 g) with *n*-BuOH–H_2O (1 : 1) ($n=2$)

○—○, distribution pattern; ×---×, activity (shortening of bleeding time after *i.p.* administration at a dose of 1 g/kg).

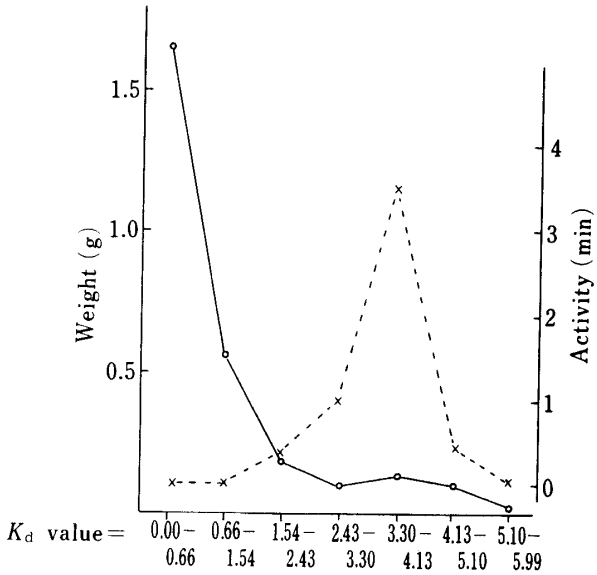


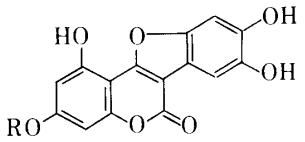
Fig. 2. Elution Pattern of Active Fraction I (3.243 g) on a Sephadex LH-20 Column

○—○, chromatogram; ×---×, activity (shortening of bleeding time after *i.p.* administration at a dose of 5 mg/kg).

(IR), mass and ultraviolet (UV)) of **1** and **2** suggested that **2** is the desmethyl derivative of **1**.

The IR spectrum of **1** showed peaks at 3550, 3425 and 3300 cm^{-1} (phenolic hydroxyl), 1690 cm^{-1} (conjugated δ-lactone), 1607 cm^{-1} (conjugated C=C) and 1160 cm^{-1} (aromatic

C–O–C). Further, **1** was reacted with acetic anhydride in pyridine to afford a tri-*O*-acetyl derivative. The proton nuclear magnetic (^{1}H-NMR) and IR spectral data showed the presence of three phenolic hydroxyl groups, one methoxyl group, 4,5-disubstituted pyrocatechol and 4,5-disubstituted resorcinol moieties in the molecule of **1**. The UV absorption spectrum of **1** was apparently identical with that of wedelolactone.[5] Accordingly **1** was concluded to be identical with wedelolactone (**1**) and **2** was concluded to be desmethylwedelolactone (**2**).



1: R = CH_3 wedelolactone
2: R = H desmethylwedelolactone

These conclusions were confirmed by direct comparison of the products with the synthetic samples of wedelolactone [5′,5,6-trihydroxyl-7′-methoxy-cumarino-(3′,4′ : 3,2)-coumarone] (1,8,9-trihydroxy-3-methoxy-6-oxo-6*H*-[*z*]benzofuro[3,2-*c*][*z*]benzopyran)[6] and desmethylwedelolactone [5′,7′,5,6-tetrahydroxyl-cumarino-(3′,4′ : 3,2)-coumarone](1,3,8,9-tetrahydroxy-6-oxo-6*H*-[*z*]benzofuro [3,2-*c*][*z*]benzopyran)[7] prepared by Wanzlick's method.[6] The antihemorrhagic activities of the synthetic compounds were equal to those of the natural products.

Wedelolactone and desmethylwedelolactone were previously isolated from *Wedelia calendulaceae* (Compositae)[8] and *Eclipta alba* L. (Compositae),[9] and they cause the blacking of *Eclipta alba* L. by being oxidized enzymatically by a polyphenoxidase.[10] The presence of these compounds in *Eclipta alba* L. had been recorded in the earlier reports, but the compounds could not be separated by fractional crystallization or chromatography over cellulose, deactivated alumina or silica gel. However, as shown in Chart 1, they were easily separated by means of chromatography on Sephadex LH-20 with methanol.

Wedelolactone and desmethyl-wedelolactone were reported to have antibacterial activity,[11] but this is the first report of their antihemorrhagic activity.

Experimental

Proton and carbon magnetic resonance spectra were recorded with a JEOL FX-90 Fourier-transform NMR spectrometer and are calibrated in parts per million (δ) downfield from tetramethylsilane as an internal standard. IR spectra were recorded on a JASCO IRA-2 grating infrared spectrophotometer. Low- and high-resolution mass spectra (MS) were recorded on a Hitachi M-80A instrument. UV spectra were recorded on Shimadzu UV-360 recording spectrophotometer. Melting points are uncorrected.

Assay of Hemostatic Activity——Hemostatic activity testing was carried out by Tajima's method on male mice weighing 18—20 g. Test material homogenized in 1% methyl-cellulose–0.9% sodium chloride aq. was given interperitoneally by injection. The bleeding time was determined by means of Tajima's procedure.[12]

Extraction——Ground herb of *Hypericum erectum* THUNB. (500 g) was extractd with 5 l of water under reflux for 30 min. The mixture was centrifuged at 2500 rpm for 30 min and the supernatant was lyophilized to afford a green powder (93.2 g).

Countercurrent Distribution with *n*-BuOH and H_2O (1 : 1) Solvent System——A portion (9.0 g) of the crude extract was distributed between the lower phase (180 ml) and upper phase (180 ml) of the solvent system described above for 2-plate transfer by transfering the lower phase. In total, 3.243 g of active fraction I ($r = 2$) was obtained by repeated countercurrent distribution.

Gel Filtration through Sephadex LH-20——Active fraction I (3.243 g) was dissolved in 6 ml of methanol and subjected to gel filtration on a Sephadex LH-20 column (4.1 × 90 cm); elution with methanol afforded 127 mg of active fraction II (K_d value = 3.12 to 4.29).

Column Chromatography on Sephadex LH-20——Active fraction III (127 mg) was dissolved in 2 ml of methanol and subjected to gel filtration on Sephadex LH-20 (80 ml) using methanol as an eluent to afford active fraction III

(51 mg) [K_d value = 3.14 to 3.15] and III′ (7 mg) [K_d value = 3.63 to 3.97]. The active fraction III and III′ were recrystallized from methanol to give colorless plates (37 mg) (**1**) and green needles (3.2 mg) (**2**), respectively. Physical properties of **1** and **2** are described below.

Identification of Wedelolactone (1) and Demethylwedelolactone (2)——**1** and **2** have following properties.

1: colorless plates. mp 327—328 °C. Ferric chloride-positive. MS *m/z*: 314 (M^+), 299, 285, 271, 243, 215, 187, 157, 131. High-resolution MS: 314.0433 (error +0.7 mMU) for $C_{16}H_{10}O_7$. ^{1}H-NMR (in d_6-DMSO) δ (ppm): 10.93 (1H, s), 9.39 (1H, s), 6.45 (1H, d, J = 2.2 Hz), 3.81 (3H, s). ^{13}C-NMR (in d_6-DMSO) δ (ppm): 162.2 (s), 158.9 (s), 157.6 (s), 155.2 (s), 154.8 (s), 148.9 (s), 145.3 (s), 144.2 (s), 113.9 (s), 104.7 (d), 101.7 (s), 98.8 (d), 98.1 (d), 95.5 (s), 93.2 (d), 56.6 (q). IR ν_{max}^{KBr} cm^{-1}: 3550, 3425, 3300, 1690, 1607, 1460, 1442, 1320, 1160, 1060, 850. UV λ_{max}^{MeOH} nm (log ε): 350 (4.36), 303 (3.91), 250 (4.28), $\lambda_{max}^{MeOH+0.1\,N\,NaOH}$ nm (log ε): 305 (4.12), 235 (sh), (4.38).

2: Green needles. mp 360 °C. Ferric chloride-positive. MS *m/z*: 300 (M^+), 271, 243, 187, 150. High-resolution MS: 300.3273 (error +0.3 m MU) for $C_{15}H_8O_7$. ^{1}H-NMR (in d_6-DMSO) δ (ppm): 10.00—11.00 (2H, br), 9.00—9.60 (2H, br), 7.17 (1H, s), 7.10 (1H, s), 6.30 (2H, m). IR ν_{max}^{KBr} cm^{-1}: 3475, 3350, 3300, 3100, 1690, 1630, 1620, 1460, 1338, 1285, 1090, 1070, 900, 850. UV λ_{max}^{MeOH} (log ε): 350 (4.36), 303 (3.91), 248 (4.28), $\lambda_{max}^{MeOH+1\,N\,NaOH}$ nm (log ε): 363 (4.36), 329 (3.95), 279 (4.11).

1 and **2** were identified by direct comparison with authentic samples of wedelolactone (**1**) and desmethylwedelolactone (**2**), respectively.

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