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Studies on Antihemorrhagic Substances in Herbs Classified as Hemostatics in Chinese Medicine. V. On Antihemorrhagic Principle in *Biota orientalis* (L.) ENDL.

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The present paper describes the isolation of an antihemorrhagic principle in *Biota orientalis* (L.) ENDL. (Cupressaceae) by a combination of partition, Sephadex LH-20 and silica gel column chromatographies, and its identification as quercitrin.

Keywords——hemostatic; antihemorrhagic principle; *Biota orientalis*; quercitrin; quercetin; Tajima's method; pharmacological study

In the previous paper,[1] we reported the isolation of the antihemorrhagic principles, wedelolactone and desmethylwedelolactone, of *Hypericum erectum* THUNB., which is one of the herbs used as a hemostatic in Chinese medicine. The present paper describes the isolation and identification of the antihemorrhagic principle in *Biota orientalis* (L.) ENDL.

Biota orientalis (L.) ENDL. (Cupressaceae), which is a component of Hakuyoutou (柏葉湯), Sokuhakusan (側柏散), Dankougetsu (断紅月) and Shiseigan (四生丸), is an important herb in Chinese medicine as a hemostatic, expectorant and cough remedy; the hemostatic action of the herb is well established.[2] Previous work on the herb has been focused on the chemical components, *e.g.*, terpenoids,[3] estloids,[4] flavonoids,[5,6] *etc.* Pharmacological studies of the extract and flavonoids of the herb to investigate the expectorant, antitussive and antibacterial actions have also been reported,[3,7] but no study on the antihemorrhagic principle of the herb has appeared.

We wish to report here the isolation and identification of the antihemorrhagic principle in the herb. In the isolation process, Tajima's method[8] with mice was used for the pharmacological measurement of the antihemorrhagic activity of the material.[9] Isolation of the active principle was achieved by a combination of partition, gel filtration on Sephadex LH-20 and column chromatography on silica gel. The procedures are summarized in Chart 1.

As shown in Chart 1, ground leaves and branches of *Biota orientalis* (L.) ENDL. were extracted with water under reflux. The extract was partitioned with water and ethyl acetate. As the activity emerged predominantly in the ethyl acetate layer (active fraction I), this fraction was subjected to Sephadex LH-20 column chromatography with methanol. The elution pattern and activities of the fractions are shown in Fig. 1.

The active fraction II (K_d value = 1.31 to 2.35) was subjected to gel filtration on Sephadex LH-20. The active fraction III thus obtained was subjected to silica gel column chromatography eluting firstly with tetrahydrofuran (THF)–$CHCl_3$ (1 : 1) and then THF. The activity was emerged only in the fraction eluted with THF. A small amount of impurity was removed from active fraction IV by gel filtration on Sephadex LH-20, and the product was recrystallized from methanol and water to afford light yellow needles.

The active principle was hydrolyzed with hesperidinase[10] to yield equimolar amounts of quercetin (**1**) and rhamnose. Thus, the active principle appeared to be identical with quercitrin

leaves and branches of *Biota orientalis* (L.) (ENDL. (400 g)

↓ extracted with H_2O

extract (35.3 g) [1 g/kg---4.33 min]

↓ partitioned with AcOEt and H_2O

active fraction I (AcOEt-layer) (3.73 g) [135 mg/kg---4.5 min]

↓ gel filtration on Sephadex LH-20 with MeOH

active fraction II (0.425 g) [50 mg/kg---3.85 min]

↓ gel filtration on Sephadex LH-20 with MeOH

active fraction III (123 mg) [50 mg/kg---3.90 min]

↓ silica gel column chromatography with THF–$CHCl_3$ and then THF

active fraction IV (73 mg) [50 mg/kg---4.67 min]

↓ gel filtration on Sephadex LH-20

active fraction V (67 mg) [50 mg/kg---5.2 min]

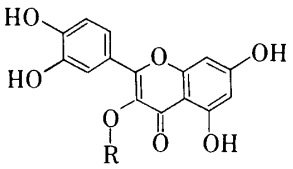
↓ recrystallized from MeOH and H_2O

light yellow powder (44.5 mg) [1.25 mg/kg---5 min]

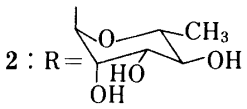
( ) indicates yield. [ ] indicates dose and activity (shortening of bleeding time).

Chart 1. Isolation of the Active Principle

(**2**).[11,12] This conclusion was confirmed by direct comparison with an authentic sample (obtained from Wako Co.). The antihemorrhagic activity of the authentic sample was equal to that of the natural product.



1 : R=H quercetin



2 : R= quercitrin

Quercitrin has been isolated from many plants, *e.g., Prunus tomentosa*,[13] *Polygonum polystachym*,[14] *Cassia obtusifolia* L.,[15] *Vaccinium myrtillus*,[16] *Lathyrus odoratus*,[17] *Wuphoria longana* LAM.,[18] *Thuja orientallis*,[19,20] *etc.* Pharmacological studies of quercitrin, describing its capillary-stabilizing effect,[21] potentiation of the effect of adrenaline,[22] effect on blood pressure,[23] effect on lymph flow,[24] mutagenicity,[25] toxity[26] and so on have appeared, but there has been no previous report on the antihemorrhagic activity.

Experimental

Proton and carbon magnetic resonance spectra were recorded with a JEOL FX-90 Fourier-transform nuclear

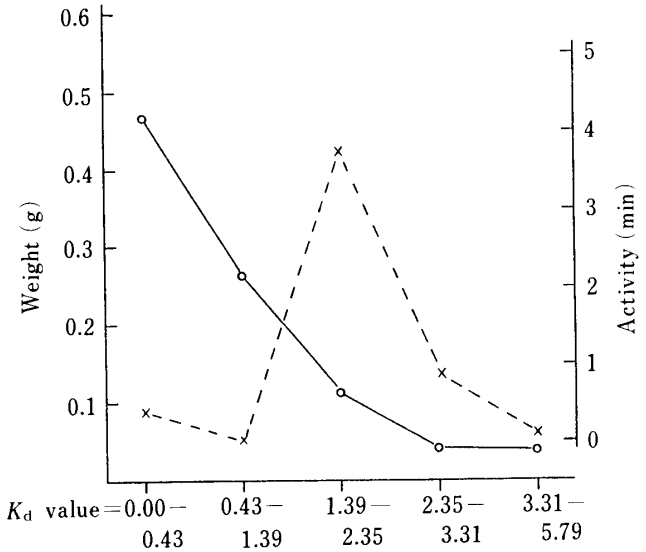


Fig. 1. Elution Pattern of the AcOEt Layer (1.436 g) on Sephadex LH-20 Gel Filtration (4.1 × 45 cm Column)

○—○, chromatogram; ×---×, activity (shortening of bleeding time after the *i.p.* administration of each fraction at a dose of 50 mg/kg).

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

Assay of Hemostatic Activity——Hemostatic tests were carried out by Tajima's method[8] on mice weighing 18 to 20 g. Test material homogenized in 1% methylcellulose–0.9% sodium chloride aq. was given interperitoneally by injection. The bleeding time was determined by Tajima's procedure.

Extraction——Ground leaves and branches of *Biota orientalis* ENDL. (500 g) were extracted with 4 l of water under reflux for 30 min. The mixture was centrifuged at 2500 rpm for 20 min and the supernatant was lyophilized to afford a yellow-brown powder (35.3 g).

Partition between Ethyl Acetate and Water——The crude extract (35.3 g) was dissolved in 700 ml of water and extracted with 700 ml of ethyl acetate four times. Upon concentration of the active fraction (ethyl acetate layer) in a rotary evaporator, 3.73 g of fraction I was obtained as a light brown gum.

Gel Filtration on Sephadex LH-20——A portion (1.436 g) of active fraction I was dissolved in 5 ml of methanol and subjected to gel filtration on Sephadex LH-20 (4.1 × 45 cm) with methanol. In total, 0.425 g of active fraction II [K_d value = 1.31 to 2.35] was obtained by repeated chromatography of active fraction I.

Column Chromatography on Sephadex LH-20——Active fraction II (0.425 g) was dissolved in 2 ml of methanol and applied to Sephadex LH-20 (3 × 48 cm) column. Elution with methanol afforded 123 mg of active fraction III [K_d value = 1.31 to 1.52].

Silica Gel Column Chromatography——Active fraction III (123 mg) was dissolved in 2 ml of THF–$CHCl_3$ (1 : 1), and subjected to silica gel column chromatography (1.5 × 6.7 cm). Elution with THF–$CHCl_3$ and then THF gave 73.5 mg of active fraction IV from the THF fractions as a light yellow gum.

Recrystallization from Methanol and Water——Active fraction V (67 mg) was obtained by gel filtration of active fraction IV on Sephadex LH-20 (2 × 24 cm) with methanol in the fractions from K_d 1.31 to 1.52. It was recrystallized from methanol and water to afford a light yellow powder (44.5 mg). The physical properties of the active principle are described below.

Identification of the Active Principle——The active compound has following the properties. mp 177 °C. *Anal.* Calcd for $C_{21}H_{20}O_{11} \cdot 2H_2O$: C, 52.07; H, 4.96; O, 42.97. Found: C, 52.39; H, 4.81; O, 42.80. MS *m/z*: 358, 302, 286, 273, 257, 254, 228, 153, 137, 128, 109. High resolution MS: 302.0445 (error +1.9 m MU) for $C_{15}H_{10}O_7$. ^{1}H-NMR (in d_6-acetone) δ (ppm): 12.70 (1H, s), 8.00—11.00 (3H, br), 7.49 (1H, d, J = 1.8 Hz), 7.39 (1H, dd, J = 7.9 and 1.8 Hz), 6.98 (1H, d, J = 7.9 Hz), 6.45 (1H, d, J = 2.2 Hz), 6.25 (1H, d, J = 2.2 Hz), 5.51 (1H, d, J = 1.3 Hz), 2.90—4.2 (7H, m), 0.92 (3H, d, J = 5.3 Hz). ^{13}C-NMR (in d_6-acetone) δ (ppm): 172.7 (s), 158.4 (s), 156.6 (s), 151.8 (s), 151.4 (s), 142.5 (s), 139.2 (s), 129.4 (s), 116.5 (s), 116.1 (d), 110.4 (d), 109.7 (d), 99.3 (d), 93.1 (s), 91.9 (d), 88.0 (d), 66.7 (d), 65.7 (d), 65.0 (d), 64.8 (d), 11.2 (q). IR ν_{max}^{KBr} cm^{-1}: 3600—2800, 1655, 1600, 1500, 1440, 1357, 1300, 1265, 1200, 1160, 1060, 1055, 995, 960, 810. UV λ_{max}^{MeOH} nm (log ε): 350 (4.19), 272 (4.33), 205 (4.53), $\lambda_{max}^{MeOH+1NNaOH}$ nm (log ε): 373 (4.12), 322 (3.98), 256 (4.31), 203 (4.54). The active compound was hydrolyzed with hesperidinase[10] to afford equimolar amounts of quercetin and rhamnose. The identify of the active compound was confirmed by direct comparison with an authentic sample.

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