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Studies on Antihemorrhagic Substances in Herbs Classified as Hemostatics in Chinese Medicine. III. On the Antihemorrhagic Principle in *Sanguisorba officinallis* L.

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The antihemorrhagic principle in *Sanguisorba officinallis* L. was isolated by a combination of countercurrent distribution, column chromatography on silica gel and preparative thin layer chromatography on silica gel, and identified as 3,3′,4-tri-*O*-methylellagic acid.

Keywords——hemostatic; *Sanguisorba officinallis*; antihemorrhagic principle; 3,3′,4-tri-*O*-methylellagic acid; ellagic acid; countercurrent distribution; Tajima's method

In the previous paper,[1] we reported that the hemostatically active principle had been isolated from Sanci Ginseng Radix, which is one of the herbs used as a hemostatic in Chinese medicine, and identified as β-*N*-oxallyl-α, β-diaminopropionic acid (neurotoxin).[2]

Sanguisorba officinallis L., which is an important herb in Chinese medicine, is also used as an antihemorrhagic agent, analgesic, astringent, *etc.*[3] However, previous work on this herb had been focused mostly on saponin[4] (Ziyu glycoside)[5] and tannin[4] components, and no chemical study on the antihemorrhagic principle in the herb has been reported. The present paper deals with the isolation of the antihemorrhagic substance from *Sanguisorba officinallis* L. During the isolation process, Tajima's method[6] using the mice was found to be useful for pharmacological measurement of antihemorrhagic activity of the material. Isolation of the principle was achieved by a combination of countercurrent distribution, column chromatography and thin layer chromatography (TLC) on silica gel. The detailed procedures are summarized in Chart 1.

roots of *Sanguisorba officinallis* L. (1 kg)
↓ extracted with H_2O and MeOH
extract (350 g) [1 g/kg—4.5 min]
↓ countercurrent distribution with C_6H_6–$CHCl_3$–MeOH–H_2O (n=4)
active fraction I (r=4) (9.2 g) [65 mg/kg—7.5 min]
↓ countercurrent distribution with n-hexane–MeOH–H_2O (n=4)
active fraction II (r=4) (1.75 g) [10 mg/kg—4.0 min]
↓ silica gel column chromatography eluted with C_6H_6–EtOH
active fraction III (290 mg) [5 mg/kg—5.8 min]
↓ preparative TLC on silica gel developed with $CHCl_3$–MeOH
active fraction IV (26.4 mg) [0.7 mg/kg—6.5 min]
↓ recrystallized from $CHCl_3$–MeOH
colorless needles (10 mg) [0.5 mg/kg—8.0 min]

Chart 1. Isolation of the Active Principle

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

Roots of *Sanguisorba officinallis* L. were ground and extracted with water and then methanol under reflux. The combined extracts were separated by countercurrent distribution using a solvent system of C_6H_6–$CHCl_3$–MeOH–H_2O (15 : 15 : 23 : 7). The results are shown in Fig. 1.

The activity was located only in the $r=4$ tube (active fraction I). Thus, this fraction was further separated by countercurrent distribution using a solvent system of *n*-hexane–MeOH–H_2O (10 : 9 : 1). Figure 2 shows the results.

The activity emerged in two tubes ($r=2$ and 4). In this work, we will deal with the $r=4$ tube, which contained the bulk of the antihemorrhagic activity of active fraction I. Work on the $r=2$ tube is still in progress. The highly active tube $r=4$ was subjected to silica gel column chromatography with benzene and methanol as eluents to afford four fractions (fractions 1—4). The thin layer chromatograms of these fractions on silica gel are shown in Fig. 3.

Most of the activity was present in fraction 3. Final purification of active fraction IV was achieved by preparative TLC on silica gel (Merck Art. 5721) with $CHCl_3$–MeOH (97 : 3). The active zone with *Rf* 0.40—0.46 was extracted with chloroform to obtain a white powder. This material was recrystallized from chloroform and methanol to give colorless needles. The compound has the following properties; mp 284 °C, ferric chloride positive, MS *m/z*: 344 (M^+), 329, 286, 258, High resolution MS: 344.0510 (error −2.0 mMU) for $C_{17}H_{12}O_8$, ^{1}H-

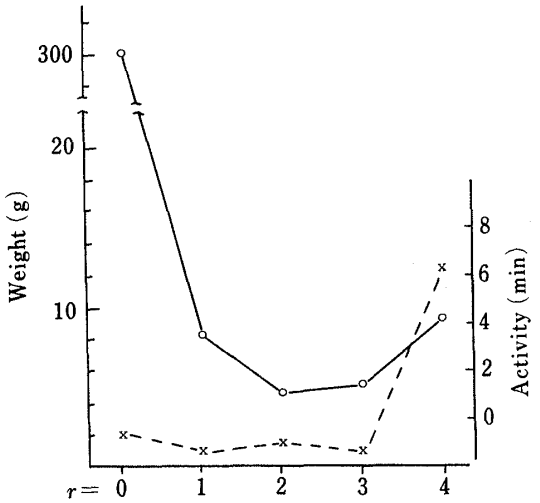


Fig. 1. Countercurrent Distribution Pattern of the H_2O-Extract (350 g) with Benzene–Chloroform–Methanol–H_2O (15 : 15 : 23 : 7) ($n=4$)

○—○, distribution pattern; ×---×, activity (shortening of time of bleeding after *i.p.* administration of each tube at a dose of 65 mg/kg).

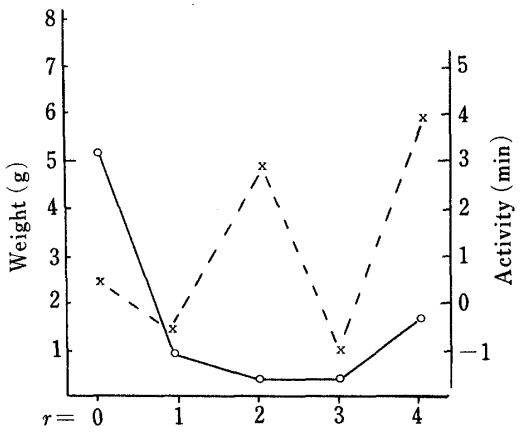


Fig. 2. Countercurrent Distribution Pattern of Active Fraction I (9.2 g) with *n*-Hexane–MeOH–H_2O (10 : 9 : 1) ($n=4$)

○—○, distribution pattern; ×---×, activity (shortening of time of bleeding after *i.p.* administration of each tube at a dose of 10 mg/kg).

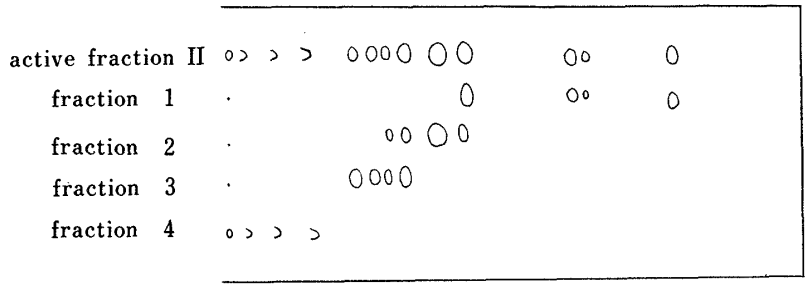
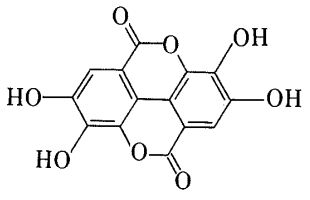


Fig. 3. Thin Layer Chromatograms of Active Fraction II and Fractions 1—4

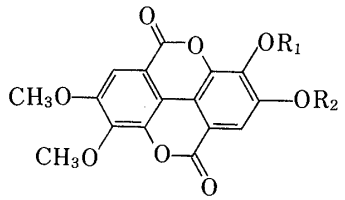
Plate: Kiselgel H60 (Merck Art. 5721).
Solvent: $CHCl_3$–MeOH (97 : 3).

NMR (0.2% solution in d_6-DMSO) δ (ppm): 10.79 (1H, s), 7.62 (1H, s), 7.53 (1H, s), 4.06 (3H, s), 4.04 (3H, s), 4.00 (3H, s), IR ν_{max}^{KBr} cm^{-1}: 3450, 1730, 1710, 1100, 1090 and UV $\lambda_{max}^{ethanol}$ nm ($\log\varepsilon$): 248 (4.48), 360 (sh) (3.85), 373 (3.92), $\lambda_{max}^{ethanol+NaOAc}$ nm ($\log\varepsilon$): 256 (4.26), 305 (3.90), 412 (3.85).

The nuclear magnetic resonance (NMR) spectrum shows the presence of three methoxyl groups (4.06, 4.04 and 4.00 ppm) in the compound. The infrared (IR) spectrum suggests the presence of α,β-unsaturated lactones (1730, 1710, 1110 and 1090 cm^{-1}). The strong absorption in the hydroxyl region (3450 cm^{-1}) in the IR spectrum and the solubility in aqueous sodium carbonate in the absence of oxygen indicates that the compound possesses a phenolic group but no carboxylic group. The above results, and the ultraviolet (UV) spectra[7] and mass (MS) spectrum[8] suggested a tri-*O*-methylellagic acid structure; this was confirmed by mild demethylation with conc. sulfuric acid to give ellagic acid (I). Only two structures were possible for tri-*O*-methylether of ellagic acid (II and III).



I



II: $R_1=H$, $R_2=CH_3$
III: $R_1=CH_3$, $R_2=H$

Distinction between the two structures was possible from the UV spectral nature of the free hydroxyl group. In the presence of sodium acetate the low-wavelength band of its UV spectrum suffered a bathochromic shift of 2 nm, suggesting that the compound possesses the free hydroxyl group in the 4-position.[7] Therefore the compound appeared to be identical with 3,3′,4-tri-*O*-methylellagic acid (III).

This conclusion was confirmed by direct comparison of III with a synthetic sample of 3,3′,4-tri-*O*-methylellagic acid prepared by Jurd's method.[9] The antihemorrhagic activity of the synthetic compound was equal to that of the natural product. 3,3′,4-Tri-*O*-methylellagic acid has been isolated from many plants, *e.g.*, *Legerstoemia subcostata* KOEHNE,[10] *Eugenia marie* A. CUNN,[11] *Nasutitermes exitiosus*,[12] *E. polyathemos*, and *E. deglupta*,[13] but not previously from *Sanguisorba officinallis* L.

Cliffton and co-workers had reported that ellagic acid given by intravenous injection causes a significant decrease in bleeding after amputation of the tail of the rat.[14] Further work is needed on the pharmacological activity relating to the antihemorrhagic action of 3,3′,4-tri-*O*-methylellagic acid.

In summary, this is the first isolation of 3,3′,4-tri-*O*-methylellagic acid as an antihemorrhagic principle from *Sanguisorba officinallis* L. A novel synthesis of the compound will be reported elsewhere.

Experimental

Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a JEOL FX-90 Fourier-transform NMR spectrometer and calibrated in parts per million (δ) downfield from tetramethylsilane (TMS) as an internal standard. IR spectra were recorded on a JASCO IRA-2 grating infrared spectrophotometer. Low resolution MS spectra were recorded on a JEOL JMS-D 100 and high resolution MS spectra on a Hitachi M-80A machine. UV spectra were recorded on a Shimadzu UV-360 recording spectrophotometer. Elemental analysis was recorded with a Perkin Elmer 240. Melting points are uncorrected.

Assay of the Hemostatic Activity——Hemostatic activity testing was carried out by Tajima's method on mice

weighing 18 g to 20 g. Test material homogenized in 1% methylcellulose–0.9% sodium chloride aqueous solution was given by intraperitoneal injection.

Extract——Ground roots of *Sanguisorba officinallis* L. (1 kg) were extracted with water (6 l) and then 3 times with methanol (6 l) for 3 h under reflux. The combined extracts were concentrated under reduced pressure to give a brown gum (350 g).

Countercurrent Distribution with the Solvent System of C_6H_6–$CHCl_3$–MeOH–H_2O (15 : 15 : 23 : 7)——A portion (50 g) of the crude extract was distributed between the lower phase (500 ml) and upper phase (500 ml) of the solvent system described above for 4 transfers by lower phase transfer. In total, 9.2 g of the active fraction I ($r=4$) was obtained by repeated countercurrent distribution.

Countercurrent Distribution with the Solvent System of *n*-Hexane–MeOH–H_2O (10 : 9 : 1)——The active fraction I (9.2 g) was successively distributed between the lower phase (250 ml) and upper phase (250 ml) of the solvent system described above by lower phase transfer for 4 transfers to afford the active fraction II (1.75 g) ($r=4$).

Silica Gel Column Chromatography——A portion (630 mg) of the active fraction II was subjected to column chromatography on silica gel (6 × 30 cm) using C_6H_6–EtOH (9 : 1) as an eluent to afford active fraction III. In total, 290 mg, of the active fraction III was obtained by repeated chromatography.

Preparative TLC——A portion (7.25 mg) of the active fraction III was subjected to preparative TLC on silica gel (Merck Art. 5721) using $CHCl_3$–MeOH (97 : 3) as a developing solvent. The zone with *Rf* 0.40—0.46 was extracted with chloroform. In total, 26 mg of the active fraction IV was obtained by repeated chromatography. This product was recrystallized from chloroform and methanol to afford colorless needles of 3,3′,4-tri-*O*-methylellagic acid (10 mg).

Demethylation of 3,3′4-Tri-*O*-methylellagic Acid——The natural product (3 mg) was heated under reflux with conc. sulfuric acid (0.5 ml) at 140 °C for 10 min, and the cooled solution was poured into water. Recrystallization of the precipitate from DMF gave yellow needles (2 mg) (mp >360 °C). The compound has the following properties; ferric chloride positive, *Anal.* Calcd for $C_{14}H_6O_8 \cdot 2H_2O$: C, 49.70; H, 2.96. Found: C, 49.33; H, 2.93, MS *m/z*: 302 (M^+), 273, 246, 228, 218, 190, 162, ^{1}H-NMR (1% solution in d_6-DMSO) δ (ppm): 8.20—12.00 (4H, br), 7.45 (2H, s), IR ν_{max}^{KBr} cm^{-1}: 3500, 3400—1900, 1690, 1615, 1580, 1335, 1108, 1050 and UV $\lambda_{max}^{ethanol}$ nm (log ε): 254 (4.68), 365 (3.97), $\lambda_{max}^{ethanol+1\ N\ NaOH}$ nm (log ε): 253 (4.55), 277 (4.65), 354 (4.11). The demethylated product was identified as ellagic acid by direct comparison with an authentic sample.

References and Notes

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