Studies on the Antihemostatic Substances in Herbs Classified as Hemosstatics in Traditional Chinese Medicine. I. On the Antihemostatic Principles in Sophora japonica L. 1)

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By following the antihemostatic activity of the material according to Tajima's method, the antihemostatic principle was isolated from dried buds of Sophora japonica L., and identified as isorhamnetin [2-(4-methoxy-4-hydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one]. The antihemostatic specificity of isorhamnetin toward antihemorrhagic compounds isolated previously from hemostatic herbs used in traditional Chinese medicine was examined.

Keywords antihemostatic principle; hemostatic; Sophora japonica L.; isorhamnetin; quercetin

In previous papers21 of our series on the chemical elucidation of the antihemorrhagic principles in herbs which are commonly used as hemostatics in traditional Chinese medicine,32 we have shown that the bud of Sophora japonica L. contains a component(s) which works antithrombostatically with respect to the antihemorrhagic action of quercetin, which is responsible for the antihemorrhagic activities of the herb.23) This study was carried out to identify the component in question.

Chart 1 shows the relative activity of each fraction obtained in the previously reported isolation processes of the antihemorrhagic principle (quercetin) from the unparched and parched herbs of Sophora japonica L.24) with respect to the antihemorrhagic activity of isolated quercetin in each case. As reported in the preceding paper,11 it was concluded by comparisons of the relative activities that the water extract and the active fraction I of each herb contain antithrombostatic components which suppress the antihemorrhagic action of quercetin. In this report, we deal with the antithrombostatic principle in active fraction I. Its antithrombostatic specificity with respect to the antithrombostatic compounds which were isolated previously from the hemostatic herbs employed in our studies21 is also presented here. Isolation of the water soluble principle(s) is in progress.

Isolation of the antithrombostatic principle from active fraction I, which was obtained by partitioning the water extract of the unparched herb of Sophora japonica L. between n-butanol and water, was achieved by two steps of gel filtration and column chromography on silica gel, as shown in Chart 2. The isolation procedures are described in detail in the experimental section.

ground buds of Sophora japonica L. (50g)
| extracted with H2O under reflux
H2O-extracts (16.4g) [630mg/kg, 0.12%] (16g) [240mg/kg, 0.24%]
| partitioned between n-butanol and H2O
active fraction I (4.79g) [85mg/kg, 0.26%] (6.43g) [38mg/kg, 0.50%]
| gel filtration on Sephadex LH-20 with MeOH
active fraction II (275mg) [1.1mg/kg, 1.35%] (576mg) [1.5mg/kg, 1.14%]
| recrystalized from MeOH
light yellow needles (268mg) [1.0mg/kg, 1.00%] (338mg)

( ) indicates yield. [ ] indicates ED50 and relative activity calculated by Eq. 1. < > indicates values of each fraction obtained from the parched herb.

relative activity = weight (mg)/ED50 of the material content (mg)/ED50 of quercetin

Chart 1. Isolation of the Antithrombostatic Principle

During the isolation process, Tajima's method41 with mice was employed for following the antithrombostatic activity of the material, as reported previously.24) The antithrombostatic activity of the sample was calculated by the use of Eq. 2, from the bleeding times of the control and the groups administered quercetin (5 mg/kg) and administered the test sample mixed with quercetin (5 mg/kg).

From the physical data (melting point, nuclear magnetic resonance (NMR), infrared (IR), ultraviolet (UV) and mass (MS) spectra of the active principle, it was suggested to be isorhamnetin. This was confirmed by a direct comparison of physical properties (melting point, NMR, UV, IR and MS spectra) with those of an authentic sample prepared by Atree and Perkin's method.5) The authentic sample suppressed the antithrombostatic action of quercetin as effectively as did the natural compound.

Isorhamnetin has been isolated from many plants, such as Dillenia indica,40) Anthyllis vulneraria,7) Asclepias syriaca,81) Aristolochia reticulata9) and Thymbraez lactifolia L.,10) but to our knowledge this is a first isolation of isorhamnetin from Sophora japonica L. The pharmacological activities of isorhamnetin include an effect on capillary permeability,11) inhibition of aggregation and secretion of human platelet in vitro,12) change of myocardial cyclic nucleotide (cAMP) content,13) and so on, but there has been no report on the antithrombostatic action of isorhamnetin in vivo. A report relating to the inhibition of platelet aggregation in vitro12) supports our finding on the antithrombostatic action of isorhamnetin. Further work is necessary to elucidate in...
detail the mechanisms of the antihemostatic effect of isorhamnetin and antihemorrhagic effect of quercetin.

In the literatures, it has been noted that the herbs used as hemostatics in traditional Chinese medicine are employed as cures for both bleeding and stagnant blood, but there has been no rationale to explain why the herbs cure stagnant blood. Our finding that isorhamnetin in Sophora japonica L. suppresses the antihemorrhagic action of quercetin is the first evidence on this point.

The dose-response relationship for antihemostatic activity of isorhamnetin is presented in the figure. It was determined by a quantitative analysis that active fractions I of the unparched and parched herbs contain about 0.068% and 0.039% isorhamnetin and 0.50% and 0.64% quercetin, respectively. The antihemorrhagic activity of the fraction of the parched herb is about twice of that of the unparched herb, as shown in Chart 1. From the dose–response curve, the suppression of the antihemorrhagic action of quercetin by isorhamnetin alone can be estimated to be about 34% and 15% at the isorhamnetin/quinetrin ratios in the above fractions, whereas the values actually observed were ca. 75% and 50% (presented in Chart 1). During the course of the isolation of isorhamnetin, other fractions did not show significant suppressive activity. So, it was concluded that this fraction contains a material which enhances the activity of isorhamnetin on quercetin.

The effect of related compounds to isorhamnetin on quercetin was tested, but, as shown in Table I, no effect was found. The antihemostatic specificity of isorhamnetin with respect to antihemorrhagic compounds was also examined (Table II). It was found that isorhamnetin clearly inhibits the actions of the examined compounds except for isorhamnetin 3-rutinoside-7-rhamnoside and quercetin, but it strengthens the hemostatic action in the case of isorhamnetin 3-rutinoside-7-rhamnoside. From these results, it was concluded that further study is necessary to clarify the mechanism of the action of isorhamnetin on hemostatics. A study on this point is on going.

In summary, it has been demonstrated that isorhamnetin is an antihemostatic principle in the bud of Sophora japonica L. and it inhibits the antihemorrhagic action of some hemostatics.

Experimental

Animals Male mice (Shizuoka Laboratory Animal Center, Shizuoka, Japan) of the ddY strain weighing 10–20 g were used throughout the study. They were housed in an air conditioned room with free access to a commercial chow (Oriental, MF) and tap water.

Assay of Hemostatic Activity Hemostatic testing was carried out according to Tajima’s method with male mice, as reported previously. Test material was homogenized in 1% methylcellulose-69% aqueous sodium chloride and given intraperitoneally by injection. The bleeding time was determined according to Tajima et al.

Calculation of ED₅₀ The ED₅₀ was computed for each assay by interpolating a plot of 25 and 75% values against the logarithm of the dose of material administered.

Statistical Analysis The data were analyzed by the use of Student’s t-Test.

### Table I. Antihemorrhagic Activities of Isorhamnetin and Related Compounds and Their Inhibitory Activities on Quercetin Action

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose (ED₅₀) (mg/kg)²</th>
<th>T₅₀ (min)ᵇ</th>
<th>T₆₀ (min)ᵇ</th>
<th>Inhibition (%)ᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quercetin</td>
<td>2.5</td>
<td>5.8 ± 0.6ᵉ</td>
<td>0.5 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>Isorhamnetin</td>
<td>10.0</td>
<td>0.4 ± 0.2</td>
<td>5.5 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>7-O-Methylisorhamnetin</td>
<td>10.0</td>
<td>0.3 ± 0.1</td>
<td>5.0 ± 1.7⁴</td>
<td></td>
</tr>
<tr>
<td>7,3′-Tri-O-methylisorhamnetin</td>
<td>10.0</td>
<td>0.2 ± 0.1</td>
<td>6.1 ± 1.4⁴</td>
<td></td>
</tr>
</tbody>
</table>

² Administered dose. ᵇ Each value represents the mean ± S.E. in five different experiments. ᶜ T₅₀ is (T₅₀ – T₀). ᵈ Inhibition (%) = 100 x (1 - T₅₀/T₆₀) (2)

### Table II. Inhibitory Effect of Isorhamnetin on Antithrombotic Actions of Hemostatic Compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose (ED₅₀) (mg/kg)⁶</th>
<th>T₅₀ (min)ᵇ</th>
<th>T₆₀ (min)ᵇ</th>
<th>Inhibition (%)ᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td>β- N-Oxallyl-α,β-diaminopropionic acid</td>
<td>50.0 (35.5)</td>
<td>5.0 ± 0.2⁶</td>
<td>3.7 ± 0.2⁶</td>
<td>26.0 ± 0</td>
</tr>
<tr>
<td>3,3′-Tri-O-methylleagic acid</td>
<td>0.5 (0.2)</td>
<td>8.0 ± 0.2⁶</td>
<td>2.9 ± 0.2⁶</td>
<td>63.8 ± 0</td>
</tr>
<tr>
<td>Quercetin</td>
<td>1.3 (1.0)</td>
<td>5.0 ± 0.4⁶</td>
<td>4.8 ± 0.2⁶</td>
<td>26.0 ± 0</td>
</tr>
<tr>
<td>Pectolinarin</td>
<td>1.3 (1.3)</td>
<td>3.6 ± 0.2⁶</td>
<td>0.7 ± 0.1</td>
<td>80.6 ± 0</td>
</tr>
<tr>
<td>Wedelolactone⁷</td>
<td>0.5 (0.3)</td>
<td>3.8 ± 0.1⁵</td>
<td>0.6 ± 0.1</td>
<td>84.2 ± 0</td>
</tr>
<tr>
<td>Desmethywedelolactone²⁸</td>
<td>0.5 (0.3)</td>
<td>4.2 ± 0.1⁵</td>
<td>1.4 ± 0.4⁴</td>
<td>96.7 ± 0</td>
</tr>
<tr>
<td>Isorhamnetin 3-rutinoside-7-rhamnoside</td>
<td>2.5 (1.5)</td>
<td>3.8 ± 0.3⁵</td>
<td>5.2 ± 0.3⁵</td>
<td>36.8 ± 0</td>
</tr>
<tr>
<td>Quercetin²⁸</td>
<td>2.5 (1.0)</td>
<td>2.8 ± 0.1⁵</td>
<td>0.1 ± 0.2</td>
<td>96.4 ± 0</td>
</tr>
</tbody>
</table>

⁶ Administered dose. ᵇ Each value represents the mean ± S.E. in five different experiments. ᶜ T₅₀ is (T₅₀ – T₀). ᵈ Inhibition (%) = 100 x (1 - T₅₀/T₆₀) (2)
ᵉ Significant difference from the control: /f p < 0.001, /g p < 0.02.  h Significant difference between T₅₀ and T₆₀.  i p < 0.001, /j p < 0.01, /k p < 0.02.
test. Values were expressed as mean ± S.E.

**Material** The herb examined in this study was a commercial product and was identified as *Sophora japonica* L. by an expert. Parched herbes were prepared by heating at 200°C for 15 min on a hot plate as reported previously.²⁻³

**Extraction and Partition between n-Butanol and H₂O** Ground buds of *Sophora japonica* L. (50 g) were extracted with 500 ml of H₂O under reflux for 0.5 h. The mixture was centrifuged at 2500 rpm for 20 min and the supernatant was lyophilized to give a brown powder (16.4 g). This extract was dissolved in 350 ml of H₂O and extracted with 350 ml of n-butanol three times. The combined organic layer was concentrated under reduced pressure to afford 4.79 g of active fraction I, as a brown gum. Finally 6.43 g of n-butanol extract was obtained from 40.3 g of parched herb prepared from 50 g of the herb.

**Gel Filtration of Active Fraction I on Sephadex LH-20** Active fraction I (4.79 g) was dissolved in 50 ml of MeOH and subjected to gel filtration on Sephadex LH-20 (3.70 × 73.0 cm), eluted with MeOH. The active fraction II (191 mg) (Kₜ value = 2.40—3.15) was obtained as yellow-brown gum.

**Silica Gel Column Chromatography of Active Fraction II** This fraction II (191 mg) was chromatographed over silica gel (2.0 × 30.0 cm) to yield 68.3 mg of active fraction III, using the lower layer of CHCl₃-MeOH-H₂O (65:35:10) as an eluent.

**Recrystallization of Active Principle** After removal of impurities from active fraction III (68.3 mg) by gel filtration through Sephadex LH-20 with methanol, recrystallization from dilute EtOH gave the active principle (26.7 mg) as light yellow needles. Finally, 15.8 mg of the principle was isolated from 40.3 g of the parched buds of *Sophora japonica* L.

**Identification of the Active Principle** The active compound (mp 301—303°C (dec.) (lit. 305—306°C (dec.))³⁵ was identified as isorhamnetin by direct comparison of spectral data with those of an authentic sample and by mixed melting point determination.

**References and Notes**

14. The conditions for high performance liquid chromatography were as follows: column, Porasil (Waters) 10 × 250 mm; flow rate, 1 ml/min; mobile phase, 0.3% EtOH–CHCl₃; detector, UV.