

Studies on the Antihemostatic Substances in Herbs Classified as Hemostatics 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-(3-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 papers²⁾ of our series on the chemical elucidation of the antihemorrhagic principles in herbs which are commonly used as hemostatics in traditional Chinese medicine,³⁾ we have shown that the bud of *Sophora japonica* L. contains a component(s) which works antihemostatically with respect to the antihemorrhagic action of quercetin, which is responsible for the antihemorrhagic activities of the herb.^{2e)} 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.^{2e)} with respect to the antihemorrhagic activity of isolated quercetin in each case. As reported in the preceding paper,¹⁾ it was concluded by comparisons of the relative activities that the water extract and the active fraction I of each herb contain antihemostatic components which suppress the antihemorrhagic action of quercetin. In this report, we deal with the antihemostatic principle in active fraction I. Its antihemostatic specificity with respect to the antihemorrhagic compounds which were isolated previously from the hemostatic herbs employed in our studies²⁾ is also presented here. Isolation of the water soluble principle(s) is in progress.

ground buds of *Sophora japonica* L. (50 g)
 | extracted with H₂O under reflux
 H₂O-ext. (16.4 g) [630 mg/kg, 0.12] < (16.6 g) [200 mg/kg, 0.24] >
 | partitioned between *n*-butanol and H₂O
 active fraction I (4.79 g) [85 mg/kg, 0.26] < (6.43 g) [38 mg/kg, 0.50] >
 | gel filtration on Sephadex LH-20 with MeOH
 active fraction II (275 mg) [1.1 mg/kg, 1.35] < (576 mg) [1.5 mg/kg, 1.14] >
 | recrystallized from MeOH
 light yellow needles (268 mg) [1.0 mg/kg, 1.00] < (338 mg) >

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

$$\text{relative activity} = \frac{\text{weight (mg)/ED}_{50} \text{ of the material}}{\text{content (mg)/ED}_{50} \text{ of quercetin}} \quad (1)$$

Chart 1. Isolation of the Antihemorrhagic Principle

During the isolation process, Tajima's method⁴⁾ with mice was employed for following the antihemostatic activity of the material, as reported previously.^{2a)} The antihemostatic 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).

Isolation of the antihemostatic 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 chromatography 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. (50 g)
 | extracted with H₂O under reflux
 H₂O-ext. (16.4 g) < (16.6 g) >
 | partitioned between *n*-butanol and H₂O
 active fraction I (*n*-butanol layer) (4.79 g) < (6.43 g) >
 | gel filtration on Sephadex LH-20 with MeOH
 active fraction II (191 mg) [20 mg/kg, 79.4%] < (146 mg) >
 | silica gel column chromatography with lower layer of
 CHCl₃-MeOH-H₂O (65:35:10)
 active fraction III (68.3 mg) [7.0 mg/kg, 62.3%] < (22.8 mg) >
 | 1) gel filtration on Sephadex LH-20 with MeOH
 | 2) recrystallized from acetone and MeOH
 light yellow needles (26.7 mg) [2.5 mg/kg, 69.0%] < (15.8 mg) >

() indicates yield. [] indicates dose and inhibitory effect on the antihemorrhagic action of quercetin (5 mg/kg), which is calculated by the use of Eq. 2 in Table II. < > indicates the yield of each fraction obtained from the parched herb.

Chart 2. Isolation of the Antihemostatic Principle

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 antihemorrhagic action of quercetin as effectively as did the natural compound.

Isorhamnetin has been isolated from many plants, such as *Dillenia indica*,⁶⁾ *Anthyllis vulneria*,⁷⁾ *Asclepias syriaca*,⁸⁾ *Aristolachia reticulata*⁹⁾ and *Thyphaceae 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 antihemostatic action of isorhamnetin *in vivo*. A report relating to the inhibition of platelet aggregation *in vitro*¹²⁾ supports our finding on the antihemostatic 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,³⁾ 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.¹⁴⁾ 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 suppressions of the antihemorrhagic action of quercetin by isorhamnetin alone can be estimated to be about 34% and 15% at the isorhamnetin/quercetin 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

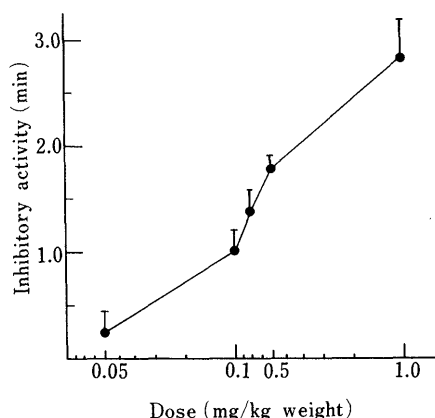


Fig. 1. Dose-Response Relationship for Antihemorrhagic Activity of Isorhamnetin

Each point represents the mean $\pm$ S.E. in five different experiments. Inhibitory activity (min) = $(T_1 - T_2) - (T_1 - T_3)$. T_1 , bleeding time of control group (10.6 ± 0.2 min); T_2 , bleeding time of the group administered quercetin at 1 mg/kg (7.6 ± 0.1 min); T_3 , bleeding time of the group administered a mixture of the corresponding amount of isorhamnetin and quercetin at 1 mg/kg.

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 quercitrin, 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.^{2a)} Test material was homogenized in 1% methylcellulose–0.9% aqueous sodium chloride and given interperitoneally by injection. The bleeding time was determined according to Tajima *et al.*⁴⁾

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*-

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

Compound	Dose (ED ₅₀) (mg/kg) ^{a)}	T _a (min) ^{b)}	T _b (min) ^{c)}
Quercetin	2.5	5.8 $\pm$ 0.6 ^{d)}	
Isorhamnetin	10.0 (2.5)	0.4 $\pm$ 0.2 (0.2 $\pm$ 0.1)	0.5 $\pm$ 0.3
7-O-Methylquercetin ¹⁵⁾	10.0	0.7 $\pm$ 0.1	5.5 $\pm$ 1.1 ^{d)}
5,7,3'-Tri-O-methylquercetin ¹⁶⁾	10.0	0.3 $\pm$ 0.4	5.0 $\pm$ 1.7 ^{d)}
7,3'-Di-O-methylquercetin ⁵⁾	10.0	0.2 $\pm$ 0.1	6.1 $\pm$ 1.1 ^{d)}

a) Administered dose. b, c) $T_a (= T_1 - T_4)$ indicates hemostatic activity. T_b is $(T_1 - T_5)$. Each value represents the mean with $\pm$ S.E. in five different experiments. T_1 , T_4 and T_5 are the bleeding times of the control group and the groups given the test compound and a mixture of test compound and quercetin (2.5 mg/kg). $T_1 = 10.6 \pm 0.4$ (min). d) Significant difference from the control: $p < 0.001$.

TABLE II. Inhibitory Effect of Isorhamnetin on Antihemorrhagic Actions of Hemostatic Compounds

Compound	Dose (ED ₅₀) (mg/kg) ^{a)}	T _a (min) ^{b)}	T _b (min) ^{c)}	Inhibition (%) ^{d)}
β -N-Oxallyl- α , β -diaminopropionic acid ^{2a)}	50.0 (35.5)	5.0 $\pm$ 0.2 ^{e,f)}	3.7 $\pm$ 0.2 ^{e,f)}	26.0 ^{h,i)}
3,3',4-Tri-O-methylellagic acid ^{2b)}	0.5 (0.2)	8.0 $\pm$ 0.2 ^{e,f)}	2.9 $\pm$ 0.2 ^{e,f)}	63.8 ^{h,i)}
Quercitrin ^{2d)}	1.3 (1.0)	5.0 $\pm$ 0.4 ^{e,f)}	4.8 $\pm$ 0.2 ^{e,f)}	4.0
Pectolinarin ^{2f)}	1.3 (1.3)	3.6 $\pm$ 0.2 ^{e,f)}	0.7 $\pm$ 0.1	80.6 ^{h,i)}
Wedelolactone ^{2c)}	0.5 (0.3)	3.8 $\pm$ 0.1 ^{e,f)}	0.6 $\pm$ 0.1	84.2 ^{h,i)}
Desmethylwedelolactone ^{2c)}	0.5 (0.3)	4.2 $\pm$ 0.1 ^{e,f)}	1.4 $\pm$ 0.4 ^{e,g)}	66.7 ^{h,i)}
Isorhamnetin 3-rutinoside-7-rhamnoside ^{2h)}	2.5 (1.5)	3.8 $\pm$ 0.3 ^{e,f)}	5.2 $\pm$ 0.3 ^{e,f)}	–36.8 ^{h,k)}
Quercetin ^{2e)}	2.5 (1.0)	2.8 $\pm$ 0.1 ^{e,f)}	0.1 $\pm$ 0.2	96.4 ^{h,i)}

a) Administered dose: the dose of a hemostatically active compound was determined by reference to its ED₅₀ and antihemorrhagic activity. b, c) $T_a (= T_1 - T_4)$ indicates hemostatic activity. T_b is $(T_1 - T_5)$. Each value represents the mean with $\pm$ S.E. in five different experiments. T_1 , T_4 and T_5 are the bleeding times of the control group and the groups given the hemostatic compound and a mixture of hemostatic compound and isorhamnetin (2.5 mg/kg). d) Inhibition was calculated by applying Eq. 2.

$$\text{Inhibition (\%)} = 100 \times (1 - T_b/T_a) \quad (2)$$

e) Significant difference from the control: f) $p < 0.001$, g) $p < 0.02$. h) Significant difference between T_a and T_b : i) $p < 0.001$, j) $p < 0.01$, k) $p < 0.02$.

test. Values were expressed as mean $\pm$ S.E.

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

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 $\times$ 73.0 cm), eluted with MeOH. The active fraction II (191 mg) (K_d 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 $\times$ 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.))⁵⁾ was identified as isorhamnetin by direct comparison of spectral data with those of an authentic sample and by mixed melting point determination.

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