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Studies on Active Substances in the Herbs Used for Oketsu ("Stagnant Blood") in Chinese Medicine. II. On the Anticoagulative Principle in Persicae Semen

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The anticoagulative principle in Persicae Semen was investigated. In the isolation process, plasma recalcification time in mice was used for the pharmacological measurement of anticoagulative activity of the material. The active principle was isolated by a combination of partition and column chromatography on silica gel, and identified as triolein.

Keywords——Persicae Semen; stagnant blood; anticoagulative principle; plasma recalcification time; triolein

In the previous paper[1] we reported that the extracts of herbs which are commonly used for Oketsu[2] ("stagnant blood") in Chinese medicine had an anticoagulative activity, and the extract of Persicae Semen (*Prunus persica* (L.) BATSCH), one of them, had particulary high activity. The present paper describes the isolation and identification of the anticoagulative principle in the herb.

Persicae Semen is an important herb in Chinese medicine as an anticoagulant, antiphlogistic, anodyne and expectorant.[3] Previous studies on this herb have focused mostly on the components, *e.g.*, amygdarin,[4] glycerides,[5] emulsin,[6] *etc.* A pharmacological study on the herb showed that the aqueous and alcoholic extracts of the herb have a weak hemolytic activity and inhibit a blood coagulation in an *in-vitro* test.[7] However no study on the anticoagulative principle in the herb has been reported.

We would like to report here the isolation and identification of the anticoagulative principle in the herb. During the isolation process, the measurement of plasma recalcification time[8] in mice was found to be useful for following the anticoagulative activity of the material.[1] Isolation of the active principle was achieved by a combination of partition and column chromatography on silica gel. The procedures are summarized in Chart 1.

Persicae Semen (50 g)
↓ extracted with H_2O
extract (17 g)
↓ partitioned with AcOEt and H_2O
active fraction I (ethyl acetate layer) (10.9 g) [0.1 g/kg---37.4][b]
↓ silica gel column chromatography eluted with
1) $CHCl_3$–*n*-hexane
2) $CHCl_3$–MeOH
3) lower layer of $CHCl_3$–MeOH–H_2O (65 : 35 : 10)
colorless oil (8.24 g) [0.1 g/kg---37.0][a]

Chart 1. Isolation of the Active Principle

( ) indicates yields. [ ] indicates dose and anticoagulative activity (%). *a*) $p<0.01$, *b*) $p<0.05$: Significance of difference from the control group. The coagulation time of the control group was 2.40 ± 0.10 min (mean ± S.E. from 5 mice).

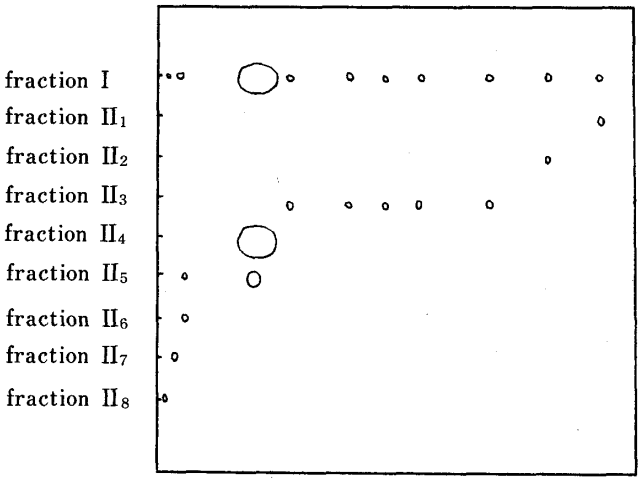
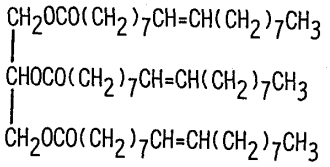


Fig. 1. Thin Layer Chromatograms of Fraction I and Fractions II_1—II_8.

Plate: Kieselgel H 60 (Merck Art. 5721). Solvent: $CHCl_3$–*n*-hexane (3 : 1). Color reagent: I_2.



triolein

As shown in Chart 1, ground Persicae Semen was extracted with water and the extract was partitioned with ethyl acetate and water. Since the activity emerged only in the ethyl acetate layer as previously reported,[1] this fraction was subjected to silica gel column chromatography, eluting firstly with *n*-hexane–$CHCl_3$, secondly with $CHCl_3$–MeOH and finally with the lower layer of $CHCl_3$–MeOH–H_2O (65 : 35 : 10) to afford eight fractions, namely fractions II_1—II_8. The thin layer chromatograms (TLC) of these fractions on silica gel are shown in Fig. 1.

High activity appeared in fraction II_4, which was analytically pure. Thus, the active principle (fraction II_4) was hydrolyzed with potassium hydroxide to afford 1 mol of glycerin and 3 mol of oleic acid. From this result and detailed analysis of the spectral data of the active principle, it was concluded to be triolein. This conclusion was confirmed by direct comparison of the compound with an authentic sample of triolein (glyceryl trioleate) (purchased from Wako Co.). The anticoagulative activity of the authentic sample was equal to that of the natural product.

Triolein had been isolated from seeds of peaches,[3] tea,[9] *Monarda fistulosa* and *M. mollis*,[10] peanuts,[11] rice bran,[12] and the pollen of *Pinus taeda*,[13] but it has not previously been found in Persicae Semen. Pharmacological studies of the effects of triolein on heparin induced lipase,[14] blood platelets[15] and calcium adsorption[16] have been reported, but this is the first study on the anticoagulative action.

In summary, this is the first report of the isolation of triolein as an anticoagulative principle from Persicae Semen.

Experimental

Proton and carbon-13 nuclear magnetic resonance (^1H- and ^{13}C-NMR) spectra were recorded with a JEOL FX-90 Fourier tansform 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. The low-resolution mass spectrum (MS) was recorded on a Hitachi M-80A instrument. Elemental analysis was done on a Perkin Elmer 240 analyzer.

Assay of Anticoagulative Activity——As previously reported, anticoagulative activity was assessed in terms of plasma recalcification time in male mice weighing 18 to 20 g.[1] Test material homogenized in 1% methylcellulose–0.9% sodium chloride aq. was administered by *i.p.* injection. The plasma coagulation time was determined according to the previously outlined procedure and the anticoagulative activity of the material was calculated by means of

equation 1. The statistical treatment of data was done as described previously.[1)]

$$\text{anticoagulative activity (\%)} = 100\left(1 - \frac{\text{average coagulative time of treated group}}{\text{average coagulative time of control group}}\right) \quad (1)$$

Extraction——Ground Persicae Semen (50 g) was extracted with 250 ml of water under reflux for half an hour. The mixture was centrifuged at 2500 rpm for 20 min, and the supernatant was lyophilized to give the extract (17 g) as a colorless oily mass.

Partition between Ethyl Acetate and Water——The crude extract (17 g) was suspended in 170 ml of water and extracted with 170 ml of ethyl acetate four times. Upon concentration of the ethyl acetate layer in a rotary evaporator, the active fraction I (10.9 g) was obtained as a colorless oil.

Silica Gel Column Chromatography——Active fraction I (10.9 g) was dissolved in 6 ml of $CHCl_3$–*n*-hexane (3 : 1) and subjected to column chromatography on silica gel (5.4 × 61 cm) eluting firstly with $CHCl_3$–*n*-hexane (3 : 1), then with $CHCl_3$–MeOH (9 : 1), and finally with the lower layer of $CHCl_3$–MeOH–H_2O (65 : 35 : 10). The active principle (Fr. II_4) (8.24 g) was obtained from the $CHCl_3$–*n*-hexane (3 : 1) fraction as a colorless oil.

Identification of the Active Principle——The active compound has the following properties; colorless oil, bp_4 217—220 °C (lit.[17)] bp_{15} 235—240 °C). *Anal.* Calcd for $C_{57}H_{104}O_6$; C, 77.28; H, 11.75. Found: C, 76.85; H, 11.94. MS *m/z*: 885 (M + 1), 746, 603, 550, 466, 339. ^{1}H-NMR (in $CDCl_3$) δ (ppm): 5.10—5.55 (7H, m), 3.99—4.55 (4H, m), 2.31 (6H, t, J = 7.5 Hz), 2.08—1.80 (12H, m), 1.00—2.08 (66H, m), 0.88 (9H, t, J = 6.5 Hz). ^{13}C-NMR in $CDCl_3$) δ (ppm): 174.4 (s), 174.0 (s), 131.6 (d), 131.4 (d), 131.1 (d), 129.4 (d), 70.5 (d), 63.5 (t), 35.5 (t), 33.4 (t), 33.0 (t), 31.1 (t), 30.6 (t), 28.7 (t), 27.1 (t), 26.4 (t), 24.1 (t), 15.5 (q). IR $\nu_{max}^{liq.\,film}$ cm^{-1}: 2920, 2850, 1740, 1460, 1180, 720. Hydrogenation of the active principle over 10% palladium on charcoal gave a single crystalline product, which was recrystallized from ethanol to afford a colorless powder (85%, mp 66—68 °C). The reduced product was identified as tristearin by direct comparison with an authentic sample. Then the active principle was hydrolyzed with potassium hydroxide to afford 1 mol of glycerin and 3 mol of oleic acid. The identify of the active principle was confirmed by direct comparison with an authentic sample.

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