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Studies on Active Substances in the Herbs Used for Oketsu ("Stagnant Blood") in Chinese Medicine. III. On the Anticoagulative Principles in *Curcumae Rhizoma*

TAKUO KOSUGE, HITOSHI ISHIDA* and HIDEHIRO YAMAZAKI

*Shizuoka College of Pharmacy, 2-2-1 Oshika,
Shizuoka 422, Japan*

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The anticoagulative principles were isolated from *Curcumae Rhizoma* by a combination of partition and column chromatographies on silica gel, and identified as 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione (curcumin), *p,p'*-dihydroxydicinnamoylmethane and *p*-hydroxycinnamoylferuloylmethane. During the isolation process plasma recalcification time in mice was used to follow the anticoagulative activity of the material.

Keywords—*Curcumae Rhizoma*; stagnant blood; plasma recalcification time; anticoagulative principle; curcumin; *p,p'*-dihydroxydicinnamoylmethane; *p*-hydroxycinnamoylferuloylmethane

In the course of studies on the isolation of the active substances in the herbs, which are commonly used for Oketsu¹⁾ ("stagnant blood") in Chinese medicine, we found that the anticoagulative principle in *Persicae Semen* is triolein.²⁾ The present paper describes the isolation and identification of the anticoagulative principles in *Curcumae Rhizoma* (*Curcuma long* L.).

Curcumae Rhizoma, which is a component of many traditional prescriptions,³⁾ is an important herb in Chinese medicine as a stomachic, uretic and anodyne for biliary calculus and menstrual pain.⁴⁾ Previous work on this herb has been mostly focused on the chemical components, *e.g.*, curcuminoids,⁵⁾ essential oils,⁶⁾ *etc.* Pharmacological studies on the choloretic action of curcumin⁷⁾ and the antibacterial activity of the essential oil⁸⁾ of the herb have been reported, but no study on the anticoagulative principle in the herb has appeared.

We describe here the isolation and identification of the anticoagulative principles in the herb. During the isolation process, plasma recalcification time⁹⁾ in mice was used for following the anticoagulative activity of the material.¹⁰⁾ Isolation of the active principles was achieved by a combination of partition and column chromatographies over silica gel. The procedures are summarized in Chart 1.

As shown in Chart 1, ground *Curcumae Rhizoma* was extracted with refluxing water. When the extract was partitioned between ethyl acetate and water, the activity emerged in the ethyl acetate layer as previously reported.²⁾ The active fraction I was subjected to silica gel column chromatography to afford three fractions. Since the highly active fraction II consisted of three yellow components, this fraction was subjected to repeated silica gel column chromatographies to obtain three active fractions, IV, V and VI. The thin layer chromatograms (TLC) of the fractions on silica gel are shown in Fig. 1.

Since the three active fractions, IV, V and VI, contained impurities which could not be removed by usual column chromatography on silica gel, they were purified by recrystallization from suitable solvents to afford three crystalline compounds, 1, 2 and 3, respectively. They were all positive to color reagents such as ferric chloride, boric acid, boric acid–oxalic acid, 1% sodium hydroxide and conc. sulfuric acid reagents.

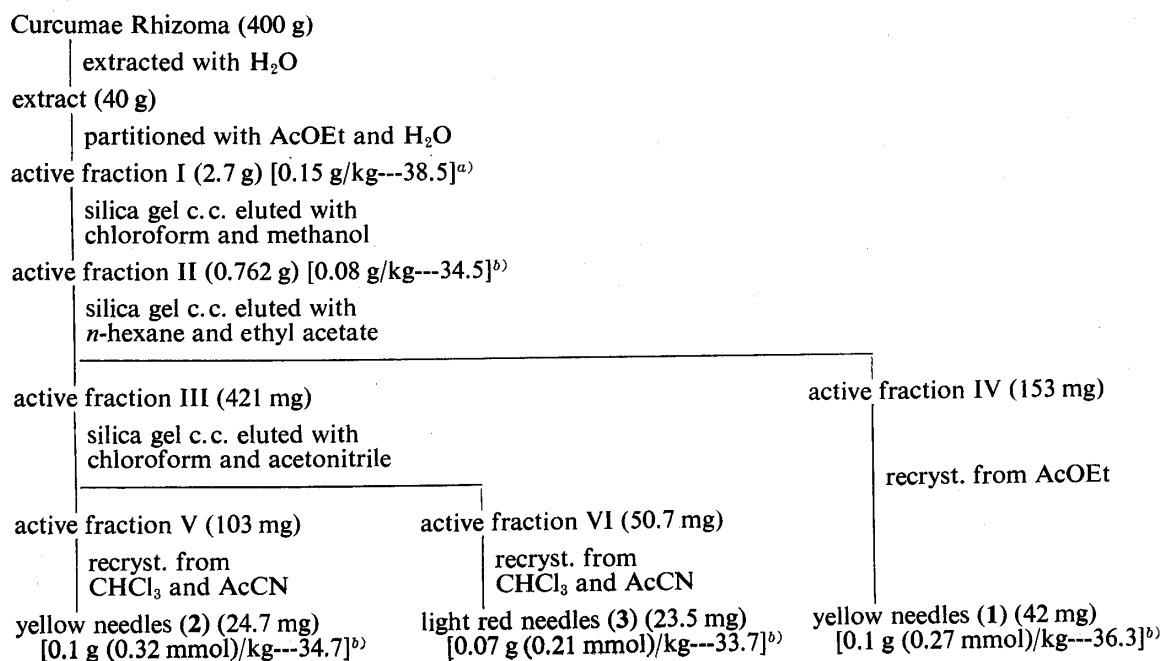


Chart 1. Isolation of the Active Principles

() 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 2.30 ± 0.12 (the mean \pm S.E. from 5 mice).

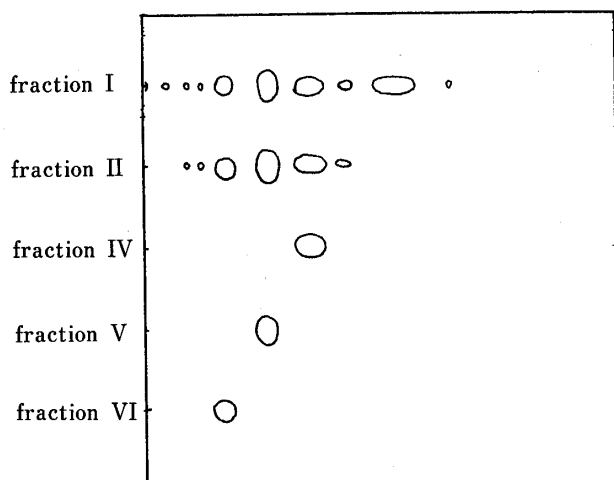
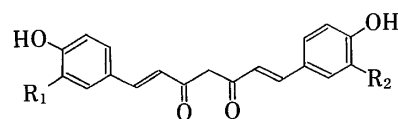


Fig. 1. Thin Layer Chromatograms of Fractions I, II, IV, V and VI

Plate: Kieselgel H 60 (Merck Art. 5721). Solvent: CHCl₃-CH₃CN (9:1). Color reagent: I₂.



- 1: R₁=R₂=OCH₃ (curcumin)
 2: R₁=R₂=H
 3: R₁=OCH₃, R₂=H

Compound **1** was identified by direct comparison with an authentic sample of 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione (curcumin (**1**)) (purchased from Wako Co.).⁵⁾ A comparison of the spectral data among **1**, **2** and **3** suggested that **2** and **3** were homologues of curcumin (**1**).

At higher field in the proton nuclear magnetic resonance (¹H-NMR) spectrum of **2**, signals at δ 7.56 (d) and 6.90 (d) were assignable to the protons on two 1,4-disubstituted benzene rings and A₂X₂-type signals at δ 7.61 (d) and 6.64 (d) were ascribable to *trans*-olefinic protons. ¹H-NMR and carbon-13 magnetic resonance (¹³C-NMR) spectra indicated that **2** had a highly symmetrical structure. Thus, compound **2** was characterized as *p,p'*-dihydroxydicinna-

moylemethane (1,7-bis(4-hydroxyphenyl)-1,6-heptadiene-3,5-dione) (2).⁵⁾

Compound 3 was hydrolyzed with sodium hydroxide to yield equimolar amounts of ferulic acid (*p*-hydroxycinnamic acid) and *p*-coumaric acid (4-hydroxy-3-methoxycinnamic acid), which were identified by direct comparison with authentic samples (purchased from Aldrich Co.). From this result and a detailed analysis of the spectral data, 3 was identified as *p*-hydroxycinnamoylferuloylmethane (1-(4-hydroxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) (3).⁵⁾

The anticoagulative activity of an authentic sample of curcumin was equal to that of the natural compound. Among the isolated compounds 3 showed the highest anticoagulative activity, and 1 showed the next highest activity.

Curcumin (1), *p,p'*-dihydroxydicinnamoylmethane (2) and *p*-hydroxycinnamoylferuloylmethane (3), which are so-called curcuminoids, have been isolated from various *Curcuma* species, e.g., *Curcuma longa* L.,⁵⁾ *Curcuma xanthorrhiza* ROXB.¹¹⁾ and *Curcuma aromatica* SALISB,¹²⁾ as well as *Anthocephalus cadamba*.¹³⁾

Pharmacological studies on the antifungal,⁸⁾ antibacterial,^{8,14)} antioxidant¹⁵⁾ and anti-inflammatory¹⁶⁾ actions of curcumin (1) have been reported. However no study on the anticoagulative action of curcumin, *p,p'*-dihydroxydicinnamoylmethane or *p*-hydroxycinnamoylferuloylmethane has been reported.

As the total anticoagulative activity of the ethyl acetate fraction of the extract of *Curcumae Rhizoma* was not account for by these isolated curcuminoids, further work is under way to isolate other active compounds in this fraction.

Experimental

Melting points are uncorrected. The infrared (IR) spectra were taken on a JASCO IRA-2 grating infrared spectrophotometer. ¹H-NMR and ¹³C-NMR spectra (chemical shifts are given in ppm from tetramethylsilane) were recorded on a JEOL FX-90 Fourier transform NMR spectrometer. Abbreviations used: s, singlet; d, doublet; dd double doublet; t, triplet; q, quartet; m, multiplet; brs, broad singlet. Low- and high-resolution mass spectra (MS) were taken on a Hitachi M-80A machine. Ultraviolet (UV) spectra were taken on a Shimadzu UV-360 recording spectrophotometer.

Assay of the Anticoagulative Activity—Anticoagulative activity was followed by measuring the plasma recalcification time⁹⁾ in male mice weighing 18 to 20 g. Test material homogenized in 1% methylcellulose–0.9% sodium chloride aq. was given by *i.p.* administration. The plasma coagulation time was determined according to the previously reported procedure.¹⁰⁾ Anticoagulative activity of the material was calculated by means of equation 1. The statistical treatment of the data was done as described previously.¹⁰⁾

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

Extraction—Ground *Curcumae Rhizoma* (400 g) was extracted with 2 l 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 crude extract (40 g) as a yellow powder.

Partition between Ethyl Acetate and Water—The crude extract (40 g) was dissolved in 400 ml of water and extracted with 400 ml of ethyl acetate four times. Concentration of the ethyl acetate layer in a rotary evaporator yielded active fraction I (2.7 g) as a brown gum.

Silica Gel Column Chromatography with CHCl₃–MeOH—Active fraction I (2.7 g) was subjected to silica gel column chromatography (4.5 × 34 cm) with CHCl₃–MeOH (95 : 5) to give active fraction II (0.765 g) as a yellow gum.

Column Chromatography on Silica Gel with *n*-Hexane and Ethyl Acetate—Active fraction II (0.765 g) was subjected to column chromatography over silica gel (24 × 45 cm) with *n*-hexane and ethyl acetate (1 : 1) as eluents to afford active fraction III (420.6 mg) and active fraction IV (153 mg) as yellow gums.

Silica Gel Column Chromatography with Chloroform–Acetonitrile—Active fraction III (420.6 mg) was subjected to column chromatography over silica gel (1.6 × 50 cm) with chloroform and acetonitrile (9 : 1) to yield fraction V (103 mg) and fraction VI (50.7 mg) as yellow gums.

Recrystallization of Fractions IV, V and VI—Active fraction IV (153 mg) was recrystallized from ethyl acetate to afford yellow needles (1) (42 mg). Recrystallization of fraction V (103 mg) and fraction VI (50.7 mg) from

chloroform and acetonitrile gave yellow needles (2) (50.7 mg) and light reddish needles (3) (23.5 mg), respectively.

Identification of Curcuminoids, 1, 2 and 3—1, 2 and 3 have the following properties. 1; yellow needles, mp 183 °C. MS m/z : 368 (M^+), 350, 342, 326, 285, 253, 192, 177. High-resolution MS: m/z Calcd for $C_{21}H_{20}O_6$: 368.1284. Obsd: 368.1300. UV λ_{max}^{MeOH} nm (log ϵ): 261 (5.02), 422 (4.76), $\lambda_{max}^{MeOH+1N NaOH}$ nm (log ϵ): 275 (4.33), 506 (4.71). IR ν_{max}^{KBr} cm^{-1} : 3550—1760, 1615, 1580, 1510, 1420, 1275, 1135, 1110, 1020, 959, 840, 800. 1H -NMR (in d_6 -THF) δ (ppm): 8.22 (2H, br s), 7.56 (2H, d, $J=15.8$ Hz), 7.04—7.16 (4H, m), 6.76 (2H, d, $J=7.9$ Hz), 6.53 (2H, d, $J=15.8$ Hz), 5.82 (1H, s), 3.88 (6H, s) and ^{13}C -NMR (in d_6 -THF) δ (ppm): 183.8 (s), 150.0 (s), 148.6 (s), 140.0 (d), 127.9 (s), 123.2 (d), 122.0 (d), 111.5 (d), 101.6 (d), 56.1 (q). 2; yellow needles, mp 232—234 °C. MS m/z : 308 (M^+), 290, 282, 225, 162. High-resolution MS: m/z Calcd for $C_{19}H_{16}O_4$: 308.1040. Obsd: 308.1034. UV λ_{max}^{MeOH} nm (log ϵ): 245 (4.12), 414 (4.69), $\lambda_{max}^{MeOH+1N NaOH}$ nm (log ϵ): 264 (4.00), 482 (4.67). IR ν_{max}^{KBr} cm^{-1} : 3500—1700, 1625, 1595, 1560, 1510, 1425, 1235, 1165, 1135, 835. 1H -NMR (in d_6 -acetone) δ (ppm): 8.82 (2H, br s), 7.61 (2H, d, $J=15.8$ Hz), 7.56 (4H, d, $J=8.4$ Hz), 6.90 (4H, d, $J=8.4$ Hz), 6.64 (2H, d, $J=15.8$ Hz), 5.97 (1H, s) and ^{13}C -NMR (in d_6 -acetone) δ (ppm): 183.2 (s), 159.1 (s), 139.7 (d), 129.6 (s), 126.6 (s), 120.9 (d), 115.5 (d), 100.2 (d). 3; light red needles, mp 181—182 °C. MS m/z : 338 (M^+), 312, 255, 223, 177, 124. High-resolution MS: m/z Calcd for $C_{20}H_{18}O_5$: 338.1167. Obsd: 338.1181. UV λ_{max}^{MeOH} nm (log ϵ): 250 (4.10), 418 (4.75). $\lambda_{max}^{MeOH+1N NaOH}$ nm (log ϵ): 274 (4.02), 496 (4.72). IR ν_{max}^{KBr} cm^{-1} : 3550—1740, 1620, 1600, 1580, 1560, 1515, 1260, 1135, 970, 960, 815. 1H -NMR (in d_6 -acetone) δ (ppm): 8.88 (1H, br s), 8.12 (1H, br s), 7.60 (2H, d, $J=15.8$ Hz), 7.55 (2H, d, $J=7.9$ Hz), 7.32 (1H, d, $J=1.7$ Hz), 7.17 (1H, dd, $J=7.9$ and 1.7 Hz), 6.89 (2H, d, $J=7.9$ Hz), 6.87 (1H, d, $J=7.9$ Hz), 6.68 (1H, d, $J=15.8$ Hz), 6.63 (1H, d, $J=15.8$ Hz), 5.96 (1H, s), 3.92 (3H, s) and ^{13}C -NMR (in d_6 -acetone) δ (ppm): 184.4 (s), 160.4 (s), 150.0 (s), 148.8 (s), 141.2 (d), 141.0 (d), 130.8 (d), 128.2 (s), 127.8 (s), 123.7 (d), 122.4 (d), 122.1 (s), 116.8 (d), 116.2 (d), 111.7 (d), 101.5 (d), 56.4 (q). 1 was identified as curcumin (1) by direct comparison with an authentic sample.

Hydrolysis of 3 with Sodium Hydroxide—A solution of 20 mg of 3 in 5 ml of 5%-sodium hydroxide aq. was heated on a water bath for 2 h. After neutralization of the reaction mixture with dil. hydrochloric acid, the products were extracted with ethyl acetate (10 ml) and separated by preparative thin layer chromatography (Merck Art. 6751) using *n*-hexane and ethyl acetate as a developing solvent to give *p*-coumaric acid (3 mg) (mp 215—216 °C recrystallized from *n*-hexane) and ferulic acid (2 mg) (mp 173—175 °C recrystallized from *n*-hexane).

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