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Studies on Anticancer Principles in Chinese Medicines. II.[1] Cytotoxic Principles in *Biota orientalis* (L.) ENDL. and *Kaempferia galanga* L.

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Deoxypodophyllotoxin and ethyl *p*-methoxy-*trans*-cinnamate were isolated as the cytotoxic principles from *Biota orientalis* (L.) ENDL. and *Kaempferia galanga* L., respectively, by tracing the inhibitory activity against HeLa cells.

Keywords——*Biota orientalis*; *Kaempferia galanga*; HeLa cells; cytotoxic principle; deoxypodophyllotoxin; ethyl *p*-methoxy-*trans*-cinnamate

As a result of our program of screening of Chinese medicines for anticancer principles, it was found that the methanol extracts of the leaves of *Biota orientalis* (L.) ENDL. ("Ce-bai-ye" in Chinese) and the rhizomes of *Kaempferia galanga* L. ("Shan-nai" in Chinese)[2] were highly cytotoxic to HeLa cells. This paper describes the isolation and characterization of the cytotoxic principles in these herbs. The isolation of the cytotoxic principles was followed by measuring the inhibitory activity on the colony-forming ability of HeLa cells.

Table I shows the cytotoxic activities and yields of the fractions from the leaves of *Biota orientalis* (L.) ENDL. (1 kg). On the basis of its yield and cytotoxic activity, deoxypodophyllotoxin is considered to account for the cytotoxic action of the methanol extract.

Table II also summarizes the experimental results on the rhizomes of *Kaempferia galanga*

TABLE I. Cytotoxic Activities and Yields of Fractions from the Leaves of *Biota orientalis* (L.) ENDL. (1 kg)

Fraction or compound	Yield (g)	IC_{50} (μg/ml)[a]
MeOH ext.	121	1.7
Benzene-sol.	40.0	5.7×10^{-1}
H_2O-sol.	79.9	17.0
Fraction 1	26.7	50.0
Fraction 2	3.2	4.8×10^{-2}
Fraction 3	1.5	9.0×10^{-1}
Fraction 4	6.8	9.0
Fraction 2-1	1.0	1.5
Fraction 2-2	0.7	1.0×10^{-2}
Fraction 2-3	0.8	7.0
Deoxypodophyllotoxin	0.4	5.7×10^{-3}
Mitomycin C		2.0×10^{-1}

a) Concentration giving 50% inhibition of colony-forming ability of HeLa cells.

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TABLE II. Cytotoxic Activities and Yields of Fractions from the Rhizomes of *Kaempferia galanga* L. (500 g)

Fraction or compound	Yield (g)	IC_{50} (μg/ml)[a]
MeOH ext.	40.5	80
Benzene-sol.	27.8	50
H_2O-sol.	13.2	500
Fraction 1	4.8	125
Fraction 2	17.3	40
Fraction 3	5.6	100
Ethyl *p*-methoxy-*trans*-cinnamate	14.4	35
Mitomycin C		0.2

a) Concentration giving 50% inhibition of colony-forming ability of HeLa cells.

L. Ethyl *p*-methoxy-*trans*-cinnamate has been already isolated from the rhizomes.[3,4] However, no one has previously correlated the constituents to the cytotoxic activity. This is therefore the first report on the action of a constituent of the rhizomes of *Kaempferia galanga* L.

Experimental

Melting points were determined on a Yanaco MP-S3 micro melting point apparatus (hot-stage type) and are uncorrected. Optical rotation was measured with a JASCO DIP-140 digital polarimeter. Low-resolution mass spectra (MS) were taken with a JEOL JMS-D100 instrument. Infrared (IR) spectra were recorded on a JASCO A-220 IR spectrophotometer. Ultraviolet (UV) spectra were recorded on a Shimadzu UV-220 spectrophotometer. Proton nuclear magnetic resonance (^{1}H-NMR) and carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectra were measured with a JEOL FX-90 NMR spectrometer. Chemical shifts are reported in δ-values downfield from internal tetramethylsilane (TMS), and the following abbreviations are used; s = singlet, d = doublet, t = triplet, q = quartet and m = multiplet. The coupling constants are quoted in Hz. Elemental analyses were done on a Perkin Elmer 240C elemental analyzer. High-performance liquid chromatography (HPLC) was performed on an ALC/GPC 201 (Waters Associates Inc.) machine equipped with a UV detector (Shimadzu SPD-1 spectrophotometer).

Cytotoxicity Test——A HeLa cell cultivation test described by Koshiura[5] was used in these experiments.

Chinese Crude Drugs——Chinese crude drugs used in these experiments were obtained from the Institute of Chinese Materia Medica, Academy of Chinese Traditional Medicine, China.

Isolation of Deoxypodophyllotoxin——The dried leaves of *Biota orientalis* (L.) ENDL. (1 kg) were extracted three times with 5 l each of methanol under reflux. The mixture was filtered and the filtrate was concentrated *in vacuo* below 40 °C to give a methanol extract (MeOH ext., 121 g). The MeOH ext. (121 g) was partitioned between benzene and water (total, 2 l each). The combined benzene layer was concentrated *in vacuo*, affording a benzene-soluble fraction (benzene-sol., 40.0 g). A portion (5.0 g) of the benzene-sol. was subjected to column chromatography on silica gel (3.2 × 40 cm, Kieselgel 60, Merck) with *n*-hexane: ethyl acetate = 2 : 1 (v/v) as the eluent, giving four fractions, fr. 1 (3.34 g), fr. 2 (0.40 g), fr. 3 (0.19 g) and fr. 4 (0.85 g). In total, 3.20 g of the active fraction No. 2 (fr. 2) was obtained by repeated chromatography. The fr. 2 (3.2 g) was subjected to preparative HPLC (column, μ PORASIL, 7.8 mm i.d. × 30 cm, Waters; solvent system, *n*-hexane : ethyl acetate = 2 : 1; flow rate, 3.0 ml/min; detection, UV absorption at 294 nm). Three fractions were obtained on the basis of two main peaks, fr. 2—1 (1.0 g), fr. 2—2 (0.7 g) and fr. 2—3 (0.8 g, eluted with methanol) by repeated chromatography. The solid (0.7 g) obtained from fr. 2—2 was recrystallized from methanol to afford colorless plates of deoxypodophyllotoxin (0.4 g).

Isolation of Ethyl *p*-Methoxy-*trans*-cinnamate——The dried rhizomes of *Kaempferia galanga* L. (500 g) were extracted three times with 2.5 l each of methanol under reflux. The mixture was filtered and the filtrate was concentrated *in vacuo* below 40 °C to give a methanol extract (MeOH ext., 40.5 g). The MeOH ext. (40.5 g) was dissolved in 500 ml of water saturated with benzene, and extracted three times with 500 ml of benzene saturated with water. The combined extracts were concentrated *in vacuo*, affording the benzene-soluble fraction (benzene-sol., 27.8 g) and the water-soluble fraction (H_2O-sol., 13.2 g). A portion (9.0 g) of the benzene-sol. was subjected to column chromatography on silica gel (450 g, Kieselgel 60, Merck) with *n*-hexane: ethyl acetate = 9 : 1 (v/v) as the eluent, giving three fractions, fr. 1 ($Rf > 0.5$, 1.5 g), fr. 2 ($Rf = 0.5$—0.25, 5.6 g), fr. 3 ($Rf < 0.25$, 1.8 g) based on monitoring by thin layer chromatography (TLC) (solvent system, *n*-hexane: ethyl acetate = 4 : 1 (v/v), Kieselgel 60 F_{254}, Merck). In total, 17.3 g of fr. 2 was obtained by repeated chromatography. The crystalline material (17.3 g) obtained from fr. 2 was recrystallized from methanol to give ethyl *p*-methoxy-*trans*-cinnamate (14.4 g).

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Characterization of the Cytotoxic Principles——The structures of the cytotoxic principles were characterized by direct comparison of their physical properties with those of authentic samples.

Deoxypodophyllotoxin——Colorless plates. mp 171 °C. $[\alpha]_D^{23}$ −117° (c=0.40, $CHCl_3$). MS m/z: 398 (M^+), 185, 181, 173. IR ν_{max}^{KBr} cm^{-1}: 1780 (C=O), 1590 (aromatic ring), 1230, 1130 (C–O–C). UV λ_{max}^{MeOH} nm (log ε): 294 (3.62). ^{1}H-NMR ($CDCl_3$) δ: 2.70—3.50 (4H, m), 3.75 (6H, s), 3.80 (3H, s), 3.90—4.60 (3H, m), 5.94 (2H, s), 6.35 (2H, s), 6.51 (1H, s), 6.67 (1H, s). ^{13}C-NMR ($CDCl_3$) δ: 32.8 (t), 33.1 (d), 43.8 (d), 47.5 (d), 56.3 (q), 60.6 (q), 72.0 (t), 101.2 (t), 108.5 (d), 108.8 (d), 110.5 (d), 128.4 (s), 130.8 (s), 136.3 (s), 137.6 (s), 146.8 (s), 147.2 (s), 152.6 (s), 174.7 (s). *Anal.* Calcd for $C_{22}H_{22}O_7$: C, 66.32; H, 5.57. Found: C, 66.33; H, 5.62.

Ethyl *p*-Methoxy-*trans*-cinnamate——Colorless prisms. mp 49—50 °C. MS m/z: 206 (M^+), 178, 161, 134, 133, 119. IR ν_{max}^{KBr} cm^{-1}; 1720, 1620, 1615, 1512, 1180. ^{1}H-NMR ($CDCl_3$)δ: 1.32 (3H, t, J=7), 3.81 (3H, s), 4.24 (2H, q, J=7), 6.29 (1H, d, J=16) 6.88 (2H, d, J=9), 7.46 (2H, d, J=9), 7.63 (1H, d, J=16). ^{13}C-NMR ($CDCl_3$)δ: 14.4 (q), 55.3 (q), 60.3 (t), 114.4 (d), 115.9 (d), 127.3 (s), 129.7 (d), 144.2 (d), 161.4 (s), 163.3 (s). *Anal.* Calcd for $C_{12}H_{14}O_3$: C, 69.88; H, 6.84. Found: C, 70.05; H, 6.90.

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