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Studies on Crude Drugs Effective on Visceral Larva Migrans. II.[1] Larvicidal Principles in Kaempferiae Rhizoma

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Larvicidal principles obtained from the methanol extract of Kaempferiae Rhizoma (rhizomes of *Kaempferia galanga*) were identified as ethyl cinnamate, ethyl *p*-methoxycinnamate, and *p*-methoxycinnamic acid. Cinnamic acid and some of its derivatives showed larvicidal activity.

Keywords——anthelmintic; *Kaempferia galanga*; cinnamic acid; *p*-methoxycinnamic acid; ethyl *p*-methoxycinnamate; *Toxocara canis*; larva; larvicide; 3-carene-5-one

Kaempferiae Rhizoma (山奈), rhizomes of *Kaempferia galanga* (Zingiberaceae), have been used as an aromatic stomachic in Chinese medicine and also as an incense. The constituents of this rhizoma hitherto reported include cineol, borneol, 3-carene, camphene, kaempferol, kaempferide, cinnamaldehyde, *p*-methoxycinnamic acid, ethyl cinnamate, and ethyl *p*-methoxycinnamate.[2] Ethyl *p*-methoxycinnamate was reported to inhibit monoamine oxidase.[3]

In the course of our continuing screening work of crude drugs and plant materials effective on visceral larva migrans, we have observed that the hot water extract of Kaempferiae Rhizoma showed moderately strong larvicidal activity against the second stage larva of dog roundworm, *Toxocara canis*, which is a common pathogenic parasite in visceral larva migrans.[4] In this paper, we report the identification of the active principles.

Materials and Methods

Melting points were taken on a Yanagimoto micro hot-stage melting point apparatus, and are uncorrected. Infrared (IR) spectra were taken on a JASCO A-202 spectrometer and are given in cm^{-1}. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were measured in $CDCl_3$ solution with tetramethylsilane as an internal standard on a JEOL PMX-60 (60 MHz) and/or FX-100 (100 MHz) spectrometer. Mass spectra (MS) were taken on a Hitachi M-80 machine. Fuji-Davison BW-820 MH (silica gel) was used for column chromatography. For thin layer chromatography (TLC), Macherey-Nagel SIL G-25 UV_{254} plates were used and spots were observed under 254 nm light and/or by spraying 1% ceric sulfate in 10% H_2SO_4 followed by heating.

Chemicals——All chemicals were purchased from Nakarai Chemicals Ltd., or otherwise synthesized by the usual procedures, and their structures were confirmed by IR, ^{1}H-NMR, and MS analyses.

Assay Method——Larvicidal activity of each fraction was tested by the method previously described.[1] For one assay, 20 second stage larvae of *Toxocara canis* were incubated with the test solution in a Corning cell well at 37 °C and the behavior of the larvae was observed under a microscope at 1, 3, 6, and 24 h. All assays were duplicated. The effect of a test material was assessed according to the state of the larvae, and larvicidal activity was evaluated on the basis of the relative movability (RM) value described in the previous paper.[1] A smaller RM value indicates stronger larvicidal activity, and when all the larvae die, this value becomes 0. Minimal lethal concentration (MLC) was determined as the lowest concentration with an RM value of 0 at 24 h of incubation.

Isolation of Active Principles——Cut rhizomes of *K. galanga* (500 g), purchased from Hokuriku Yakugyo Ltd., were extracted with two 800 ml portions of hexane and three 800 ml portions of MeOH for 2 h under reflux,

successively. The MeOH extract, after concentration to about 400 ml under reduced pressure, was extracted with three 200 ml portions of hexane to give 4.55 g of hexane-soluble fraction (fr. I). The residual MeOH layer was concentrated to dryness and partitioned in $CHCl_3$: MeOH : H_2O = 6 : 3 : 1 to give 5.96 g of chloroform-soluble (fr. II) and 3.13 g of aqueous methanol-soluble (fr.III) fractions. Fraction II was subjected to silica gel column chromatography and eluted with benzene (II-1, 1.88 g and II-2, 2.61 g), benzene : acetone = 9 : 1 (II-3, 220 mg, benzene : acetone = 1 : 1 (II-4, 140 mg), and acetone and MeOH (II-5, 700 mg), successively. The RM values of the fractions at a concentration of 1 mg/ml were 0, 0, 0, 90, and 106, respectively. A portion (500 mg) of fraction II-1 was rechromatographed on silica gel with benzene : hexane = 1 :1 to give compound **1** (50 mg) and compound **2** (300 mg). Fraction II-2 was ethyl *p*-methoxycinnamate (compound **2**). Fraction II-3 deposited colorless crystals (33 mg), which gave colorless needles of compound **3** on a recrystallization from H_2O–MeOH.

Ethyl Cinnamate (Compound **1**): Colorless oil. IR (film): 1715. ^{1}H-NMR: 1.32 (3H, t, J=7 Hz), 4.21 (2H, q, J=7 Hz), 6.31 (1H, d, J=15 Hz), 7.17—7.47 (5H), 7.54 (1H, d, J=15 Hz).

Ethyl *p*-Methoxycinnamate (Compound **2**): Colorless masses, mp 43—48 °C. IR ($CHCl_3$): 1700. ^{1}H-NMR: 1.30 (3H, t, J=7 Hz), 3.75 (3H, s), 4.16 (2H, q, J=7 Hz), 6.17 (1H, d, J=15 Hz), 6.77 (2H, d, J=8 Hz), 7.35 (2H, d, J=8 Hz), 7.51 (1H, d, J=15 Hz). MS m/z (%): 206 (M^+, 64), 161 (100).

p-Methoxycinnamic Acid (Compound **3**): Colorless needles from MeOH–H_2O, mp 175—177 °C. IR (KBr): 1680. ^{1}H-NMR: 3.78 (3H, s), 6.21 (1H, d, J=15 Hz), 6.80 (2H, d, J=8 Hz), 7.40 (2H, d, J=8 Hz), 7.65 (1H, d, J=15 Hz).

The mother liquor (180 mg) of fraction II-3 was chromatographed on silica gel to give fractions II-3-1 (14 mg), II-3-2 (63 mg), II-3-3 (39 mg), and II-3-4 (55 mg). The RM values of these fractions at a concentration of 1 mg/ml were 77, 86, 0, and 78, respectively. The major constituent of the active fraction (II-3-3) was *p*-methoxycinnamic acid (compound **3**). However, preparative TLC analysis revealed that a less polar zone (II-3-3b) which absorbed ultraviolet (UV) light also showed the activity.

To identify this active principle, another 800 g of rhizomes were fractionated as described above. The corresponding fraction (II-3-3b, 75 mg, RM = 35 at 0.1 mg/ml) thus obtained was subjected to medium-pressure liquid chromatography on LiChroprep Si-60B with benzene : ethyl acetate = 19 : 1 to give 3-caren-5-one[5] (23 mg). However, this compound was not active, and the larvicidal activity was found in a slightly less polar fraction (12 mg, RM = 96 at 0.1 mg/ml and 0 at 0.2 mg/ml), which was still found to be a mixture of several compounds. Moreover, the activity of this fraction was weaker than that before fractionation (fr. II-3-3b). Therefore, further purification was abandoned.

Results and Discussion

Isolation and Identification of Larvicidal Principles

In a preliminary experiment on the isolation of larvicidal principles, cut rhizomes of *K. galanga* were successively extracted with hexane, chloroform, methanol, and water under reflux. The larvicidal activity of each (hexane ext., RM = 43; chloroform ext., RM = 0; methanol ext., RM = 0; water ext., RM = 94 at 1 mg/ml after a 24 h incubation) showed that the activity was concentrated in the chloroform and the methanol extracts.

Therefore, the rhizomes were newly extracted with hexane and methanol successively and

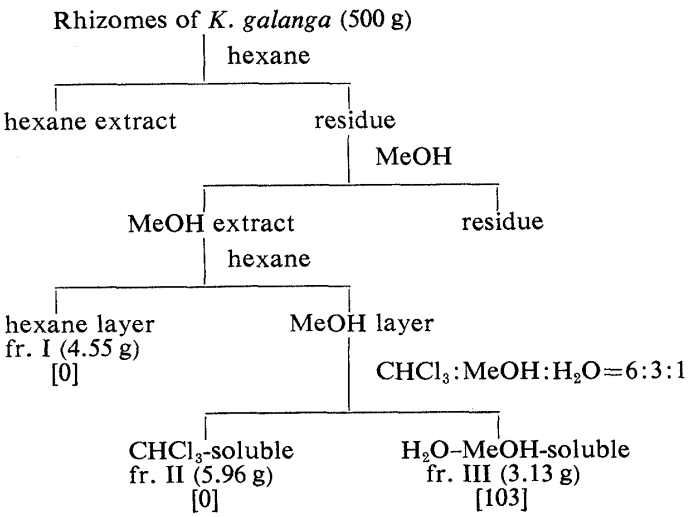


Chart 1. Extraction of Larvicidal Principles from *K. galanga*

the methanol extract was divided into hexane-soluble (fr. I), chloroform-soluble (fr. II), and aqueous methanol-soluble (fr. III) fractions (Chart 1). Among these fractions, fractions I and II showed strong larvicidal activity. Because their TLC patterns were very similar to each other, fraction II was fractionated by silica gel column chromagraphy (monitoring by TLC and by measuring RM value at a concentration of 1 mg/ml), resulting in the isolation of three active constituents. These were identified as ethyl cinnamate, ethyl *p*-methoxycinnamate, and *p*-methoxycinnamic acid by direct comparisons with authentic specimens. The MLC against larva of *T. canis* were 4.0, 0.5, and 0.3 mM, respectively. As ethyl cinnamate and ethyl *p*-methoxycinnamate are major constituents of the methanol extract, most of the larvicidal activity of Kaempfeiae Rhizoma should be attributable to these two compounds.

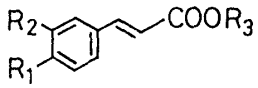
The mother liquor from *p*-methoxycinnamic acid (fr. II-3-3) suggested the presence of another active constituent, but this could not be isolated.

Larvicidal Activity of Cinnamic Acid Derivatives

Since cinnamic acid derivatives were obtained as larvicidal principles of *K. galanga*, some cinnamic acid derivatives were examined for larvicidal activity against dog roundworm larva (Table I). Among five free acids tested, cinnamic and *p*-methoxycinnamic acids showed the strongest activity with MLC of 0.3 mM. When the acids were converted to the sodium salts, the larvicidal activity was almost lost. They were inactive at a concentration of 10 mM.

Larvicidal activities of alkyl (methyl to butyl) esters of cinnamic and *p*-methoxycinnamic acids were weaker than those of the free acids. On the other hand, for *p*-hydroxy- and *p*-acetoxy-cinnamic acids, alkyl esters, especially propyl and butyl esters, were more active than the corresponding free acids. The MLC's of the butyl esters of these acids were 0.1 and 0.08 mM, respectively. For 3,4-methylenedioxycinnamic acid, the propyl and butyl esters were again more active than the free acids. Their MLC's were 0.5 and 0.6 mM, respectively.

TABLE I. MLC of Cinnamic Acid Derivatives against *T. canis*



R_3	$R_1=H$ $R_2=H$	$R_1=OH$ $R_2=H$	$R_1=OMe$ $R_2=H$	$R_1=OAc$ $R_2=H$	R_1 R_2 $=-OCH_2O-$
H	0.3	1.0	0.3	0.6	>10
Me	3	1.5	5	0.4	>10
Et	4	0.8	0.5	0.2	1.5
Pr	5	0.2	0.5	0.15	0.5
Bu	2.0	0.1	1.0	0.08	0.6

MLC: 24 h incubation, mM.

TABLE II. MLC of Cinnamic Acid Analogues against *T. canis*

Compound	MLC (mM)
Cinnamic acid	0.3
Cinnamaldehyde	0.5
Cinnamyl alcohol	2.5
Phenylpropionic acid	1.0
Phenylpropionaldehyde	2.0
Phenylpropanol	>10

MLC: 24 h incubation.

In a previous paper,[1] we have shown that aliphatic alcohols with C_{10} to C_{14} alkyl chains are strong larvicides against *T. canis*. For example, the MLC of tetradecanol is 15 μM. Consequently, cinnamic esters of C_6 to C_{16} alcohol were also tested. However, all of them were inactive at a concentration of 10 mM.

Next, some analogues of cinnamic acid were tested for larvicidal activity (Table II) and the following structure–activity relationships were revealed. When cinnamic acid was reduced to cinnamaldehyde and cinnamyl alcohol, larvicidal activity was decreased in that order, suggesting that the higher the oxidation state of the functional group on the side chain, the stronger is the larvicidal activity. Reduction of the side chain double bond of cinnamic acid again led to a decrease of larvicidal activity. Thus, the activity of phenylpropionic acid was about one-third of that of cinnamic acid. The larvicidal activity of phenylpropionaldehyde was weaker than that of the corresponding acid. Phenylpropanol did not show larvicidal activity up to a concentration of 10 mM.

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