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Studies on Crude Drugs Effective on Visceral Larva Migrans. V.[1]
The Larvicidal Principle in Mace (Aril of *Myristica fragrans*)

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The larvicidal principle in mace, aril of *Myristica fragrans* (Myristicaceae), was identified as a diarylnonanoid, malabaricone C (**1**), whose minimal lethal concentration against second-stage larva of *Toxocara canis* was 6 μM.

Keywords——mace; nutmeg; Papua mace; *Myristica fragrans*; *Myristica argentea*; malabaricone C; diarylnonanoid; larvicidal activity; *Toxocara canis*; visceral larva migrans

Mace, the aril of *Myristica fragrans* HOUTT. (Myristicaceae), has been widely used as a spice and is an important medicine in Ayurvedic treatment.[2] Among a number of constituents of mace as well as nutmeg (the seed kernels of *Myristica fragrans*) so far isolated,[3] such as essential oils, fats, glycerides, cyclic and acyclic bis-phenylpropanoids, and pigments, bis-phenylpropanoids are particularly interesting in their pharmacological actions[3a]; they are hallucinogenic, and they inhibit the growth of silkworm larvae, and prostaglandin biosynthesis. Recently Namba *et al.*[4] indicated that some bis-phenylpropanoids in mace showed significant antibacterial activity against *Streptococcus mutans*. Papua mace, sometimes used as a substitute spice for mace, is the aril of a different plant, *Myristica argentea* WARB., which also contains bis-phenylpropanoids.[5]

In the course of our continuing screening work on crude drugs, plant materials, and spices effective against diseases caused by nematodes,[6] we have observed that the hot water extract of mace showed considerable larvicidal activity against second-stage larva of dog roundworm, *Toxocara canis*, which is a common pathogenic parasite in visceral larva migrans. Here we report the identification of the active principle against nematodes, which has not previously been isolated from mace.

Results and Discussion

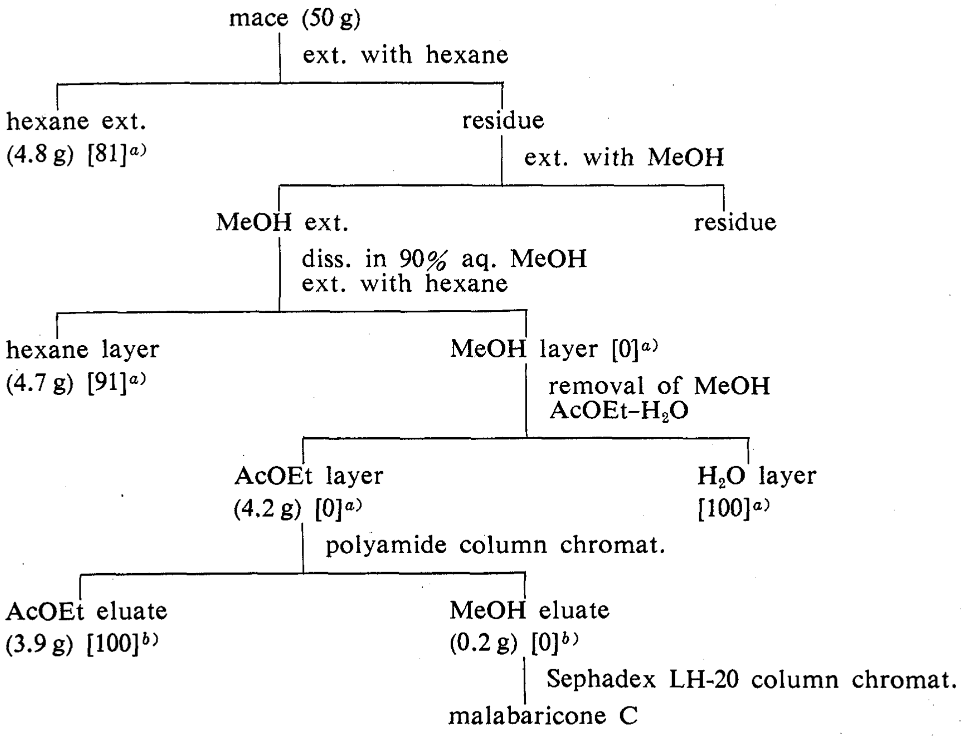
Comparison of the larvicidal activity of hot water extracts of mace, nutmeg, and Papua mace from different sources against second-stage larva of *Toxocara canis* (Table I) indicated that mace is the most active, suggesting that it has the highest content of the active principle(s). A preliminary experiment on its isolation also suggested that the active principle is not soluble in hexane or chloroform, but can be extracted into methanol.

Therefore mace was first extracted with hot hexane, then with methanol, and the active principle was transferred from methanol to ethyl acetate (Chart 1). Chromatographic separation of this fraction over silica gel was first attempted, but was found to give an unsatisfactory result, because the activity was largely lost. Attempted separation of phenolic components by the use of sodium hydroxide solution was also fruitless. The activity was lost almost completely.

Table I. Larvicidal Activity of Water Extract of Mace, Nutmeg, and Papua Mace against *Toxocara canis* (RM Value[a] at 10 mg/ml)

Spice	(origin)	Time (h)[b]			
		1	3	6	24
Mace	(Indonesia)	100	100	100	0
	(Sir Lanka)	100	84	25	0
	(commercial)[c]	100	99	71	0
Nutmeg	(Sri Lanka)	100	100	96	0
	(commercial)[c]	100	100	100	22
Papua mace	(Papua New Guinea)	100	100	100	75

a) See ref. 6. *b*) Hours of incubation. *c*) Powdered, purchased at a supermarket in Kanazawa city.



( ), yield; [ ], RM value after 24 h incubation; *a*), 1 mg/ml; *b*), 0.1 mg/ml.

Chart 1. Isolation of the Larvicidal Principle from Mace

The following procedure was found to be practical for isolation of the active principle. The active principle was adsorbed on a polyamide column from ethyl acetate solution and eluted with methanol; it was easily purified by chromatography on Sephadex LH-20. By these two chromatographies it was purified without decomposition, and finally crystallized as pale yellow prisms from benzene, mp 125—126 °C. The *in vitro* minimal lethal concentration (MLC) of this compound against *T. canis* (after 24 h incubation) was 6 μM. All other fractions were almost inactive.

The active principle has the formula $C_{21}H_{26}O_5$. It gave greenish-brown color with ferric chloride and gave a tetraacetate on acetylation. In the ^{1}H-nuclear magnetic resonance (^{1}H-NMR) spectrum it exhibited signals of six aromatic and sixteen aliphatic protons. Decoupling experiments indicated that there are two aromatic moieties, 4-alkylcatechol and 2,6-dihydroxyalkylphenone. When the double-doublet at δ 6.55 (H_c) was irradiated, the signals at δ 6.74 (H_a) and 6.70 (H_b) changed into sharp singlets, and when the doublet at δ 6.36 (H_d) was irradiated a triplet at $\delta 7.17$ (H_e) changed into a singlet. The chemical shift and coupling pattern of the former protons are almost identical with those of the aromatic protons of 4-methylcatechol,7a) and those of the latter are the same as those of 2,6-dihydroxyacetophenone.7b) This assignment was supported by the infrared (IR) spectrum (1605, $1630\,cm^{-1}$) which is in accord with the carbonyl absorption of 2,6-dihydroxyacetophenone.8) The ultraviolet (UV) absorption (λ_{max} 223, 273, and 343 nm) can also be explained by the sum of the absorptions of the above two moieties.

The aliphatic methylene protons at $\delta 3.12$ (H_f) and 1.67 (H_g), and at $\delta 2.46$ (H_h) and 1.54 (H_i) are coupled each other, respectively. Since H_f and H_h are assigned as the methylene protons adjacent to the carbonyl and the aromatic group, H_g and H_i are the methylene protons next to these groups, respectively. The remaining broad singlet of 8 H at $\delta 1.30$ is therefore assigned to four methylene groups between H_g and H_i. These spectral data lead to the structure **1** for the active principle.

The assigned structure is identical with a known diarylnonanoid, malabaricone C, isolated from *Myristica malabarica* by Connolly *et al.*9) This plant is sometimes used in India as a substitute medicine for *M. fragrans*. The identity of the spectral data and melting point of the two samples undoubtedly indicates that our active principle is malabaricone C.

Malabaricone C is not a very minor constituent of mace (yield was *ca.* 0.1%). The reason why this compound was missed in previous investigations may be its instability to bases and to silica gel chromatography. For example, on contact with a dilute sodium hydroxide solution it rapidly changed (probably due to aerial oxidation) into a compound that, after acidification, was immobile on thin layer chromatography (TLC).

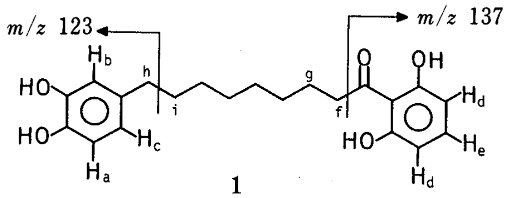


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Chart 2

Judging from the very strong activity and the content of malabaricone C in mace, we conclude that the larvicidal activity of mace is attributable to this compound. Comparison of the larvicidal activities of several bis-phenylpropanoids in mace supported this conclusion [MLC's of dehydrodiisoeugenol, 5′-methoxydehydrodiisoeugenol, 2,3-dihydro-7-methoxy-3-methyl-2-(3,4-methylenedioxyphenyl)-5-(1*E*-propenyl)benzofuran, 2,3-dihydro-7-methoxy-3-methyl-2-(3-methoxy-4,5-methylenedioxyphenyl)-5-(1*E*-propenyl)benzofuran, and *erythro*-2-(4-allyl-2,6-dimethoxyphenoxy)-1-(3,4-dimethoxyphenyl)propan-1-ol were >1 mM, after a 24 h incubation].10) Although dehydrodiisoeugenol and 5′-methoxydehydrodiisoeugenol are known to be bacteriocidal to *Streptococcus mutans*,4) they were almost inactive against *T. canis*.

Experimental

Melting points were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. UV spectra were measured with a Hitachi 323 spectrophotometer, IR spectra were taken on a JASCO A-202 spectrometer, ^1H- and ^{13}C-NMR spectra were recorded on a JEOL GX-400 spectrometer, and mass spectra (MS) and high-resolution mass spectra (HRMS) were measured with a Hitachi M-80 mechine at an ionization voltage of 20 eV and 70 eV, respectively. For TLC, Macherey-Nagel precoated TLC plates SIL G-25 UV_{254} were used.

Assay Method——Larvicidal activity of each fraction was tested by the method previously described.6) 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 after the start of incubation. All assays were done in duplicate. The effect of each test material was assessed according to the state of the larvae, and the larvicial activity was evaluated in terms of the relative mobility (RM) value as described in the previous paper.[6] A smaller RM value indicates a stronger larvicidal activity, and when all the larvae die, the value becomes 0. MLC was determined as the lowest concentration with an RM value of 0 after 24 h of incubation.

Isolation of Malabaricone C from Mace——Indonesian mace (50 g) was roughly ground and extracted with hexane (200 ml) for 1 h, then with methanol (200 ml × 2) for 3 h under reflux. After concentration of the combined methanol extract to 150 ml, water (15 ml) was added, and the whole was extracted several times with hexane (100 ml), then concentrated to dryness. The residue was distributed between ethyl acetate (100 ml) and water (100 ml), and the dried (over Na_2SO_4) organic layer was passed through a column of polyamide (Woelm, for TLC), then the column was washed with the same solvent. Elution of the column with methanol yielded a product (200 mg) which was purified by chromatography on a Sephadex LH-20 (Pharmacia Fine Chemicals) column with methanol to give malabaricone C (**1**) (40 mg), mp 125—126 °C (lit. mp 123—124 °C),[9] as pale yellow prisms from benzene. UV (EtOH) λ_{max} nm (ε): 223 (31000), 273 (21000), 343 (5000). IR ($CHCl_3$) cm^{-1}: 1605, 1630. ^{1}H-NMR ($CDCl_3 + CD_3OD$): δ 1.30 (8H, br s), 1.54 (2H, quin, $J=7.3$ Hz, H_i), 1.67 (2H, quin, $J=7.3$ Hz, H_g), 2.46 (2H, t, $J=7.3$ Hz, H_h), 3.12 (2H, t, $J=7.3$ Hz, H_f), 6.36 (2H, d, $J=8.2$ Hz, H_d), 6.55 (1H, dd, $J=8.2$, 1.5 Hz, H_c), 6.70 (1H, d, $J=1.5$ Hz, H_b), 6.74 (1H, d, $J=8.2$ Hz, H_a), 7.17 (1H, t, $J=8.2$ Hz, H_e). ^{13}C-NMR ($CDCl_3 + CD_3OD$): δ 24.5 (t), 29.0 (t), 29.3 (t), 29.3 (×2, t), 31.5 (t), 35.2 (t), 44.7 (t), 107.8 (×2, d), 110.4 (s), 115.0 (d), 115.4 (d), 120.3 (d), 135.6 (s), 135.8 (d), 141.9 (s), 144.0 (s), 161.8 (×2, s), 208.6 (s). MS *m/z* (%): 358 (M^+, 26), 248 (24), 220 (13), 165 (27), 149 (9), 137 (100), 123 (70). HRMS *m/z*: Calcd for $C_{21}H_{26}O_5$ (M^+): 358.1778. Found: 358.1780. *Anal.* Calcd for $C_{21}H_{26}O_5$: C, 70.37; H, 7.31. Found: C, 70.54; H, 7.45.

Acetylation of Malabaricone C——Acetylation of malabaricone C (10 mg) in Ac_2O (1 ml) and pyridine (1 ml) gave a tetraacetate (10 mg) as an oil. ^{1}H-NMR ($CDCl_3$): δ 2.25 (×2), 2.275, 2.279. MS *m/z* (%): 526 (M^+, 1), 358 (100).

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