Biologically Active Constituents of *Melaleuca leucadendron*: Inhibitors of Induced Histamine Release from Rat Mast Cells

Tomoko TSURUGA, Yui-To CHUN, Yutaka EBIZUKA and Ushio SANKAWA*

Faculty of Pharmaceutical Sciences, The University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113, Japan. *Received June 24, 1991*

Chloroform and methanol extracts of the fruits of *Melaleuca leucadendron* strongly inhibited histamine release from rat mast cells induced by compound 48/80 or concanavalin A. Ursolic acid, a triterpene, was the most active compound contained in the chloroform extract and two stilbenes, piceatannol and oxyresveratrol, were isolated as active compounds from the methanol extract. Several other stilbenes and related compounds were examined to obtain information on the structure activity relationships of stilbenes.

Keywords *Melaleuca leucadendron*; stilbene; piceatannol; oxyresveratrol; ursolic acid; anti-allergy; mast cell

Melaleuca leucadendron L. (Myrtaceae) is a large tree growing both wild and cultivated in tropical Asia. The leaves of the tree have been used to produce an essential oil, cayuput oil,[1] while the tiny fruits of this plant have been used as a medicinal drug in traditional Indonesian Jamu medicine.[2] The fruits are called Borong-Borong in Indonesia, which means 'many holes'. The Indonesian name derives from the hole of the tiny fruit. This Jamu drug is used as a tonic and is available in the herbal medicine shops in Java and Bahli islands where Jamu herbal drugs are used in daily health care. During a survey study of Indonesian Jamu medicinal drugs we purchased the material at Sukabumi, a city in east Java. The aqueous ethanol extract of the fruits that was prepared in small scale was subjected to random screening with *in vitro* bioassay systems which were routinely used in our studies on medicinal plants.[3] It was highly active in a bioassay testing the inhibitory effect on histamine release from rat mast cells. The mast cell bioassay system has been used to find potential anti-allergy compounds[4] and has good correlation with passive cutaneous anaphylaxis (PCA) test which is a standard bioassay to evaluate anti-allergy activity.[4a] The *in vitro* mast cell bioassay has been extensively used in our bioassay oriented phytochemical studies on medicinal plants. Flavonoids, coumarins, lignans and sesquiterpenes were found as potential anti-allergy compounds which were highly active in the mast cell bioassay as well as PCA test.[4] Since no reports were available on the constituents of the fruits of *M. leucadendron*, we investigated the active constituents of the medicinal drug with *in vitro* monitoring of the mast cell bioassay. To clarify the chemical properties of active constituents the fruits of *M. leucadendron* (200 g) were successively extracted with hexane, chloroform, acetone and methanol. The inhibitory effects of the extracts to the mast cell bioassay are shown in Table I. Except for the hexane extract all the other extracts exhibited significant inhibitory activities on histamine release. Following this observation, 5 kg of this plant material was successively extracted with chloroform, acetone and methanol to isolate and identify bio-active constituents in the extracts.

Extensive separation of the chloroform extract by column chromatographies afforded six known triterpenoids, betulinic acid,[5] acetyl betulinic acid,[5] betulinaldehyde,[6] pyracrenic acid,[7] ursolic acid and ursolaldehyde,[8] and one known sesquiterpene, globulol.[9] Ursolic acid was contained 5.6% in the chloroform extract and inhibited compound 48/80 induced histamine release 95% and 26% at concentrations of 10^{-3} and 10^{-4} M, respectively. Since the activities of the other terpenoids are not significant, ursolic acid can be regarded as the main active compound that contributes to the bioactivity of the chloroform extract.

The methanol extract was fractionated with silica-gel column chromatography and the biological activities of obtained fractions were monitored by the mast cell bioassay. (Chart 1). Five compounds were so far isolated from active fractions and tentatively named ML-1—ML-5. ML-1 and ML-5 were identified as quercetin and gallic acid, respectively. ML-2 was found to be a phenolic compound and identified as piceatannol (**1**), a stilbene by the comparison of spectral data.[10] The chemical and spectral properties of ML-3 were similar to those of piceatannol (**1**) and it was finally identified as oxyresveratrol (**2**) by the comparison of its spectral data with those reported.[11] The proton nuclear magnetic resonance (^{1}H-NMR) spectrum of ML-4 suggested it to be a dimeric stilbene and finally identified it as scirpusin B (**3**).[10b]

Quercetin (ML-1) inhibited concanavalin A (Con A) induced histamine release nearly 100% at a concentration of 10^{-4} M and the result is well in accord with those reported elsewhere.[4,12] Monomeric stilbenes, **1** and **2**, were highly active and in particular the IC_{50} value of piceatannol (**1**) was 9.6 μM in an experiment using Con A as a histamine releaser. The activities of monomeric stilbenes are comparable to those of active flavonoids. To obtain information on structure activity relationships of stilbenes we investigated inhibitory activities of available stilbenes and related compounds. The results are summar-

TABLE I. Inhibitory Effects of *Melaleuca leucadendron* Extracts on Histamine Release from Rat Mast Cells Induced by Compound 48/80 or Con A

	Inhibition % of histamine release			
	Inducer			
	Compound 48/80		Con A	
	50 μg/ml	500 μg/ml	50 μg/ml	500 μg/ml
Hexane ext.	19	84	6	47
Chlololform ext.	30	100	5	88
Acetone ext.	40	100	2	95
Methanol ext.	28	70	14	81
DSCG	15	40	14	32

DSCG: disodium cromoglycate.

© 1991 Pharmaceutical Society of Japan

NII-Electronic Library Service

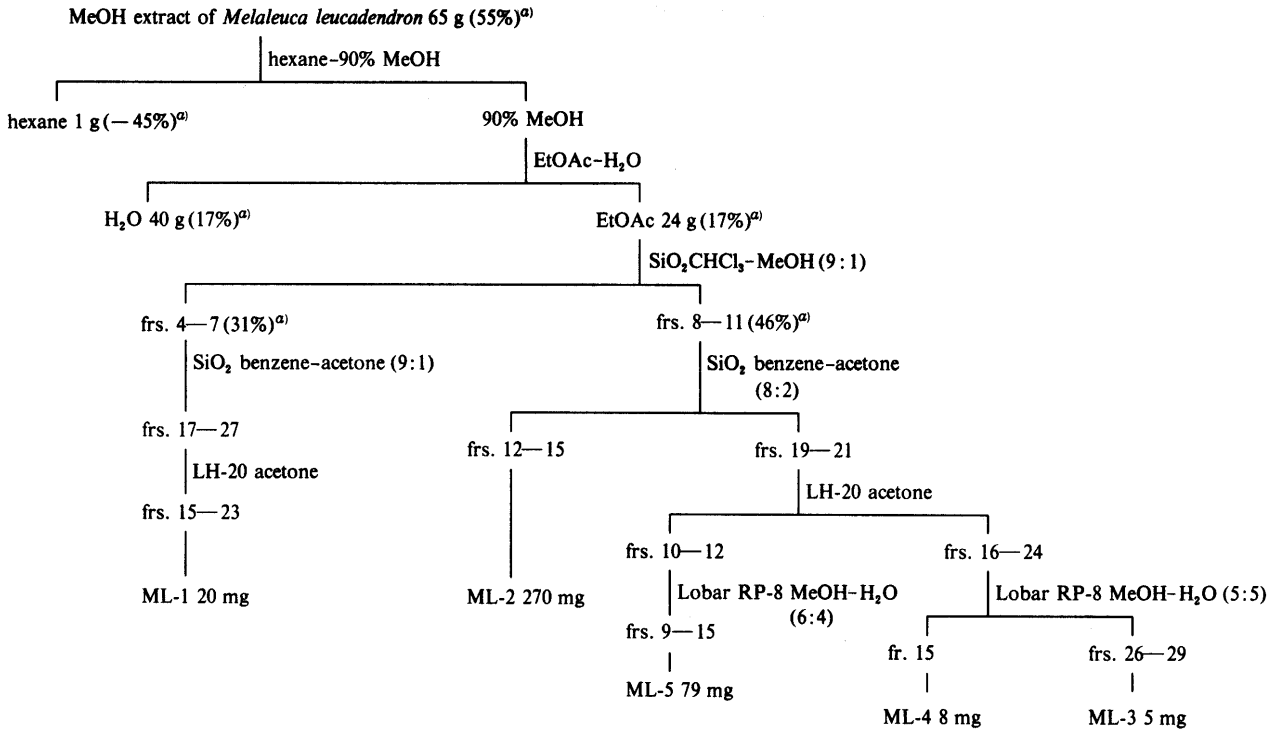


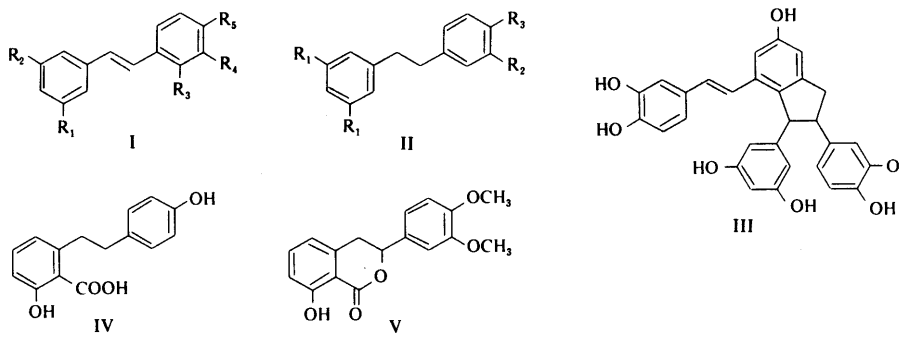
Chart 1. Fractionation of the MeOH Extract of *Melaleuca leucadendron*

a) Inhibition % for histamine release from mast cells induced by compound 48/80 at a substrate concentration of 50 μg/ml.

TABLE II. Inhibitory Effects of Stilbenes on Histamine Release from Rat Mast Cells Induced by Compound 48/80 or Con A

	R_1	R_2	R_3	R_4	R_5	Inhibition % of histamine release				IC_{50} μM
						Inducer				
						Compound 48/81		Con A		
						10^{-3} M	10^{-4} M	10^{-3} M	10^{-4} M	
Ia (**1**)	OH	OH	H	OH	OH	86	40	108	103	9.6
Ib (**2**)	OH	OH	OH	H	OH	31	26	103	84	22
Ic (**4**)	OH	OH	H	OH	OMe	74	28	108	108	22
Id (**5**)	H	H	H	OMe	OH	12	2	107	92	35
Ie (**6**)	OH	O-G	H	OH	OH	11	14	32	23	
If (**7**)	OH	O-G	H	OH	OMe	12	14	32	15	
IIa (**8**)	OH	OH	OH			76	14	107	97	27
IIb (**9**)	H	OH	OMe			−15	−6	49	25	
IIc (**10**)	H	OMe	OH			−46	−21	38	8	
III (**3**)						25	10	84	22	
IV (**11**)						90	27	105	60	53
V (**12**)						−8	−4	97	84	9.5

O-G = *O*-glucoside. Ia, piceatannol (**1**); Ib, oxy-resveratrol (*2*); Ic, rhapontigenin (**4**); Id, 4-hydroxy-3-methoxystilbene (**5**); Ie, piceatannol glucoside (**6**); If, rhaponticin (**7**); IIa, dihydropiceatannol (**8**); IIb, 3,4-dihydroxydihydrostilbene (**9**); IIc, 4-hydroxy-3-methoxydihydrostilbene (**10**); III, scirpusin B (**3**); IV, lunuralic acid (**11**); V, monomethylphylodulcin (**12**).



NII-Electronic Library Service

ized in Table II. In general, monomeric stilbenes (**1**, **2**, **4**, **5**) were highly active except for glucosides (**6**, **7**). A very interesting compound is monomethyl phylodulcin (**12**) which showed selective inhibition to Con A induced histamine release, but it did not inhibit histamine release induced by compound 48/80. The inhibitory effect of piceatannol (**1**) on *in vitro* anti-immunoglobulin E (IgE)-induced histamine release from human basophils was reported quite recently and the result is well in accord with our observation.[13] The two benzene rings of stilbene structure are somewhat similar to those of flavonoid whose anti-allergic activity has been firmly established.[12] This indicates that the presence of two benzene rings with an appropriate distance may be essential to their common inhibitory effect on induced histamine release from mast cells.

Experimental

^{1}H- and ^{13}C-NMR spectra were measured on a JEO JNM FX-100 or a FX-400, mass spectra (MS) on a JEOL JMS-DX300, infrared (IR) on a JASCO model 701G and ultraviolet (UV) on a Hitachi spectrophotometer model 100-60. Melting points were determined on a Yanagimoto micro-melting point apparatus and uncorrected. The fruits of *M. leucadendron* were purchased in a Jamu shop in Skabumi, Indonesia. Samples for reference and testing of bioactivity were obtained from the following sources. Betulinic acid: Prof. H. Ageta, Showa College of Pharmacy; betulinaldehyde: Dr. H. Miles, Mississippi State University; piceatannol (**1**), rhapontigenenin (**4**), piceatannol glucoside (**6**), rhapontici-cin (**7**): Prof. I. Nishioka, Kyushu University; scirpusin B (**3**): Dr. H. Taguchi, Tsumura Co.; 4-hydroxy-3-methoxystirbene (**5**), 3,4-dihydroxy-dihydrostirbene (**9**), 4-hydroxy-3-methoxydihydrostirbene (**10**), mono-methylphylodulcin (**12**): Prof. M. Yamato, Okayama University. The mast cell bioassay was purformed as described in previous papers.[4]

Small Scale Extraction Dried fruits of *M. leucadendron* (200 g) were each extracted twice successively each twice with 500 ml hexane, $CHCl_3$, acetone and MeOH. The solvents were removed *in vacuo* to give hexane (6 g), $CHCl_3$ (13 g), acetone (12 g) and MeOH (7 g) extracts, which were submitted to the *in vitro* mast cell bioassay. (Table I)

Large Scale Extraction, Fractionation and Separation Dried fruits of *M. leucadendron* (5 kg) were extracted each twice with $CHCl_3$, acetone and MeOH. On evaporation of solvents *in vacuo*, $CHCl_3$, acetone and MeOH extracts were obtained in yields of 453, 287 and 179 g, respectively. A part of $CHCl_3$ extract (36 g) was chromatographed over silica-gel with benzene–acetone (99 : 1—90 : 10) and 106 eluted fractions were combined into four active fractions, frs. 20—29, 44—49, 50—55 and 56—66. Upon repeated fractionation with silica-gel frs. 20—29 gave globulol (570 mg), betulinaldehyde (83 mg), acetylbetulinic acid (32 mg) and ursolaldehyde (5 mg). Fractions 44—49 were further separated with silica-gel column to give pyracrenic acid (160 mg). From frs. 50—55 and 56—60 gave crystals of betulinic acid (2.1 g) and ursolic acid (2.0 g), respectively.

A part of MeOH extract (65 g) was subjected to fractionation as shown in Chart 1. The MeOH extract was partitioned between hexane and 90% aqueous MeOH, and after removing the solvent the MeOH fraction was again partitioned with EtOAc–H_2O. The EtOAc fraction was subjected to silica-gel column chromatography and the active fractions were combined into two fractions, frs. 4—7 and frs. 8—11, which were further separated with silica-gel, Sephadex LH-20 and Lobar RP-8 columns to give compounds ML-1—ML-5. ML-1 and ML-5 were readily identified as quercetin and gallic acid, respectively.

Piceatannol (ML-2, 1)[10] Slightly brown plate from MeOH–$CHCl_3$, mp 236—237 °C. MS *m/z* (rel. int.): 244 (M^+, $C_{14}H_{12}O_4$, 100), 243 (10). Upon acetylation with pyridine and acetone **1** gave a tetraacetate of mp 120—122 °C (H_2O–EtOH). ML-2 was identified as piceatannol by the comparison of spectral data of those of authentic samples of piceatannol and its acetate.

Dihydropiceatannol (8) **1** (50 mg) was hydrogenated with Pt–H_2 in AcOEt for 2 h at r.t. The product was purified with Lobar RP-8 column with MeOH–H_2O (5 : 5) and recrystallized from MeOH–H_2O to give dihydropiceatannol (18 mg) of mp 158—160 °C. MS *m/z* (rel. int.): 246 (M^+, $C_{14}H_{14}O_4$, 100), 245 (14). ^{1}H-NMR (MeOH-d_4, 100 MHz) δ: 2.67 (4H, s, $CH_2 \times 2$), 6.0—6.2 (3H, m, C-2′, C-4′ and C-6′), 6.47 (1H, dd, J=2.0, 7.5 Hz, C-6), 6.60 ((1H, d, J=2.0 Hz, C-2), 6.65 (1H, d, J=7.5 Hz, C-5).

Oxyresveratrol (ML-3, 2)[11] Pale brown powder. MS *m/z* (rel. int.): 244 (M^+, $C_{14}H_{12}O_4$, 100), 243 (12). ML-3 was identified by the comparison of spectral data with those reported.

Scirpusin B (ML-4, 3)[10b] Pale yellow powder. MS *m/z* (rel. int.): 486 (M^+, $C_{28}H_{22}O_8$, 100), 378 (85), 244 (16), 242 (27).

Acknowledgments This work is part of a survey study on Indonesian Jamu medicinal drugs which was supported by Overseas Scientific Survey Grant No. 60041044. The author (U.S.) thanks Professor I. Kitagawa of Osaka University for giving him the opportunity of join a team surveying on Jamu medicinal drugs in Indonesia. We also thank PT Esai Indonesia for their cooperation on the survey study and supply of information and material. Thanks are due to Prof. H. Ageta of Showa College of Pharmacy, Dr. H. Miles of Mississippi State University, Dr. H. Taguchi of Tsumura Co., Prof. I. Nishioka of Kyushu University and Prof. T. Yamato of Okayama University for generous gifts of samples.

References

1) L. M. Perry, "Medicinal Plants of East and Southeast Asia," the MIT Press, Cambridge, Massachusetts, 1980, p. 284.
2) S. Maddsiwojo and H. Rajakmangunsnadarso, "Cabe Duyang Warisan Nanek Moyang I," P N Balai Pustaks, Jakarta, 1985.
3) *a*) U. Sankawa, H. Otsuka, Y. Kataoka, Y. Iitaka, A. Hoshi and K. Kuretani, *Chem. Pharm. Bull.*, **29**, 116 (1981); *b*) T. Nikaido, T. Ohmoto, H. Noguchi, T. Kinoshita, H. Saitoh and U. Sankawa, *Planta Medica*, **43**, 18 (1981); *c*) F. Kiuchi, M. Shibuya and U. Sankawa, *Chem. Pharm. Bull.*, **30**, 754 (1982); *d*) *Idem*, *ibid.*, **30**, 2279 (1982); *e*) F. Kiuchi, M. Shibuya, T. Kinoshita and U. Sankawa, *ibid.*, **31**, 3391 (1983); *f*) K. Ichikawa, T. Kinoshita, A. Itai, Y. Iitaka and U. Sankawa, *Hetetrocycles*, **22**, 2071 (1984).
4) *a*) Y.-T. Chun and U. Sankawa, *Shoyakugaku Zasshi*, **43**, 314 (1989); *b*) J.-B. Wu, Y.-T. Chun, Y. Ebizuka and U. Sankawa, *Chem. Pharm. Bull.*, **33**, 4091 (1985); *c*) T. Tsuruga, Y. Ebizuka, J. Nakajima, Y. T. Chun, H. Noguchi, Y. Iitaka and U. Sankawa, *Tetrahedron Lett.*, **25**, 4129 (1984); *d*) T. Tsuruga, Y. Ebizula, J. Nakajima, Y.-T. Chun, H. Noguchi, Y. Iitaka, H. Seto and U. Sankawa, *Chem. Pharm. Bull.*, **39**, 3265 (1991).
5) E.-H. Hui and M.-M. Li, *Phytochemistry*, **15**, 563 (1976); E. Wenkart, G. V. Baddeley, I. R. Brufitt and L. M. Moreno, *Org. Mag. Res.*, **11**, 337 (1978).
6) J. Bhattacharyya, U. Kokpol and D. H. Miles, *Phytochemistry*, **15**, 432 (1977); H. Ohtsuka, S. Fujioka, T. Komiya, M. Goto, Y. Hiramatsu and H. Fujimura, *Chem. Pharm. Bull.*, **29**, 3099 (1981).
8) S. Seo, Y. Tomita, and K. Tori, *J. Am. Chem. Soc.*, **103**, 2075 (1981).
9) M. Grahan, *Aust. J. Chem.*, **13**, 372 (1981); G. Bkuchi, W. Hofheinz and J. V. Paukastelis, *J. Am. Chem. Soc.*, **91**, 6473 (1969).
10) *a*) K. Hata, K. Baba and M. Kozawa, *Chem. Pharm. Bull.*, **27**, 984 (1989); *b*) K. Takasugi, H. Taguchi, T. Endo and I. Yosioka, *ibid.*, **26**, 3050 (1988).
11) M. Takasugi, L. Munoz, T. Masamune, A. Shirata and K. Takahashi, *Chem. Lett.*, **1978**, 1241.
12) J. C. Foreman, *J. Allergy Clin. Immunol.*, **73**, 769 (1984).
13) Y. Inamori, M. Ogawa, H. Tsujibo, K. Baba, M. Kozawa and H. Nakamura, *Chem. Pharm. Bull.*, **39**, 805 (1991).

NII-Electronic Library Service