A Mosquito Larvicidal Diterpenoid Isolated from *Podocarpus totara* D. Don ex Lambert[1]

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Abstract Totarol, a diterpenoid phenol isolated from *Podocarpus totara* (D. Don ex Lambert) root bark, was found to be active against second- and fourth-instar *Culex pipiens* Coquillett with the 24 h LC_{50} values of 0.25 and 0.37 µg/mL, respectively. The mosquito larvicidal activity against *C. pipiens* increased when bioassays were extended to 48 h. Structural elucidation of totarol was by means of ^{1}H-NMR, ^{13}C-NMR, and GC-MS analysis.

Key Words *Podocarpus totara,* totarol, mosquito larvicidal activity

The mosquito-borne diseases malaria, filariasis, dengue, yellow fever and Japanese encephalitis, contribute significantly to disease burden, death, poverty and social debility in tropical countries. Among these diseases, malaria continues to be a major public health problem in most countries of the tropical world. WHO (1992) reported that 2200 million people were exposed to malarial infections in some 90 countries or areas, and malaria was also the cause of an estimated 1.4 to 2.6 million deaths worldwide each year, with more than 90% in Africa alone. However, control of malarial and other mosquito-borne diseases is becoming increasingly difficult because the effectiveness of vector control has declined due to reduced efficiency of insecticides caused by emergence of resistance in mosquito against the current used insecticides (WHO 1995). It is also important to recognize that only a limited number of pesticides are available for use in public health. Therefore, efforts might be initiated to find an alternative for the currently used insecticides to control mosquito-borne diseases.

The use of botanical derivatives in mosquito control as an alternative to synthetic insecticides currently used in the world offers a more environmentally-safe method of insect control than the use of synthetic chemicals. Herein, we reported that *P. totara* extract was active against mosquito larvae, and totarol [1] (Fig. 1) was the most active constituent in the extract.

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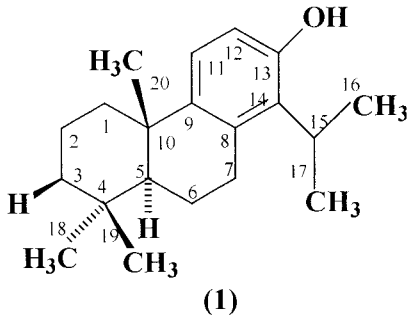

(1)

Fig. 1. Structure of totarol (1).

Materials and Methods

Plants and insects. The root bark of *P. totara* was collected at Royal Botanic Garden in Sydney, Australia in 1998. Second- and fourth-instar *Culex pipiens pallens* Coquillett (F-256) were acquired from Korea Food and Drug Administration (KFDA), Korea for use in this study.

Extraction and isolation. The dried root bark (1 Kg) of *P. totara* was crushed and extracted twice with methanol (4 L) at room temperature and filtered (Toyo filter paper No. 2, Japan). The combined filtrate was evaporated *in vacuo* at 40°C to yield about 10.4% (based on the weight of the root bark). The extract (20 g) was sequentially partitioned into hexane (2.4 g), chloroform (4.6 g), ethyl acetate (3.2 g), and water-soluble (9.8 g) portions for subsequent bioassay against *C. pipiens.* The organic solvent portions were concentrated to dryness by rotatory evaporation at 40°C, and the water portion was freeze-dried. The active hexane portion was chromatographed on a silica gel column (Merck 70-230 mesh, 300 g, 4.5 i.d. × 60 cm) and successively eluted with hexane-ethyl acetate, ethyl acetate, and ethyl acetate-methanol. The active fractions eluted with hexane-ethyl acetate (10:1) were chromatographed on a silica gel column and eluted with hexane-ethyl acetate (15:1). Column fractions were collected and analyzed by TLC (hexane-ethyl acetate, 15:1). Fractions with a similar TLC pattern were combined. For further separation of the mosquito larvicidal substance(s), a Waters Delta Prep 4000 HPLC (Waters Co., Milford, MA) was used. The column was a 29 i.d × 300 mm Bondapak C18 (Waters Co., Milford, MA) using methanol-water (3:7) at a flow rate of 7 mL/min and detection at 260 nm. Compound 1 (5.4 mg) was isolated.

Structural determination of the active isolate was based on spectral analysis. Electron impact mass spectra (EI-MS) were obtained by gas chromatography-mass spectrometry (GC-MS). GC-MS was performed on Shimadzu CLASS 5000 system (Shimadzu Scientific Instruments Inc., Rydalmere, New South Wales, Australia) interfaced with Shimadzu QP-5000 GC/MS system fitted with a capillary column (CBP-5, 25 m × 0.25 mm i.d.). Chromatography conditions were as follows: column temperature, raised from 70°C to 80°C at 1°C min^{-1}, to 190°C at 10°C min^{-1}, and then to 250°C at 30°C min^{-1}; injector temperature, 250°C; detector temperature, 280°C; carrier gas, He at 30 cm min^{-1}. ^{1}H- (400MHz) and ^{13}C-NMR (100MHz) spectra were

measured in DMSO-d_6 at room temperature on a Bruker AMX-400 (Bruker Instruments Inc., Fremont, CA) with TMS as internal standard. Mass spectra on a JEOL JMS-DX30 spectrometer (JEOL Ltd., Seoul, South Korea).

Bioassay. All bioassays were conducted in an environmentally-controlled room maintained at 30 ± 1°C and 80 ± 5% RH. Larval testing was done on acetone serial dilutions of totarol. One hundred mililiters of each test solution was placed into a Pyrex dish (100 by 50 mm) (Fisher Scientific, Pittsburgh, PA) along with 30 second or fourth instars. Each dilution along with an untreated control group (acetone carrier and water) was replicated three times. Mortality recorded at 24 h after treatment, and median lethal concentration (LC_{50}) and LC_{95} values were calculated by probit analysis (Finney 1971). Control mortality was accounted for by Abbott's (1925) formula.

Results and Discussion

The methanol extract of *P. totara* collected in Australia was larvicidal to second-instar *C. pipiens* up to 8000 µg/mL for 100% mortality. A bioassay-directed fractionation of the extract resulted in the isolation of an active compound, totarol, as shown in Fig. 1.

Totarol. MS *m/z* (rel.int.): 286[M] + (43), 271 (100), 256 (15), 243 (13), 236 (17), 229 (12), 215 (9), 201 (31); ^{1}H NMR (DMSO-d_6): δ7.10 (1H, *d,* H-11), 7.01 (1H, *d,* H-12), 3.46 (1H, *br,* H-15), 3.04 (1H, *dd,* H-7β), 2.81 (1H, *ddd,* H-7α), 2.25 (1H, *br d,* H-1β), 1.89 (1H, *dd,* H-6α), 1.67 (3H, *d,* H-17), 1.64 (3H, *d,* H-16), 1.49 (1H, *ddd,* H-2α), 1.40 (1H, *br d,* H-3β), 1.32 (1H, *ddd,* H-1α), 1.26 (1H, *d,* H-5), 1.21 (3H, *s,* Me-20), 1.14 (1H, *ddd,* H-3α), 0.93 (3H, *s,* Me-18), 0.89 (3H, *s,* Me-19); ^{13}C NMR (DMSO-d_6) δ 19.82 (C-6), 19.88 (C-2), 20.72 (C-16), 20.76 (C-17), 21.68 (C-19), 25.53 (C-20), 28.08 (C-15), 29.22 (C-7), 33.35 (C-18), 37.93 (C-10), 40.03 (C-1), 41.90 (C-3), 50.11 (C-5), 115.11 (C-12), 123.07 (C-11), 131.72 (C-14), 133.77 (C-8), 141.91 (C-9), 154.99 (C-13).

A toterane diterpene, totarol, is the major constituent of the heartwood of *P. totara.* Its structure was confirmed by Barltrop and Rogers (1958). Further studies on *P. totara* showed that the constituents of the plant were totarol, 16-hydroxytotarol, sugiol, podocarpic acid, methyl podocarpate, pododacric acid, β-sitosterol, and two unidentified compounds (Cambie and Mander 1962). Totarol shows potent antimicrobial activity (Kubo et al. 1992); the mode of antimicrobial action of totarol has been determined to be inhibition of the electron transport chain, especially near the CoQ complex (Haraguchi et al. 1996). We found that totarol had potential larvicidal-activity against the second- and fourth-instar *C. pipiens* with the 24 h LC_{50} values of just 0.25 and 0.37 µg/mL, respectively. The activity increased when bioassays were extended to 48 h as shown in Table 1. This LC_{50} values of totarol was 25-fold, 106-fold, and 240-fold stronger than that of obacunone, nomilin, and limonin, respectively (Jayaprankasha et al. 1997). Two recent reports have shown that an extract of *Tagetes minuta* L. had the strong biocidal effect on both of the larvae and adults of *Aedes aegypti* L. and *Anopheles stephensi* Liston (Perich et al. 1994), and the insecticidal components isolated from the plant extract were four thiophenes, 5-(but-3-ene-1-ynyl)-2,2′-bithiophene, 5-(but-3-ene-1-ynyl)-5′-methyl-2,2′-bithiophene, 2,2′,5′,5″-terthiophene, and 5-methyl-2,2′,5′,2″-terthiophene (Perich et al. 1995). This plant extract and the isolated thiopenes may be replacements for the currently used larvicides and potentially contribute to the development of a new biorational insecticide

Table 1. Regression parameters for mortality response of two different instars of *Culex pipiens* exposed to totarol (dose in mg/L)

Instar, (h)	LC_{50}	FL	LC_{95}	FL	Slope
2nd, (24)	0.25	0.21-0.32	0.55	0.51-0.60	2.08
(48)	0.042	0.036-0.049	0.12	0.09-0.16	2.49
4th, (24)	0.37	0.32-0.43	1.38	0.75-1.88	1.17
(48)	0.13	0.11-0.18	0.54	0.45-0.77	2.01

FL, fiducial limits (95%).

against target mosquitoes. We suggest totarol as a lead compound for the synthesis of new larvicidal agents as well.

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