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ORIGINAL ARTICLE

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Termite repellent sesquiterpenoids from *Callitris glaucophylla* heartwood

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Abstract Fractions of methanol and ethanol extracts from the heartwood of white cypress pine (*Callitris glaucophylla* Thompson et Johnson) were investigated for their repellent activity against subterranean termite *Coptotermes formosanus* Shiraki worker using a two-choice semicircular filter paper test at 0.5% (w/w) concentration. Fraction CY-E2 composed of (−)-citronellic acid, guaiol, α-, β-, and γ-eudesmol isomers as well as an unknown compound, showed the highest statistically significant repellency (97.8% ± 2.2 SEM) of all fractions tested. Bioactivity-guided fractionations using high-performance liquid chromatography led to the isolation of two, oxygenated eudesmane-type sesquiterpenes with α-methylene moieties, both termite-repellent compounds. These compounds were subsequently identified as ilicic acid methyl ester (IAME) and costic acid by means of spectroscopic analyses, electron impact mass spectrometry, and nuclear magnetic resonance spectroscopy. We report the isolation of both IAME and costic acid from *C. glaucophylla* heartwood for the first time.

Key words *Callitris glaucophylla* · Termite repellent activity · Sesquiterpene · Ilicic acid methyl ester · Costic acid

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Introduction

Callitris glaucophylla Thompson et Johnson, also called white cypress pine, grows naturally, especially in the eastern forest region south of the tropic of Capricorn in Australia.[1] Its timber has been used as building material for decks, groundsills, and outside walls because of its biological durability. Australia is known to be one of the most termite-infested countries and particulary by the termite pest *Coptotermes lacteus* Froggatt. Nevertheless, many old Australian houses built of *Callitris* species wood still stand.[2] Over the last four decades, several scientific reports on *Callitris* species have attempted to elucidate their antitermitic and antifungal activities. In the extracts of *Callitris columellaris* (−)-citronellic acid was found to be highly toxic to the termite *Nasutitermes exitiosus* Hill as well as the fungi *Coniophora olivacea* (Fr.) Karst. and *Coriolus sanguineus* (L.) G. Cunn. Moreover, eudesmols in the same extracts were recognized as potent fungicides.[3] It was also reported that the neutral fraction of the petroleum ether extract from *C. columellaris* contained mainly guaiol, β-eudesmol, and unknown sesquiterpene lactones. Furthermore, it was the major fraction responsible for repelling the termite *Mastotermes darwiniensis* Froggatt.[4,5] The composition of most monoterpenoids and sesquiterpenoids found in wood extracts of *Callitris* species have only recently been reviewed in detail.[6,7] No report has thus far dealt with the contribution of minor constituents of such wood extracts to the overall antitermitic activity of *Callitris* species.

In a previous study, we reported that a sesquiterpene γ-lactone columellarin from *C. glaucophylla* had termiticidal properties against the subterranean termite *Coptotermes formosanus* Shiraki worker.[8] Such a sesquiterpene lactone possesses an α-methylene γ-lactone moiety that is reportedly more effective in conferring various biological activities such as repellency against other insects, killing function against cytotoxic T lymphocyte,[9] and antihyperlipidemic activity,[10] in comparison with corresponding compounds having an α-methyl γ-lactone moiety. Similarly, our study demonstrated that columellarin had higher termiticidal

activity against *C. formosanus* than dihydrocolumellarin. The latter compound possesses an α-methyl structure. However, neither compound from *C. glaucophylla* heartwood extracts had termiticidal activity as high as that of the commercial antitermitic agent alkyl ammonium chloride.[8]

In the current study, we evaluate the repellent potential of several fractions of *C. glaucophylla* heartwood extracts against *C. formosanus* worker and describe the isolation and identification of a termite repellent sesquiterpenic acid and its methyl ester.

Experimental

Plant material

A *Callitris glaucophylla* tree (ca. 100 years old with a 30 cm diameter), was collected from a sawmill at Cecil Plains, Queensland (Australia). Its rectangular timber material, dried by natural seasoning, was imported into Japan by Koyamashoten Co. Ltd. and was stored at room temperature until processing in our laboratory.

Extraction and fractionation

An air-dried heartwood block (10 × 10 × 50 cm) of *C. glaucophylla* was chipped using a 10 mm diameter edge drill. The obtained wood chip (100 g) was extracted with 1 liter of methanol or ethanol for 24 h at room temperature in the dark. The solvents were subsequently evaporated in vacuo at 35°C yielding oily extractives designated CY-M (8.35 g) and CY-E (6.70 g) for methanol and ethanol, respectively. Extractive CY-M (ca. 1.0 g) was then subjected to preparative high-performance liquid chromatography (HPLC) at room temperature on a glass column (8 mm i.d. × 300 mm) packed with octadecyl silica (ODS) gel (Develosil Lop-ODS, Nomura Chemical, Aichi, Japan) and eluted with 3 ml/min of isocratic 80% aqueous methanol. Peaks detected at 210 nm were collected as fractions. Fraction solvents were subsequently evaporated in vacuo at 35°C to yield six residual fractions of CY-M that were designated CY-M1 (11.7%), CY-M2 (19.8%), CY-M3 (14.9%), CY-M4 (16.9%), CY-M5 (9.6%), and CY-M6 (11.6%). Similarly, CY-E (ca. 1.2 g) was subjected to preparative HPLC at room temperature on the same sized column packed with silica gel (Develosil Spherical Porous Silica Lop 60, Nomura Chemical) and eluted with 1 ml/min of isocratic dichloromethane : ethanol (49 : 1). Peaks detected at 210 nm were collected as fractions and the solvents evaporated in vacuo at 35°C to yield four residual fractions of CY-E that were designated CY-E1 (26.4%), CY-E2 (22.0%), CY-E3 (21.7%), and CY-E4 (18.0%). Furthermore, fraction CY-E2 (ca. 250 mg) was rechromatographed by eluting with 2 ml/min of isocratic 80% aqueous methanol on the ODS-HPLC system to give four residual subfractions of CY-E2, namely, CY-E2-1 to -4 with percentages relative to CY-E2 of 15.7%, 5.4%, 3.0%, and 56.2%, respectively.

Gas chromatography/mass spectrometry analysis

Analyses of the fractions and subfractions were performed on a Shimadzu QP5050A gas chromatography/mass spectrometry (GC/MS) system operated under the following conditions; column: DB-5ms (J&W Scientific) fused silica capillary column (30 m long × 0.25 mm × 0.25 μm film thickness composed of 5% phenylmethylpolysiloxane) connected to a quadrupole detector operated in electron impact (EI) mode (70 eV); carrier gas: helium (40 ml/min); injector and interface temperatures were set at 250°C; split ratio: 1/25; oven temperature program: 80°C (isothermal for 2 min) with an increase of 5°C/min to 250°C (isothermal for 9 min).

Isolation and identification of compounds **1** and **2**

Compound **1** (20.4 mg) was isolated from fraction CY-M4 (150 mg) using a HPLC equipped with an aqueous gel permeation chromatography (GPC) column (Shodex Asahipak GF-310HQ, 7.6 mm i.d. × 300 mm, Showa Denko, Tokyo, Japan) eluting with 0.8 ml/min of isocratic 80% aqueous methanol and detection at 210 nm. Compound **2** (47.9 mg) was isolated from subfraction CY-E2-4 (ca. 120 mg) using the same procedure mentioned above and the residue was designated as CY-E2-4r. Proton nuclear magnetic resonance (^{1}H NMR), homonuclear chemical shift correlation spectroscopy (^{1}H-^{1}H COSY), homonuclear Hartmann-Hahn spectrum (HOHAHA), nuclear Overhauser enhancement spectroscopy (NOESY), ^{13}C NMR, distortionless enhancement by polarization transfer (DEPT), ^{13}C-^{1}H COSY, ^{1}H-detected multiple-bond heteronuclear multiple quantum coherence (HMBC), and correlation spectroscopy via long-range coupling (COLOC) spectra of both isolated compounds were recorded on a JEOL JNM alpha-500 spectrometer, using $CDCl_3$ as solvent with tetramethylsilane (TMS) as internal standard.

*Ilicic acid methyl ester (**1**)*

EIMS (70 eV) *m/z*: 266 $[M]^+$, $C_{16}H_{26}O_3$. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 0.91 (3H, H-14), 1.10 (3H, H-15), 1.13 (1H, H-1), 1.22 (1H, H-6), 1.27 (1H, H-9), 1.33 (1H, H-5), 1.36 (1H, H-3), 1.40 (1H, H-1), 1.43 (1H, H-8), 1.44 (1H, H-9), 1.57 (1H, H-2), 1.58 (1H, H-2), 1.60 (1H, H-8), 1.81 (1H, H-3), 1.92 (1H, H-6), 2.52 (1H, H-7), 3.75 (3H, O-Me), 5.56 (1H, H-13), 6.13 (1H, H-13). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 18.7 (C-14), 20.1 (C-2), 22.5 (C-15), 26.4 (C-6), 27.3 (C-8), 34.6 (C-10), 40.5 (C-7), 40.9 (C-1), 43.4 (C-3), 44.5 (C-9), 51.7 (O-Me), 55.0 (C-5), 72.1 (C-4), 122.4 (C13), 145.7 (C-11), 167.8 (C-12).

*Costic acid (**2**)*

EIMS (70 eV) *m/z*: 234 $[M]^+$, $C_{15}H_{22}O_2$. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 0.75 (3H, H-14), 1.30 (1H, H-6), 1.35 (1H, H-9), 1.42 (1H, H-1), 1.43 (1H, H-1), 1.46 (1H, H-8), 1.50 (1H, H-9), 1.60 (1H, H-2), 1.62 (1H, H-2), 1.65 (1H, H-8),

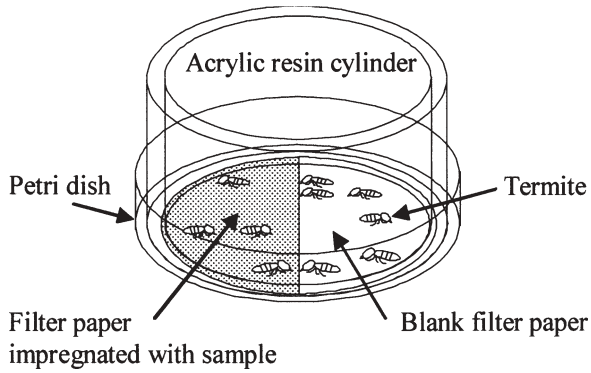


Fig. 1. Repellent activity test equipment against *Coptotermes formosanus* composed of two semicircular filter papers, a glass Petri dish bottom and an acrylic resin cylinder

1.66 (1H, H-6), 1.90 (1H, H-5), 2.01 (1H, H-3), 2.31 (1H, H-3), 2.54 (1H, H-7), 4.42 (1H, H-15), 4.72 (1H, H-15), 5.70 (1H, H-13), 6.33 (1H, H-13). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 16.4 (C-14), 23.4 (C-2), 27.2 (C-8), 29.9 (C-6), 35.9 (C-10), 36.8 (C-3), 39.2 (C-7), 41.0 (C-9), 41.8 (C-1), 49.8 (C-5), 124.9 (C-13), 105.5 (C-15), 145.2 (C-11), 150.6 (C-4), 172.9 (C-12).

Repellent activity test

Repellency of each sample was assayed using a short-term filter paper test under dry conditions as shown in Fig. 1. The test procedure was a modification of the long-term wet condition test method of Ohtani et al.[11] The crude extractives (CY-M and CY-E), prepared fractions and subfractions (CY-M1 to -M6, CY-E1 to -E4, CY-E2-1 to -4), compound **1**, and **2**, as well as authentic compounds: (−)-citronellic acid [(−)-CA, Aldrich, Milwaukee, WI, USA], geranic acid (GA, Aldrich), guaiol (GU, crude crystal separated from *C. glaucophylla*), β-eudesmol (β-EU, Wako Pure Chemical, Osaka, Japan), dihydrocolumellarin (DCO, pure crystal isolated from *C. glaucophylla*), and columellarin (CO, pure crystal isolated from *C. glaucophylla*) were dissolved in ethanol at a concentration of 23.6 mg/ml. A 50-μl aliquot (1.18 mg dissolved solids) of sample solution was diluted in 1 ml of diethyl ether. This test sample solution was distributed dropwise all over a semicircular filter paper (Advantec No. 3, 70 mm diameter) with an average weight of 0.24 g to give 0.5% (w/w) solids to each filter paper half. Soaked filter papers were air-dried for a few minutes using a blower. Antitermitic agents, namely, the commercial wood preservative alkyl ammonium chloride (AAC, Dainihon Wood Preserving, Aichi, Japan) and wood oil (HO, Tohosangyo, Mie, Japan) prepared from Japanese *Cupressaceae* species hinoki (*Chamaecyparis obtusa* Endl.) by steam distillation were used in filter paper halves to serve as positive test controls. Solvent controls were also prepared in the same way by treatment with ethanol and diethyl ether. Blank tests were conducted with the use of a pair of the blank filter paper halves. An impregnated/control filter paper half was subsequently joined with a blank filter paper half to make a circular filter paper that was placed in a glass Petri dish. To prevent warping, an acrylic resin cylinder (60 mm i.d. × 50 mm) was laid on the outer edge of each combined filter paper half. Workers of *Coptotermes formosanus* were used as test insects. These were obtained from a laboratory colony maintained for more than 10 years at the Research Institute for Sustainable Humanosphere, Kyoto University, Japan. Ten workers of *C. formosanus* were haphazardly placed at the center of each Petri dish. The Petri dishes were then placed in an incubator with an observation window at 28°C for 30 min. After every 10 min, the number of termites on each filter paper was counted. Three replicates were performed for each sample. The repellent activities of the samples were calculated using Eq. 1.

$$\text{Repellent activity}\,(\%) = \left[(B - S)/A\right] \times 100, \qquad (1)$$

where B is the number of termites on the blank filter paper, S is the number of termites on the sample filter paper, and A is the number of termites under test.

Statistical comparisons of repellent activities between fractions or compounds were made by using a *t*-test that indicated significant differences from the control test with the blank semicircular filter paper at 0.01 and 0.05 levels.

Results and discussion

The yields of the methanol and ethanol extracts of *Callitris glaucophylla* heartwood were 8.4% and 6.7% (w/w), respectively. The methanol extracts were fractionated by ODS column chromatography whereas those of ethanol were fractionated by silica gel column chromatography. GC/MS analysis using a DB-5ms capillary column revealed the accomplishment of crude separation from each extract according to its polarity. Table 1 shows the relative composition of each essential oil fraction. Identification of the constituents in each fraction was determined by the comparison of their mass spectra and fragmentation patterns with those of authentic compounds in the NIST mass spectral database (1998 Edition for CLASS Software, National Institute of Standards and Technology).

The repellent activities of CY-M, -M1 to -M6, CY-E, -E1 to -E4, CY-E2-1 to -4, and CY-E2-4r were evaluated by the choice repellent test against *Coptotermes formosanus* using two joined semicircular filter papers (Fig. 2). Predominantly essential oil fractions CY-M4 and CY-E2 exhibited extremely high repellent activity (85.0% ± 8.2 SEM; P = 0.002 and 97.8% ± 2.2 SEM; P = 0.001, respectively). Fractions CY-M1 and -E4 exhibited negligible activity. The components of both fractions were undetectable by GC/MS analysis in this study and absorbed at the benzenoid band in ultraviolet spectral range. Our presumption was that these fractions consisted of mainly phenolic compounds. Activity of CY-E2 was remarkably higher than that of the commercial antitermitic agent AAC. The CY-M4 fraction mainly

Table 1. Relative composition of fractions from *Callitris glaucophylla* extracts

RI[a]	Compound	CY-M	M3	M4	M5	M6	E	E1	E2	E3	E2-1	E2-2	E2-3	E2-4	E2-4r
1314	(−)-CA	19.0	19.4				18.5		28.1	47.7		83.6	2.5		
1347	Neric acid	0.9					0.8		1.8	3.4		5.4			
1411	GA	3.1					4.2	4.1	0.7		10.8	0.1			
1472	Eudesma-1,4(15),11-triene	0.3					0.8								
1488	β-Selinene	0.7					0.6	1.0							
1495	α-Selinene	0.5					0.4	0.5							
1596	GU	10.0				55.5	9.5	0.7	17.2	2.3				23.6	39.8
1631	γ-EU	1.4				5.2	1.3		2.5	0.9			2.7	4.0	7.3
1655	β-EU	7.1				19.9	7.6		14.8	4.3			2.7	22.8	38.7
1664	α-EU	5.1				19.4	5.2		5.7	7.1			1.5	7.5	12.4
1798	Unknown 1	1.1	7.7				1.2	2.1							
1817	Unknown 2	6.4	31.3	12.0			5.7	9.2							
1862	Compound **2**	2.0	9.0	7.0			3.3		23.5	7.7				36.8	
1899	DCO	7.6			52.4		7.4	15.9							
1907	Unknown 3	1.4		4.7			1.4	4.0	0.9				26.4		
1935	Unknown 4	4.4		13.5			4.6	9.3	0.2				2.2		
1939	Unknown 5	4.6	22.7	12.6			4.4	8.1	0.2				4.4		
1948	CO	11.9			47.6		11.1	28.2	0.9		58.9	0.2	7.4		
1957	Unknown 6	2.0	5.1	9.6			2.7	6.1							
1966	Compound **1**	5.3		15.3			4.9		3.3	19.8		1.0	12.9		
1972	Unknown 7	3.7		14.4			3.4	6.3	0.4				4.8		

Results are given as percentages in each fraction
(−)-CA, (−)-citronellic acid; GA, geranic acid; GU, guaiol; EU, eudesmol; DCO, dihydrocolumellarin; CO, columellarin
[a]Retention indices on DB-5ms column

contained compounds **1** and **2** and six unknown oxygenated sesquiterpenes with molecular masses ranging from *m*/*z* 220 to 266 as determined by EIMS. Although fraction CY-M3 contained several constituents (unknowns 2, 5, 6, and compounds **1** and **2**) that were also present in fraction CY-M4, it exhibited relatively lower activity. Disregarding potentiation, synergistic, and/or antagonistic effects, the constitutents (i.e., unknowns 3, 4, and 7 and compound **1**) that were not common between CY-M3 and CY-M4 may account for the observed activity differences between the two fractions. We therefore isolated compound **1**, the most predominant constituent (0.19% of air-dried *C. glaucophylla* heartwood) of the relevant components of CY-M4, and subsequently identified it as ilicic acid methyl ester (IAME, Fig. 3). The NMR spectra of compound **1** were consistent with literature data on IAME.[12,13] On the other hand, fraction CY-E2 consisted mainly of (−)-CA, GU, α-, β-, and γ-eudesmol (EU) isomers as well as compound **2**. Furthermore, fraction CY-M6, composed of a mixture of GU and the (α-, β-, γ-) EU isomers, showed lower activity than fraction CY-E2. These results pointed to the possibility that the rest of the constituents (i.e., (−)-CA and/or **2**) of fraction CY-E2 enhanced its activity. Eventually, we identified compound **2** (0.32% of air dried *C. glaucophylla* heartwood) as costic acid (Fig. 3). Its mass fragments were consistent with literature data.[14,15] Ilicic acid methyl ester has been isolated from *Ambrosia ilicifolia* (Gray) Payne,[12] *Artemisia herba-alba* subsp. *valentina* Lam.,[13] *Artemisia phaeolepis* Krasch.,[16] and *Tithonia diversifolia* (Hemsl.) A. Gray,[17] whilst costic acid has been isolated from costus root oil (*Saussurea* spp.),[14] *Eupatorium capillifolium* (Lam) Small,[15] and *Artemisia hedinii* Ostenf.[18] We report herein the isolation of both compounds from *C. glaucophylla* heartwood for the first time.

Figure 2 shows results of the repellent activity test against *C. formosanus* for IAME and costic acid in addition to six authentic compounds present in *C. glaucophylla* heartwood extracts. IAME was previously reported as a strong deterrent agent against storage pests such as *Sitophilus granarius* (Linnaeus) adult, *Trogoderma granarium* Everts larva, and *Tribolium confusum* Jacquelin du Val larva and adult.[19] This compound demonstrated high repellency (66.7% ± 7.5 SEM) against *C. formosanus*, but its individual activity was lower than that of the parent fraction CY-M4 in this test. Costic acid was reported as a strong repellent against another storage pest *Tribolium castaneum* (Herbst) larva and as an inducer of different kinds of deformities including severe deficiencies in development of the cephalic capsule, lack of cuticle deposition, and abnormal disposition of legs and wings.[20] In this experiment, costic acid exhibited termite repellency (68.9% ± 10.1 SEM) equivalent to that of IAME, GA, DCO, and CO, while (−)-CA, GU, and β-EU showed even higher repellency individually.

In conclusion, whole terpenoid fractions of *C. glaucophylla* heartwood extracts consisting of monoterpene carboxylic acids, sesquiterpene alcohols, sesquiterpene carboxylic acids and their derivatives, and sesquiterpene lactones showed repellent activity against *C. formosanus*. The bioactivity-guided fractionations yielded IAME and costic acid as termite repellent constituents. It was confirmed that the antitermitic activity of both compounds compared reasonably with that of hinoki wood oil and exceeded that of AAC used as positive controls. We have isolated both compounds from *C. glaucophylla* heartwood for the first time. In our previous article,[8] none of the fractions and compounds of *C. glaucophylla* heartwood extracts had termiticidal activity higher than that of the commercial

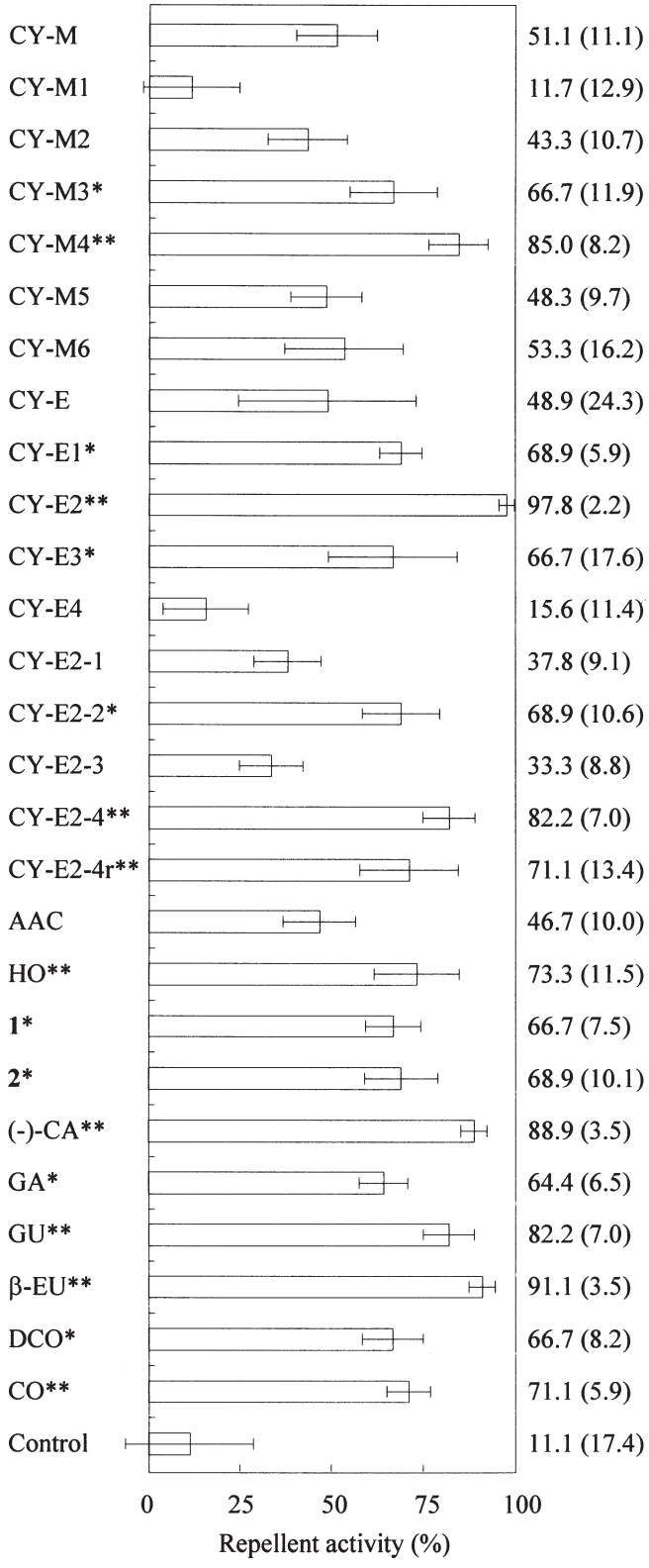
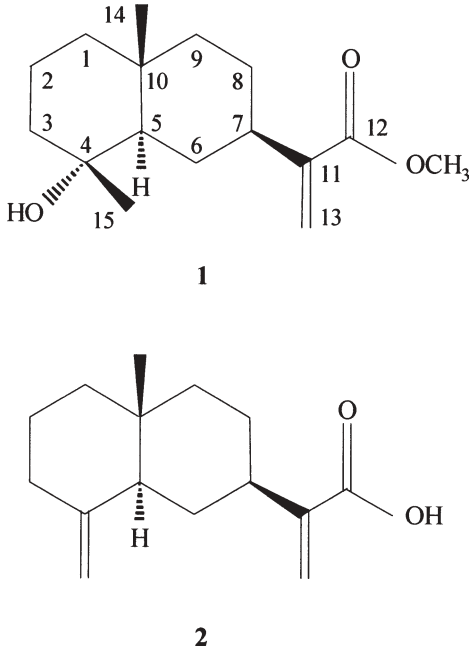


Fig. 2. Repellent activity of fractions from *Callitris glaucophylla* methanol or ethanol extracts, compounds **1** and **2**, and authentic compounds against *C. formosanus*. *Error bars* indicate ± SEM of 9 replications. *Asterisks* denote the significant differences from the control at *: $P < 0.05$, **: $P < 0.01$, respectively. Values given are means with SEM in parentheses. *AAC*, alkyl ammonium chloride; *HO*, hinoki steam-distilled oil



1

2

Fig. 3. Structures of **1** and **2**

antitermitic agent AAC. However, in this study we have found extremely high repellent fractions relatively superior to AAC against *C. formosanus* in the extracts of *C. glaucophylla* heartwood. The durability of *C. glaucophylla* could thus be attributed to its repellency rather than to toxicity.

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