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Biological performance of *Eucalyptus camaldulensis* leaf oils from Thailand against the subterranean termite *Coptotermes formosanus* Shiraki

Received: April 16, 2008 / Accepted: August 20, 2008

Abstract The antitermitic activities of leaf oils and their constituents, taken from three clones of *Eucalyptus camaldulensis* Dehnh. in Thailand, against *Coptotermes formosanus* Shiraki were investigated in contact and noncontact tests. The termiticidal mechanism was also examined. Antitermitic tests demonstrated that *E. camaldulensis* leaf oils were both contact toxicants and fumigants to *C. formosanus* with LC₅₀ values ranging between 12.68 and 17.50 mg/g by the contact method, and between 12.65 and 17.50 mg/petri dish (100 cm³) by the noncontact method. *p*-Cymene and γ -terpinene were primarily responsible for the contact toxicity and 1,8-cineole was responsible for fumigation. From the investigation of termiticidal mechanism, *E. camaldulensis* leaf oils exhibited the inhibition of acetylcholinesterase activity and showed the common symptoms of a neurotoxic mode of action against *C. formosanus*.

Key words Antitermitic activity · *Coptotermes formosanus* Shiraki · Essential oil · *Eucalyptus camaldulensis*

Introduction

Termites seriously damage a variety of materials, not only wooden products but also noncellulosic materials. Damage to wooden structures and other cellulosic materials caused by termites has been estimated to exceed \$3 billion annually in the United States.¹ Among many species of termites, *Coptotermes formosanus* Shiraki is the subterranean termite responsible for most destruction of wooden materials in

countries such as Japan, Taiwan, and southern parts of the United States. Thus, it has been used for bioassays in many research studies.²⁻⁴

Synthetic chemicals are currently used for controlling termites. However, the conventional termiticides, such as aldrin, chlordane, dieldrin, endrin, heptachlor, and mirex, which are the chemicals identified as persistent organic pollutants (POPs), have been known to cause negative impacts on global environments, including health hazards to humans and other nontarget species. The application of these chemicals now tends to be restricted.⁵ To avoid those impacts, there is a trend toward identifying naturally occurring pesticides. Many phytochemicals are known to possess antitermitic or repellent activities. Among them, plant essential oils may provide potential alternatives to termite controlling agents currently in use because they constitute a rich source of bioactive chemicals. Recently, essential oils have been the subject of increasing attention as sources of effective and safe insecticides in which the main components are monoterpenes and sesquiterpenes, and some of them have been found to be termiticidal.^{6,7}

Eucalyptus camaldulensis Dehnh. is the most well-known *Eucalyptus* species for cultivation in Thailand. It is mainly planted for use as pulpwood and utilized as the main raw material even at ages of 3–5 years because of the high growth rate. During the process of papermaking, large amounts of waste such as leaves are generated and disposed of. The utilization of these waste materials is crucial from an ecological viewpoint. Siramon and Ohtani⁸ found that the leaf essential oils of *E. camaldulensis* from Thailand have antioxidant properties, and suggested the potential of these materials as alternatives to synthetic antioxidants.

In this study, we investigated the bioactivity of the essential oils from *E. camaldulensis* leaves against *C. formosanus*. The antitermitic activities of the essential oils and each component against *C. formosanus* were examined using two different testing methods, namely, contact and noncontact methods. The termiticidal mechanism of these essential oils and their main constituents were also partly investigated.

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Part of this report was presented at the 58th Annual Meeting of the Japan Wood Research Society, Tsukuba, March 2008

Materials and methods

Chemicals

Acetylthiocholine iodide, dithiobisnitrobenzoic acid, α -pinene, α -terpineol, terpinen-4-ol, 1-decanol, methanol, and phosphate buffer solution were purchased from Nacalai Tesque (Kyoto, Japan). 1,8-Cineole was purchased from Wako (Osaka, Japan). *p*-Cymene and γ -terpinene were purchased from Tokyo Kasei Kogyo (Tokyo, Japan).

Preparation of essential oil

Three clones S1, S2, and S3 of *Eucalyptus camaldulensis* were selected through several generations based on their pulp yields and adaptability to circumstance, etc. by Siam Forestry, Kanchanaburi Province, Thailand. The leaves of each clone were collected in mid-April (summer season) 2007.

About 1 kg of *E. camaldulensis* fresh leaves was extracted by water and steam distillation for 5 h (until no more essential oil was obtained). The essential oils were collected, dried over anhydrous sodium sulfate, and stored in sealed vials at low temperature before analysis. The moisture content of the leaves was determined by the weights before and after drying at 105°C for 12 h in an oven.

Gas chromatography-mass spectrometry analysis

The chemical compositions of the essential oils were analyzed by gas chromatography-mass spectrometry (GC-MS). The analysis used a GC-17A gas chromatograph (GC) coupled to a QP5050A mass spectrometer (Shimadzu, Kyoto, Japan), and the GC used a fused-silica capillary column TC-1 (0.25 mm i.d. \times 15 m, 0.25 μ m film thickness; GL Sciences, Tokyo, Japan) as described by Siramon and Ohtani.⁸ Identification of the compounds was based on comparison of GC-MS data with the NIST database library, and most compounds such as *p*-cymene, γ -terpinene, α -pinene, 1,8-cineole, terpinen-4-ol, and α -terpineol obtained as reagents were directly compared with authentic compounds. The calculated chemical compositions of the oils were based on the peak areas of GC chromatogram using 1-decanol as an internal standard.

Antitermitic activities

Coptotermes formosanus was collected in May 2007 and June 2008 from fields in Kochi Prefecture, Japan. The termites collected were reared in an incubator at 25°C. Distilled water and filter papers were used as foodstuffs.

The antitermitic activities of the essential oils and their constituents against the termites were examined using contact and noncontact methods. The contact method developed by Ohtani et al.⁹ with some modification was employed to evaluate the contact toxicity of the *E. camaldulensis* leaf oil. Each oil sample (10, 15, or 20 mg) was

dissolved in 1 ml of methanol and then applied to 1 g of filter paper (Advantec No.2, 8.7 cm in diameter). A piece of filter paper treated with methanol only was used as a control. After the solvent was removed from the treated filter papers by air drying at ambient temperature, 30 active termites (28 workers and 2 soldiers) above the third instar were placed on each piece of filter paper in a petri dish (8.7 cm in diameter \times 1.7 cm in height). Distilled water was added occasionally onto each filter paper and the covered petri dishes were incubated in darkness at 25°C. Three replicates were made for each test sample. The mortality of the termites was counted daily for 14 days and the 50% lethal concentrations (LC₅₀) were estimated from linear correlation between the sample concentrations and termite mortality.

The noncontact method developed by Ohtani et al.¹⁰ was employed to distinguish the respiratory poisoning functions from termiticidal activity of essential oils. A small glass dish (2.8 cm in diameter \times 1.5 cm in height) in which a treated paper disk with either 10 mg or 20 mg of essential oils was held, was placed on the center of a filter paper in a larger petri dish (100 cm³ in volume). Twenty-eight workers and two soldiers were introduced into the outer part from the inner small dish and these samples were incubated in darkness at 25°C for 7 days. Distilled water was supplied occasionally.

Termiticidal activities of each component in *E. camaldulensis* leaf oils, namely, *p*-cymene, γ -terpinene, 1,8-cineole, terpinen-4-ol, α -pinene, and α -terpineol were tested by the aforementioned two methods with the authentic chemicals.

Determination of concentration of volatile components in the headspace air

A paper disk treated with 0.1 g of oil sample was placed in a 320-ml closed bottle. The bottle was left to stand for 1 h at room temperature. The air inside the bottle was evacuated through charcoal tubes (organic gas sampler, Shibata Scientific) with a minipump (MP-Σ30, Shibata Scientific) at a flow rate of 0.2 l/min for 5 min. Thereafter, the charcoal tubes were taken out and soaked in 5 ml acetone for 12 h. The liberated volatile substances were then analyzed and quantified by GC-MS.

Analysis of the termiticidal mechanism

The termiticidal mechanism of the essential oils from *E. camaldulensis* leaves against *C. formosanus* was investigated by the three procedures described below.

Three worker termites were placed in small glass dishes, and the dishes were placed on ice for 2 min. The oil samples or their main constituents (0.4 μ l) were applied topically to the termite abdomens using a Hamilton 701-N micro-syringe (Hamilton, Reno, NV, USA) for estimation of the contact toxicity against termites. The mortality of the termites was counted daily for 3 days. The behavior of the treated termites and the damage of tissues of dead termites were

Table 1. Tree data, oil yields, and chemical compositions of three clones of *Eucalyptus camaldulensis*

Clone sample	Tree age (years)	Tree height (m)	DBH (cm)	Oil yield (%) ^a	Chemical composition of oil						Total (%) ^b
					<i>p</i> -Cymene (%) ^b	γ -Terpinene (%) ^b	1,8-Cineole (%) ^b	Terpinen-4-ol (%) ^b	α -Pinene (%) ^b	α -Terpineol (%) ^b	
S1	1	6	3.1	1.07	19.16	33.03	27.22	–	5.25	2.91	87.57
S2	1.5	6.5	3.7	2.23	17.50	42.49	33.60	3.92	–	–	97.51
S3	1	6	3.1	1.95	18.79	75.50	–	3.90	–	–	98.19

DBH, Diameter at breast height

^aBased on dry leaves^bBased on chromatogram peak areas. Dashes indicate no detection

observed with a microscopic CCD camera controlled by computer.

To clarify the effect of volatile components on the termites, three worker termites were put in a 30-ml glass vial and covered. Each piece of filter paper with oil samples of 1.2, 3.3, 5.7, 11.4 mg was hanged inside the vial without touching the termites. The behavior of the termites was observed by the same manner mentioned above.

Determination of acetylcholinesterase (AChE) activity of the termites was conducted based on a modified method of Ellman et al.¹¹ Whole bodies of termites were homogenized [approximately 20 mg of termites per milliliter of phosphate buffer (pH 8.0, 0.1 M)]. A 0.4-ml aliquot of this homogenate was added to a cuvette containing 2.6 ml of phosphate buffer (pH 8.0, 0.1 M). Dithiobisnitrobenzoic acid (DTNB) 100 μ ml was added and the absorbance of the mixture was measured at 412 nm by ultraviolet-visible (UV-VIS) spectrophotometry. Acetylthiocholine iodide (20 μ l, 0.075 M) was added. Changes in absorbance were recorded and the changes in absorbance per min were calculated. The rates of hydrolysis of acetylthiocholine iodide were calculated as follows;

$$R = \frac{\Delta A}{1.36 \times 10^4} \times \frac{1}{(400/3120)C_0} = 5.74 \times 10^{-4} \left(\frac{\Delta A}{C_0} \right)$$

where *R* is the rate in moles of substrate hydrolyzed per minute per gram of tissue, ΔA is the change in absorbance per minute, and *C*₀ is the original concentration of tissue (mg/ml).

Statistical analysis

The Scheffe multiple comparison procedure of the SAS statistical program was employed to evaluate differences in percent mortality for the antitermitic tests. Results with *P* < 0.05 were considered statistically significant. All results were obtained from three independent experiments and were expressed as mean \pm standard deviation.

Results

Essential oil yield by water distillation

The leaf essential oils of *Eucalyptus camaldulensis* were obtained with yields ranging from 1.07% to 2.23% based on

Table 2. Antitermitic activities of three *E. camaldulensis* leaf oils against *Coptotermes formosanus* by contact method after 7 and 14 days

Sample	Concentration (mg/g)	Termite mortality (%) ^a	
		7 days	14 days
S1	10	8.2 \pm 0 b	24.8 \pm 0.7 c
	15	86.7 \pm 1.2 a	97.8 \pm 0 a
	20	91.1 \pm 0.6 a	98.9 \pm 0 a
S2	10	1.1 \pm 0 b	12.2 \pm 0.6 c
	15	23.3 \pm 0 b	37.8 \pm 1 bc
	20	76.7 \pm 0.6 a	93.3 \pm 0.6 a
S3	10	8.3 \pm 0 b	18.3 \pm 0 c
	15	46.7 \pm 0 ab	73.3 \pm 1.5 ab
	20	87.8 \pm 0.6 a	98.9 \pm 0 a
Control	0	5.3 \pm 0 b	7.6 \pm 0 c

^aMeans (*n* = 3) using 30 termites per replicate. Values followed by different letters (a–c) among the samples in each day (7 and 14 days) are significantly different by the Scheffe test (*P* < 0.05)

dry leaves. The results of GC-MS analysis of these essential oils are shown in Table 1. Six compounds were identified; three were major components (γ -terpinene, *p*-cymene, 1,8-cineole) and three were minor components. Among the major compounds, γ -terpinene had the highest content in all oil samples followed by 1,8-cineole and *p*-cymene. However, 1,8-cineole was not detected in the S3 oil sample. For the minor compounds, it was observed that terpinen-4-ol was only detected in the S2 and S3 oil samples. The other minor components, α -pinene and α -terpineol, were only found in the S1 oil sample.

Antitermitic activities

The essential oils from *E. camaldulensis* leaves exhibited antitermitic activities against *Coptotermes formosanus* by both contact and noncontact methods. The antitermitic activities by contact and noncontact methods are shown in Tables 2 and 3, respectively. The activities by contact method were decreased according to the following order: S1 > S3 > S2 (termite mortality 91.1%, 87.8%, and 76.7%, respectively) at 20 mg/g after 7 days. However, they did not show any significant difference by statistical analysis. Termiticidal activity of each essential oil increased according to the dosage from 10 to 20 mg/g. S1 gave the highest termite mortality at 10 mg/g followed by S3 and S2. S2 had a relatively lower effect than the other two.

With the noncontact method, all samples were able to give higher termite mortalities at 20 mg/petri dish (100 cm³) than at 10 mg/petri dish (100 cm³). The termite mortality with S2 by the noncontact method was the highest [100% at 20 mg/petri dish (100 cm³) after 7 days] with statistical significance ($P < 0.05$), but was the lowest by the contact method. This suggests that the antitermitic function of S2 is due to its respiratory toxicity (fumigation). The LC₅₀ values of *E. camaldulensis* leaf oils against *C. formosanus* by both methods are shown in Table 4. The LC₅₀ values of *E. camaldulensis* leaf oils range between 12.68 and 17.50 mg/g for the contact method, and between 12.65 and 17.50 mg/petri dish (100 cm³) for the noncontact method.

To evaluate the effects of chemical composition of *E. camaldulensis* leaf oils on antitermitic activities, six authentic compounds detected in the oils were tested in the same manners as mentioned above. The LC₅₀ values of the six components (*p*-cymene, γ -terpinene, 1,8-cineole, α -

pinene, terpinen-4-ol, and α -terpineol) by both methods are shown in Table 5. The purity (ca. 85%) of the authentic 1,8-cineole used here was low, and this may explain why the LC₅₀ value of 1,8-cineole is relatively high compared with those of the essential oils. The results for the contact method showed that the toxicity of the major components and the minor components except α -pinene were similar, but α -terpineol and terpinen-4-ol showed quite high toxicity by the noncontact method. *p*-Cymene was the most toxic to termites among the major compounds, followed by γ -terpinene and 1,8-cineole. On the other hand, the results from the noncontact method showed that 1,8-cineole was the most toxic, followed by γ -terpinene and *p*-cymene. Among the minor compounds, α -terpineol was the most toxic to termites by both methods, followed by terpinen-4-ol and α -pinene.

Volatile components in the headspace air

The volatile components in the headspace air after keeping *E. camaldulensis* leaf oils and main constituents in the glass bottle for 1 h are shown in Table 6. 1,8-Cineole showed the highest concentration in the headspace air among the three main components. S1 and S2 had the high content of 1,8-cineole; therefore, they gave the largest concentration of volatile substances in the headspace air.

Analysis of the termiticidal mechanism

Topical treatment with 0.4 μ l (ca. 0.3 mg) of γ -terpinene on the abdomen of termites gave 100% mortality within 10 min.

Table 3. Antitermitic activities of three *E. camaldulensis* leaf oils against *C. formosanus* by noncontact method after 7 days

Sample	Concentration (mg/petri dish) ^a	Termite mortality (%) ^b
S1	10	38.3 \pm 0.7 bc
	20	60.0 \pm 0 ab
S2	10	32.0 \pm 1.2 bc
	20	100.0 \pm 0 a
S3	10	40.0 \pm 0.7 bc
	20	53.3 \pm 0 abc
Control	0	7.8 \pm 0 c

^aVolume: 100 cm³

^bMeans ($n = 3$) using 30 termites per replicate. Values followed by different letters (a–c) among the samples are significantly different by the Scheffe test ($P < 0.05$)

Table 4. LC₅₀ values of three *E. camaldulensis* leaf oils against *C. formosanus* after 7 days

Sample	LC ₅₀	
	Contact method ^a	Noncontact method ^b
S1	12.68	15.39
S2	17.50	12.65
S3	15.41	17.50

LC₅₀, 50% lethal concentration

^aData given in units of mg/g of filter paper

^bData given in units of mg/petri dish (100 cm³)

Table 5. LC₅₀ values of six constituents of *E. camaldulensis* leaf oils against *C. formosanus* by contact and noncontact methods after 7 days

Sample	LC ₅₀	
	Contact method ^a	Noncontact method ^b
<i>p</i> -Cymene	3.78	92.22
γ -Terpinene	5.89	54.28
1,8-Cineole	6.74	51.98
α -Pinene	44.86	21.33
Terpinen-4-ol	3.30	1.70
α -Terpineol	1.46	0.81

^aData given in units of mg/g of filter paper

^bData given in units of mg/petri dish (100 cm³)

Table 6. Concentration of the volatile components in the headspace air using 0.1 g of *E. camaldulensis* oils and three major constituents at 1 h

Sample	1,8-Cineole (ppm) ^a	γ -Terpinene (ppm) ^a	<i>p</i> -Cymene (ppm) ^a	Total (ppm) ^a
S1	0.335	0.016	0.025	0.376
S2	0.312	0.019	0.019	0.349
S3	0	0.004	0.002	0.006
1,8-Cineole	0.129	0	0	0.129
γ -Terpinene	0	0.021	0	0.021
<i>p</i> -Cymene	0	0	0.016	0.016

^appm in the headspace air

On the other hand, *p*-cymene and S3 gave 50% mortality within 1 day, and all termites treated with S1, S2, and 1,8-cineole were alive with the same treatment mentioned above. The toxicity of S3 in this test may be due to its high content of γ -terpinene. No damaging traces on the bodies were observed on the ventral sides where all samples were applied. This means that these chemicals do not damage the tissue of the termite body or cause inflammatory symptoms.

The behavioral observation of termites treated with the vaporized components under atmospheric conditions showed the common symptoms of a neurotoxic mode of action. The termites treated with each sample exhibited the common symptoms of convulsions, tremors (including hyperextension of legs), and knockdown within a few minutes, and then became paralyzed, moribund, and finally died within 2 h for S2, 1 day for S1 at the minimum concentration of 40 mg/l (1.2 mg in 30 ml of vial), whereas the control termites treated with ethanol were all alive. The authentic compounds of the main constituents exhibited the same symptoms as above, and all termites died within 1 h with γ -terpinene and within 1 day for *p*-cymene and 1,8-cineole at 40 mg/l.

The essential oils from *E. camaldulensis* leaves exhibited AChE inhibitory activity against *C. formosanus*, as shown in Table 7. The concentrations of AChE in all termites treated with the essential oils by contact and noncontact methods were lower than that in untreated termites (control). Especially the essential oil of S1 decreased the production of AChE from 4.46 μ M (control) to 2.73 μ M. Among the major compounds, 1,8-cineole affected the AChE activity the most. Among the minor compounds, α -terpineol showed the largest influence.

Table 7. Acetylcholinesterase activity of *C. formosanus* treated with three *E. camaldulensis* leaf oils and their constituents

Treating chemicals	Dosage	Treatment method	Concentration of acetylcholinesterase (μ M)
S1	20 ^a	Contact	4.20
S2	20 ^a	Contact	3.57
S3	20 ^a	Contact	3.17
S1	20 ^b	Noncontact	2.73
S2	20 ^b	Noncontact	4.36
S3	20 ^b	Noncontact	3.58
<i>p</i> -Cymene	20 ^a	Contact	4.57
γ -Terpinene	20 ^a	Contact	5.29
1,8-Cineole	20 ^a	Contact	3.94
α -Pinene	20 ^a	Contact	4.29
Terpinen-4-ol	20 ^a	Contact	4.23
α -Terpineol	20 ^a	Contact	4.10
<i>p</i> -Cymene	20 ^b	Noncontact	4.76
γ -Terpinene	20 ^b	Noncontact	4.64
1,8-Cineole	20 ^b	Noncontact	4.01
α -Pinene	20 ^b	Noncontact	4.77
Terpinen-4-ol	20 ^b	Noncontact	3.75
α -Terpineol	20 ^b	Noncontact	3.72
Control	0	–	4.46

^aData given in units of mg/g of filter paper

^bData given in units of mg/petri dish (100 cm³)

Discussion

It is demonstrated from the results that the essential oils from *Eucalyptus camaldulensis* leaves are both fumigants and contact toxicants against *Coptotermes formosanus*. The antitermitic functions are strongly influenced by the chemical compositions of the oils. The variations in the chemical ingredients of *E. camaldulensis* leaf oils occur according to tree age, sample size, and the leaf harvesting period. It has also been reported that the composition of essential oils from several plants could vary significantly depending on species, chemotypes, geographical origin, season, and extraction procedure.⁸ The antitermitic function of S1 and S3 are due to their contact toxicity, but S2 shows respiratory toxicity (fumigation). *p*-Cymene and γ -terpinene are primarily responsible for the contact toxicity (through direct exposure or ingestion) of *E. camaldulensis* leaf oils. However, it may be difficult to completely eliminate the fumigant toxicity from the contact toxicity, because fumigation often occurs in the contact testing method.¹² 1,8-Cineole is effective in the noncontact testing method due to fumigation, likely via the respiratory system. Lee et al.¹³ reported that 1,8-cineole was the most active monoterpene among the miscellaneous groups against the stored product insects. Essential oils and especially their main components, such as monoterpenoids, offer promising alternatives to classical fumigants.¹⁴

The compounds that inhibit or inactivate AChE cause acetylcholine to accumulate on the cholinergic site. The accumulated acetylcholine brings about continuous stimulation of cholinergic nerve fibers throughout the central and peripheral nervous system, followed by paralysis and death.¹⁵ Inhibition of AChE activity is one of the important mechanisms of insecticidal actions. Monoterpenes in several essential oils have been shown to be competitive inhibitors of AChE. Miyazawa et al.¹⁶ reported the inhibition of AChE from bovine erythrocytes by certain monoterpenoids, such as *p*-cymene and γ -terpinene. Mills et al.¹⁷ found the AChE in lice was inhibited by two major constituents (1,8-cineole and terpinen-4-ol) of tea tree oil. The essential oils from *E. camaldulensis* leaves were found to be AChE inhibitors for termites, but their termiticidal effects were not likely to be wholly explained by AChE inhibition.

Some essential oils are reported to exhibit a neurotoxic mode of action, irrespective of the routes of administration (oral, topical, respiratory, through epidermis). The common symptoms of neurotoxicity include hyperactivity, convulsions, tremors, and paralysis.³ Our observations on the termite behavior treated with vaporized *E. camaldulensis* leaf oils suggest that all oil samples at minimum doses induce a neurotoxic mode of action and finally kill all termites within a few days. The physiological actions of essential oils on insects are little known, but treatments with various essential oils or their constituents cause symptoms similar to a neurotoxic mode of action.¹⁸ Previous reports by several groups found that some essential oils inactivated an octopamine receptor binding site and disrupted cAMP production of invertebrates, and then induced symptoms

similar to those of neurotoxicity against insects.¹⁹ The termites treated with *E. camaldulensis* leaf oils seem to partly exhibit the symptoms relating to an inhibition of octopamine functions. Thus, it is assumed that these oils have multiple physiological pathways for termiticidal function. Therefore, *E. camaldulensis* leaf oil could be used as an alternative plant-derived agent for control of termites.

Acknowledgments The authors thank Siam Forestry Co. Ltd., Kanchanaburi Province, Thailand, for providing the *Eucalyptus* leaf samples.

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