Variations in insecticidal activity and chemical compositions of leaf essential oils from *Cryptomeria japonica* at different ages

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Abstract

The larvicidal effects of the essential oils extracted from the leaves of *Cryptomeria japonica* at different ages (58, 42, and 26 years old) against 2 mosquito species, *Aedes aegypti* and *Aedes albopictus*, were studied. The analysis of major constituents of these essential oils was also investigated. Results obtained from the larvicidal tests, using essential oil from the leaves of 58-year-old *C. japonica* was found to be most effective against both *A. aegypti* and *A. albopictus* larvae, indicating tree age has significant influence on mosquito larvicidal activity. In addition, the eleven pure constituents from *C. japonica* leaf essential oil were also tested individually against the two mosquito larvae. Among them, α -terpinene, γ -terpinene, *p*-cymene, 3-carene, terpinolene, and β -myrcene shows strong larvicidal effect against the two mosquito larvae. Among these pure constituents, 3-carene exhibits the best larvicidal effect against *A. aegypti* and terpinolene shows an excellent inhibitory action against *A. albopictus* larvae. The results of this study show that the leaf essential oil and its effective constituents might be considered as a potent source for the production of fine natural larvicides.

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1. Introduction

The acquisition of new strategies or natural products for mosquito larval control has intensified over the past decades because of concerns over chemical contaminations to the environments. The use of plant essential oils as mosquito control agents can be effective, and it has been shown to minimize the impact that most pesticidal compounds impose on the environment (Fatope et al., 1993). Mosquitoes are the major vector for the spreading of malaria, dengue fever, yellow fever, filariasis, and several diseases

(James, 1992). Mosquitoes also cause allergic responses on humans that include local skin and systemic reactions such as angioedema (Peng et al., 1999). *Aedes aegypti* and *Aedes albopictus* are two main species of mosquitoes responsible for dengue fever in Taiwan. In recent years, dengue fever has increased significantly in Taiwan resulting in significant research interest in mosquito control, especially through the use of compounds from indigenous plants (Albuquerque et al., 2004; Araújo et al., 2003; Cheng et al., 2004; Cheng et al., 2008; Jantan et al., 2005; Kuo et al., 2007; Tawatsin et al., 2001).

The Japanese cedar (*Cryptomeria japonica* D. Don) is well known in Taiwan as an important plantation tree species because of its durability for exterior construction and its beautiful yellowish-red heartwood color (Chang and

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Cheng, 2001). Due to its industrial importance, its constituents have been investigated in numerous studies (Lee and Lin, 1986; Nagahama et al., 2001; Shieh et al., 1981; Su et al., 1994, 1995, 1996). Our previous studies have demonstrated that leaf essential oil from *C. japonica* has excellent antitermite (Cheng and Chang, 2002), antifungal (Cheng et al., 2005), and silverfish repellent and insecticide (Wang et al., 2006) effects. However, there have not been any thorough investigations on larvicidal effect of the constituents of *C. japonica* leaf essential oil. The relationship between the leaf essential oils with various ages from *C. japonica* and their larvicidal effect is not quite understood and needs to be clarified in terms of the determination of harvest year. Therefore, in this study we examined the larvicidal effectiveness of leaf essential oil from *C. japonica* at different ages and its individual constituent against *A. aegypti* and *A. albopictus* larvae. In addition, the yields of essential oils obtained from water distillation were compared and their constituents determined and identified by GC–MS analyses.

2. Methods

2.1. Plant materials

Leaves of 58, 42, and 26 years old *C. japonica* D. Don were collected in June 2001 from the Experimental Forest of National Taiwan University, located in Nantou County of central Taiwan. The species was identified and the voucher specimens (CJL002–CJL004) were deposited at the Laboratory of Wood Chemistry, School of Forestry and Resource Conservation, National Taiwan University.

2.2. Mosquito larvae

Larvae of *A. aegypti* and *A. albopictus* from the Kaoshiung strain were reared in the Department of Parasitology, Chang-Gung University at 27 °C with a photoperiod of 12:12 (L:D) in 80 ± 10% RH. A 10% yeast suspension was used as food source.

2.3. Essential oil extraction

Leaves of *C. japonica* were subjected to water distillation in a Clevenger-type apparatus for 6 h (Cheng et al., 2005), followed by determination of their oil contents. The essential oils obtained were separated from the aqueous phase and stored in airtight containers at 4 °C prior to further analysis.

2.4. Analysis of essential oils

Analyses of the volatile constituents were determined using a PolarisQ Ion Trap GC/FID/MSⁿ system (Thermo, USA), equipped with a fused silica column (30 mm × 0.25 mm i.d.) and coated with a DB-5MS (*df* = 0.25 μm) (Agilent J&W Scientific). The GC settings were as follows:

initial oven temperature was held at 50 °C for 2 min, then increased from 50 °C to 250 °C at the rate of 5 °C/min. Helium was used as the carrier gas at 1 ml/min. The injector temperature was maintained at 270 °C. The samples (1.0 μl, 1/100, v/v, in ethyl acetate) were injected neat with 1:60 split ratio. The mass spectra were recorded over the 50–650 amu range at one scan per second, with ionization energy of 70 eV and ion source temperature at 200 °C. The Kovats retention indices were calculated for all volatile constituents using a homologous series of *n*-alkanes C₉–C₂₂ on the DB-5MS column. Quantification was performed using percentage peak area calculations and the identification of individual compartments was done using the Wiley/NBS Registry of Mass Spectral Database and NIST MS Search, literature (Adams, 2001) and several authentic compounds. The relative concentration of each compound in essential oil was quantified based on the peak area integrated by the analysis program.

2.5. Chromatography and characterization of essential oils

An aliquot of the essential oil from leaves (68 g) was subjected to chromatography over silica gel (700 g) and eluted sequentially with *n*-hexane/EtOAc gradients and finally with EtOAc. Fractions were tracked by TLC and compounds with similar *R_f* values were pooled together to give 10 subfractions (LO1–LO10). 16-Kaurene (4.97 min retention time) was isolated and purified from LO1 by semipreparative HPLC (Zorbax column, CHCl₃/EtOAc/*n*-C₆H₁₄ (5:10:85) mobile phase, 2.5 ml/min flow rate). The structure of 16-kaurene was established from one- and two-dimensional NMR and MS experiments as well as by direct comparisons of the ¹H NMR data with literature values (Lee and Lin, 1986). β-Eudesmol (Su et al., 1995) (9.70 min retention time) and elemol (Lee and Lin, 1986; Su et al., 1995) (10.1 min retention time) were isolated and purified from LO7 by semipreparative HPLC (Luna Silica (2) column, EtOAc/*n*-C₆H₁₄ (15:85) mobile phase, 3.0 ml/min flow rate). The spectral data are consistent with those reported in the literature.

2.6. Mosquito larvicidal bioassay

The method of Rafikali and Nair (2001) and Cheng et al. (2004) was used to conduct the mosquito larvicidal effect test. Ten fourth-instar mosquito larvae were placed in 24.5 ml of degassed distilled water, followed by addition 500 μl of dimethyl sulfoxide (DMSO) solution containing the test essential oil or the known compound in a 30 ml cup, with gentle shaking to ensure a homogeneous test solution, and each cup was left at the ambient temperature. A total of 3 essential oils and 11 known compounds were tested in this manner. Concentrations of 200, 100, 50, 25, and 12.5 μg/ml of essential oil were tested and each compound was tested at 100, 50, 25, 12.5, and 6.25 μg/ml. The control was prepared with 24.5 ml of degassed distilled water and 500 μl of DMSO solution. Each test was

replicated 4 times. For comparison, commercial chlorpyrifos (*o,o*-diethyl-*o*-3,5,6-trichloro-2-pyridyl phosphorothioate), an organophosphorus pesticide was used as positive control. The toxicity of chlorpyrifos was determined at 6.25, 3.125, 1.56, 0.78, and 0.39 µg/ml.

Mortality was recorded after 24 h of exposure and the larvae were starved within this period. The percentage of mortality was corrected for control mortality using Abbott's formula and the results were plotted on log/probability paper using the method of Finney (1971). Toxicity and effect were reported as LC₅₀ and LC₉₀, representing the concentrations in µg/ml with 50 and 90% larvae mortality rate in 24 h, respectively.

2.7. Statistical analyses

To determine whether there was a statistical significant difference among *C. japonica* essential oils at different age against the two strains of mosquito larvae, Scheffe's method of SAS was used to analyze the difference of the percentage of mortality. Results with $P < 0.05$ were considered to be statistically significant.

3. Results and discussion

3.1. Yields and constituents of essential oils

The yields of leaf essential oils of *C. japonica* ranged from 1.91% to 2.37% (w/w) based on dry weight (Table 1). The results indicated that the yields of leaf essential oils of *C. japonica* at three different ages (58, 42, and 26 years old) were 2.37, 1.91, and 2.19%, respectively. The leaf of 58-year-old *C. japonica* shows the highest yield (2.37%) and there is no significant relationship between age of the tree and yield.

The chemical compositions of the 3 essential oils are presented in Table 1. A total of 30 compounds were identified representing about 95.71–97.08% of the oils. The leaf essential oils of 58, 42, and 26 years old contain 20.44%, 19.53%, and 20.69% 16-kaurene, respectively, as the major constituent. Elemol is the second highest constituent, containing 19.05%, 18.41%, and 18.30% in leaf essential oils of 58, 42, and 26 years old, respectively. All identified components were separated into the five categories of monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, oxygenated sesquiterpenes, and diterpene hydrocarbons. Upon integrating the areas of the individual categories, we found that oxygenated sesquiterpenes accounted for the largest fraction at 37.83–39.07% for leaf essential oils of three different ages, followed by monoterpene hydrocarbons (23.10–28.13%), diterpene hydrocarbons (20.28–21.36%), oxygenated monoterpenes (8.40–11.50%), sesquiterpene hydrocarbons (1.17–1.60%) (Table 1). From the results, we also found that the yield of major constituents as shown above does not show any significant difference among all three ages tested. Similar composition results have been observed in the Lee and Lin (1986) study

in which the main constituents of leaf essential oil from *C. japonica* were 16-kaurene (27.71%) and elemol (26.90%). However, our results are different from those observed by Shieh et al. (1981) and Wang et al. (2006). For examples, Shieh et al. (1981), who reported that the main constituents of leaf essential oil from *C. japonica* were β-eudesmol (14.67%), α-eudesmol (14.67%), and elemol (11.62%). Wang et al. (2006) reported that elemol (18.22%) was the dominant compound, and the second most abundant compound was 16-kaurene (11.63%). This discrepancy may be due to the different seasons in which samples were collected and the different ages of tree.

3.2. Mosquito larvicidal effect of essential oils

Mosquito larvicidal effects of 58, 42, and 26 years old of *C. japonica* leaf essential oils against fourth-instar *A. aegypti* are shown in Fig. 1. Results showed that leaf essential oils of 58- and 26-year old tree induced 100% larval mortality against *A. aegypti* in 24 h with a dosage of 200 µg/ml, while 42 year old of leaf essential oil induced 97.5% larval mortality. Among them, leaf essential oil of 58-year old tree was found to be the most effective since it still induced 77.5% larval mortality when dosage was decreased to 50 µg/ml. Fig. 2 shows the mosquito larvicidal effects for leaf essential oils of 58-, 42-, and 26-year old *C. japonica* against *A. albopictus*. At a dosage of 200 µg/ml, oils from three different ages induced 100% larval mortality of *A. albopictus* in 24 h. When dosage was decreased to 50 µg/ml, the larval mortality of *A. albopictus* for three different ages was in the decreasing order of 58-year (57.5%) > 42-year (52.5%) > 26-year (47.5%). Table 2 shows the LC₅₀ values for leaf essential oils from three different ages against *A. aegypti* and *A. albopictus* larvae. The results indicated that oils from 58-year old tree has the most effective larvicidal effect against *A. aegypti* in 24 h (LC₅₀ = 28.4 µg/ml; LC₉₀ = 111.8 µg/ml), followed by 42-year (LC₅₀ = 39.2 µg/ml; LC₉₀ = 123.0 µg/ml) and 26-year (LC₅₀ = 56.7 µg/ml; LC₉₀ = 153.9 µg/ml), respectively. Similar results have been observed for *A. albopictus* larvae (Table 2), leaf essential oil from 58-year old tree has the most effective larvicidal effect against *A. albopictus* in 24 h (LC₅₀ = 51.2 µg/ml), followed by 26-year (LC₅₀ = 56.8 µg/ml) and 42-year (LC₅₀ = 57.9 µg/ml), respectively. The control treatment had no effect on the larvae. These results indicated that leaf essential oil provided higher inhibitory performance against *A. aegypti* than *A. albopictus*. In addition, leaf essential oil obtained from the tree with different ages has significant influence on mosquito larvicidal activity.

Araújo et al. (2003) also found that *Hyptis martiusii* leaf essential oils induced 100% mortality of *A. aegypti* larvae after 1 day but it takes a very high dose 500 mg/l. Cavalcanti et al. (2004) reported that the larvicidal activity of essential oils from Brazilian plants with LC₅₀ values against the larvae of *A. aegypti* that ranged from 60 to 69 µg/ml. In other investigation, Zhu et al. (2006) reported

Table 1
Yields and chemical compositions of leaf essential oils from *C. japonica* at different ages

No.	Compounds	RT ^a	KI ^b	Relative content (%)			Identification method ^c
				58	42	26	
1	α -Thujene	7.03	931	1.21	1.28	0.92	MS, KI
2	α -Pinene	7.24	939	4.77	4.92	4.40	MS, KI, ST
3	Camphene	7.61	953	0.46	0.46	0.46	MS, KI, ST
4	Sabinene	8.31	977	10.21	10.83	6.81	MS, KI
5	β -Myrcene	8.83	994	1.14	1.16	1.03	MS, KI, ST
6	3-Carene	9.37	1013	1.24	1.03	0.77	MS, KI, ST
7	α -Terpinene	9.56	1020	0.77	1.19	0.86	MS, KI, ST
8	<i>p</i> -Cymene	9.82	1030	2.31	1.88	2.65	MS, KI, ST
9	D-Limonene	9.95	1034	2.09	2.03	2.35	MS, KI, ST
10	γ -Terpinene	10.83	1064	1.82	2.35	2.01	MS, KI, ST
11	Terpinolene	11.68	1091	0.82	1.00	0.84	MS, KI, ST
12	Linalool	12.01	1102	0.28	0.35	0.35	MS, KI, ST
13	<i>cis</i> -2- <i>p</i> -Menthen-1-ol	12.65	1126	0.27	0.31	0.31	MS, KI
14	<i>trans</i> -2- <i>p</i> -Menthen-1-ol	13.19	1145	0.25	0.26	0.27	MS, KI
15	(–)-Terpinen-4-ol	14.05	1174	6.19	6.50	8.34	MS, KI, ST
16	α -Terpineol	14.65	1193	0.33	0.35	0.47	MS, KI, ST
17	<i>cis</i> -Piperitol	14.82	1199	– ^d	0.07	0.18	MS, KI
18	<i>trans</i> -Piperitol	15.16	1212	0.19	0.18	0.24	MS, KI
19	Bornyl acetate	17.35	1290	0.89	1.11	1.34	MS, KI, ST
20	α -Murolene	22.89	1503	0.24	0.22	0.18	MS, KI
21	γ -Cadinene	23.23	1518	0.22	0.20	0.15	MS, KI
22	δ -Cadinene	23.45	1527	1.14	1.05	0.84	MS, KI
23	Elemol	24.29	1563	19.05	18.41	18.30	MS, KI, ST
24	Cedrol	25.38	1608	0.57	–	–	MS, KI, ST
25	Unidentified	25.76	1626	0.79	0.97	0.85	
26	γ -Eudesmol	26.11	1642	6.27	6.40	7.16	MS, KI, ST
27	δ -Cadinol	26.27	1649	1.38	1.48	1.29	MS, KI, ST
28	α -Eudesmol + β -Eudesmol	26.66	1667	11.80	11.54	11.83	MS, KI, ST
29	Bifloratriene	32.27	1981	0.73	0.75	0.67	MS, KI
30	16-Kaurene	34.60	2059	20.44	19.53	20.69	MS, KI, ST
	Identified constituents (%)			97.08	96.84	95.71	
	Unidentified constituents (%)			0.79	0.97	0.85	
	Monoterpene hydrocarbons (%)			26.84	28.13	23.10	
	Oxygenated monoterpenes (%)			8.40	9.13	11.50	
	Sesquiterpene hydrocarbons (%)			1.60	1.47	1.17	
	Oxygenated sesquiterpenes (%)			39.07	37.83	38.58	
	Diterpene hydrocarbons (%)			21.17	20.28	21.36	
	Oil yield (% w/w)			2.37	1.91	2.19	

^a Retention time (min).

^b Kovats retention index relative to *n*-alkanes (C₉–C₂₂) on DB-5MS column.

^c MS, NIST and Wiley libraries spectra and the literature; KI, Kovats index on a DB-5MS column in reference Adams (2001); ST, co-injection with authentic standard compounds.

^d Not detected.

that amyris oil demonstrated the greatest inhibitory effect with LC₅₀ values in 24 h of 58 µg/ml for *A. aegypti*, 78 µg/ml for *A. albopictus*, and 77 µg/ml for *Culex pipiens pallens*. Comparisons of the aforesaid results indicated that the leaf essential oils of *C. japonica* have excellent mosquito larvicidal effects.

3.3. Mosquito larvicidal effect of constituents in essential oils

To further understand the relationship between the constituents of *C. japonica* leaf essential oil and larvicidal effect, the 11 pure compounds were tested using the same method as described in Section 2. Table 3 shows the LC₅₀ and LC₉₀ values for 11 compounds in *C. japonica* leaf essential oils against

A. aegypti and *A. albopictus* larvae. Among these constituents, 3-carene demonstrated the best *A. aegypti* larvicidal effect at 24 h with an LC₅₀ value of 25.3 µg/ml (LC₉₀ = 69.4 µg/ml), followed by γ -terpinene (LC₅₀ = 26.8 µg/ml), α -terpinene (LC₅₀ = 28.1 µg/ml), terpinolene (LC₅₀ = 32.1 µg/ml), β -myrcene (LC₅₀ = 35.8 µg/ml), *p*-cymene (LC₅₀ = 37.1 µg/ml), 16-kaurene (LC₅₀ = 57.0 µg/ml), and α -pinene (LC₅₀ = 79.1 µg/ml), respectively. On the other hand, terpinolene showed the strongest inhibitory effect against *A. albopictus* larvae (LC₅₀ = 22.0 µg/ml, LC₉₀ = 55.5 µg/ml), followed by α -terpinene, γ -terpinene, *p*-cymene, 3-carene, β -myrcene, 16-kaurene, and α -pinene with an LC₅₀ value of 22.4, 22.8, 24.1, 27.0, 56.5, and 74.0 µg/ml in 24 h, respectively. (–)-Terpinen-4-ol, elemol, and β -eudesmol,

Fig. 1. Mosquito larvicidal effects of leaf essential oils from *C. japonica* at different ages against fourth-instar larvae of *A. aegypti* in 24 h. Means ($n = 4$) using 10 fourth-instar mosquito larvae per replicate. Numbers followed by different letters (a–h) are significantly different at the level of $p < 0.05$ according to the Scheffe's test.

Fig. 2. Mosquito larvicidal effects of leaf essential oils from *C. japonica* at different ages against fourth-instar larvae of *A. albopictus* in 24 h. Means ($n = 4$) using 10 fourth-instar mosquito larvae per replicate. Numbers followed by different letters (a–e) are significantly different at the level of $p < 0.05$ according to the Scheffe's test.

Table 2

LC₅₀ and LC₉₀ values (µg/ml) of the leaf essential oils from *C. japonica* at different ages against the yellow fever mosquito *A. aegypti* and *A. albopictus* larvae in 24 h

Ages	<i>A. aegypti</i>		<i>A. albopictus</i>	
	LC ₅₀	LC ₉₀	LC ₅₀	LC ₉₀
58	28.4	111.8	51.2	178.0
42	39.2	123.0	57.9	177.5
26	56.7	153.9	56.8	187.0

other major constituents, which belong to oxygenated monoterpene category, did not show any significant inhibition against those larvae tested, the exact LC₅₀ value (>100.0 µg/ml) was unable to be calculated in the range of concentration used. From the results, it was noted that the main larvicidal effect might be coming from the minor terpene hydrocarbon constituents (e.g. 3-carene, terpinolene, α -terpinene and γ -terpinene).

It has been reported that cinnamaldehyde, cinnamyl acetate, and eugenol all had excellent larvicidal effect against *A. aegypti* larvae in 24 h with an LC₅₀ value of 29, 33, and 33 µg/ml, respectively (Cheng et al., 2004). In another

Table 3

LC₅₀ and LC₉₀ values (µg/ml) of 11 compounds in *C. japonica* leaf essential oil against the yellow fever mosquito *A. aegypti* and *A. albopictus* larvae in 24 h

Compounds	<i>A. aegypti</i>		<i>A. albopictus</i>	
	LC ₅₀	LC ₉₀	LC ₅₀	LC ₉₀
α -Terpinene	28.1	76.4	22.4	58.8
γ -Terpinene	26.8	68.7	22.8	57.4
<i>p</i> -Cymene	37.1	>100.0	25.9	66.3
3-Carene	25.3	69.4	24.1	62.3
α -Pinene	79.1	>100.0	74.0	>100.0
(-)-Terpinen-4-ol	>100.0	>100.0	>100.0	>100.0
16-Kaurene	57.0	95.0	56.5	92.5
Terpinolene	32.1	83.6	22.0	55.5
β -Myrcene	35.8	>100.0	27.0	75.4
β -Eudesmol	>100.0	>100.0	>100.0	>100.0
Elemol	>100.0	>100.0	>100.0	>100.0
Chlorpyrifos ^a	1.3	3.4	1.1	2.3

^a Positive control.

investigation, Araújo et al. (2003) found that 1,8-cineole induced 100% larval mortality of *A. aegypti* after 1 day with a dosage of 100 mg/l. Comparisons of these data revealed that 3-carene, terpinolene, and terpinene (α - and γ -terpinene) examined in this study exhibited great larvicidal performance.

4. Conclusions

According to GC–MS analyses of the leaf essential oils from *C. japonica* at three different ages, there is no significant relationship between age and yield. Meanwhile, the major constituents of the leaf essential oils of *C. japonica* are 16-kaurene and elemol. Results obtained from the larvicidal tests, using leaf essential oil from 58 year old *C. japonica* was found to be most effective against both *A. aegypti* and *A. albopictus* larvae than 42- and 26-years old tree. In addition, we found that the main larvicidal effect might be coming from the minor constituents (e.g. 3-carene, terpinolene, α -terpinene and γ -terpinene). In short, our findings suggested that the essential oil from *C. japonica* leaves and its effective constituents may be explored as a potential natural larvicide. Further investigations for the mode of the constituents' actions, effects on non-target organisms and field evaluation are necessary. These results obtained are useful in search of more selective, biodegradable and naturally produced larvicidal compounds.

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