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Chemical compositions and larvicidal activities of leaf essential oils from two eucalyptus species

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ABSTRACT

In the current study, the mosquito larvicidal activity of leaf essential oils and their constituents from two eucalyptus species (*Eucalyptus camaldulensis* and *Eucalyptus urophylla*) against two mosquito species, *Aedes aegypti* and *Aedes albopictus*, was investigated. In addition, the chemical compositions of the leaf essential oils were analyzed using gas chromatography–mass spectrometry. Results from the larvicidal tests revealed that essential oil from the leaves of *E. camaldulensis* had an excellent inhibitory effect against both *A. aegypti* and *A. albopictus* larvae. The 12 pure constituents extracted from the two eucalyptus leaf essential oils were also tested individually against two mosquito larvae. Among the six effective constituents, α-terpinene exhibits the best larvicidal effect against both *A. aegypti* and *A. albopictus* larvae. Results of this study show that the leaf essential oil of *E. camaldulensis* and its effective constituents might be considered as a potent source for the production of fine natural larvicides.

1. Introduction

Mosquitoes play a predominant role in the transmission of malaria, dengue fever, yellow fever, filariasis, and several diseases which are today among the greatest health problems in the world (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, where the number of dengue fever cases has increased significantly in recent years. The control of mosquito larvae worldwide depends primarily on continued applications of organophosphates such as temephos, fenthion and insect growth regulators such as diflubenzuron and methoprene (Yang et al., 2002). Although effective, repeated use of these controlling agents has fostered several environmental and health concerns, including disruption of natural biological control systems, outbreaks of other insect species, widespread development of resistance and undesirable effects on non-target organisms (Yang et al., 2002). These problems have highlighted the need for new strategies for mosquito larval control.

Essential oils from plants may be an alternative source of mosquito larval control agents, since they constitute a rich source of bioactive compounds that are biodegradable into nontoxic products and potentially suitable for use in integrated management programs. In fact, many researchers have reported on the effectiveness of plant essential oils against mosquito larvae, and the recent examples are studied by Chantraine et al. (1998), Cheng et al. (2003, 2004), Jantan et al. (2005), and Thomas et al. (2004).

Eucalyptus is one of the world's most important and most widely planted genera. It includes more than 700 species and belongs to the family of Mitraceae (Menut et al., 1995). Many species of the genus *Eucalyptus* from the Myrtaceae family are used in China folk medicine for a variety of medical conditions. For examples, hot water extracts of dried leaves of *Eucalyptus citriodora* are traditionally used as analgesic, anti-inflammatory and antipyretic remedies for the symptoms of respiratory infections, such as cold, flu, and sinus congestion (Silva et al., 2003). Its main uses are the production of essential oils, which are used for medicinal and pharmaceutical purposes (Ghisalberti, 1996; Leung and Foster, 1996). In addition, *Eucalyptus camaldulensis* and *Eucalyptus urophylla* are also known to contain bioactive products that display antibacterial (Cimanga et al., 2002), antifungal (Su et al., 2006), analgesic and anti-inflammatory effects (Silva et al., 2003), antioxidative and

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antiradical (Siramon and Ohtani, 2007) activities. However, there have not been any thorough investigations on larvicidal effects of leaf essential oils and their constituents from *E. camaldulensis* and *E. urophylla*. Therefore, in this study we analyzed the constituents of leaf essential oils of *E. camaldulensis* and *E. urophylla* and studied their mosquito larvicidal effectiveness against *A. aegypti* and *A. albopictus* larvae. In addition, the yields of essential oils obtained from hydrodistillation were compared and their constituents determined by GC–MS analyses.

2. Methods

2.1. Plant materials

Fresh leaves from 6-year-old tree of *E. camaldulensis* and *E. urophylla* were collected in June 2006 from the Chung Hwa Pulp Corporation, located in Hualien County of eastern Taiwan. The species was identified and the voucher specimens (ECL001, EUL001) were deposited at the Laboratory of Wood Chemistry, School of Forestry and Resource Conservation, National Taiwan University.

2.2. Essential oil isolation

Fresh leaves (200 g) of *E. camaldulensis* and *E. urophylla* were subjected to hydrodistillation using a modified Clevenger-type apparatus for 6 h (Cheng et al., 2005). The yields were averaged over four experiments and calculated according to dry weight of the plant materials. Essential oils were stored in airtight containers prior to analysis by gas chromatography–flame ionization detection (GC–FID) and gas chromatography–mass spectrometry (GC–MS).

2.3. Analysis of essential oils

Analyses of the volatile constituents were determined using a Finnigan Trace GC with an FID and GC–MS equipped with a DB-5MS column (30 m × 0.25 mm i.d., 0.25 μm film thickness, J & W Scientific). The GC settings were as follows: initial oven temperature was held at 40 °C for 2 min, and ramped at 3 °C/min to 140 °C, then increased from 140 to 250 °C at the rate of 10 °C/min. Helium was used as the carrier gas at a flow rate of 1 ml/min. The injector temperature was maintained at 250 °C. The samples (1 μl) were injected neat with split ratio of 1:10. 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 230 °C. The Kovats retention indices were calculated for all volatile constituents using a homologous series of *n*-alkanes C_9–C_{17} on the DB-5MS column. Quantification was performed using percentage peak area calculations using the GC–FID, and the identification of individual components was done using their relative retention indices and the Wiley/NBS registry of mass spectral database and NIST MS Search, literature (Adams, 2001; Giamakis et al., 2001; Su et al., 2006) and several authentic reference compounds. The quantity of compounds was obtained by integrating the peak area of the spectrograms.

2.4. Mosquito larvae

The fourth-instar larvae of *A. aegypti* and *A. albopictus* served as the test organisms. The larvae colonies of the mosquito collected from the Kaoshiung strain were reared in the Department of Parasitology, Chang-Gung University at 27 °C with a photoperiod of 12 h with light and 12 h in the dark in 80 ± 10% relative humidity. Ten percentage yeast suspension was used as food source.

2.5. Determination of mosquito larvicidal activity

The method of Momin and Nair (2001) was modified and employed 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 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 two essential oils and 12 known compounds were tested in this manner. Concentrations of 400, 200, 100, 50, and 25 μg/ml of essential oil were tested and each compound was tested at 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 four times. For comparison, commercial chlorpyrifos (*o*,*o*-diethyl-*o*-3,5,6- trichloro-2-pyridyl phosphorothioate), an organophosphorus pesticide was used as a 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 LC_{50} and LC_{90}, representing the concentrations in μg/ml with 50 and 90% larvae mortality rate in 24 h, respectively.

2.6. Statistical analyses

The percentages of mortality was determined and transformed to arcsine square root values for analysis of variance (ANOVA). The Scheffe's test was utilized to analyze for significant difference among the test essential oils and compounds against the two strains of mosquito larvae. Results with $P < 0.05$ were considered to be statistically significant.

3. Results and discussion

3.1. Yields and chemical constituents of essential oils

The yields of leaf essential oils from the hydrodistillation of *E. camaldulensis* and *E. urophylla* were 0.57% (10.41 ml/kg) and 2.19% (30.11 ml/kg) according to their dry weight, respectively (Table 1).

Table 1 shows the constituents identified, percentage composition and their Kovats index (KI) values listed in order of elution from the DB-5MS capillary column. A total of 20 compounds amounting to 97.58% in the *E. camaldulensis* leaf essential oil were identified (Table 1). Among these 81.41% were monoterpene hydrocarbons, 12.55% were oxygenated monoterpenes, and it also contained 0.50% sesquiterpene hydrocarbons and 3.12% oxygenated sesquiterpenes. The major constituents in the *E. camaldulensis* leaf essential oil were α-pinene (22.52%), *p*-cymene (21.69%), α-phellandrene (20.08%), 1,8-cineole (9.48%), γ-terpinene (9.36%), and limonene (4.56%). The results differ from Shieh (1996), Tsiri et al. (2003), and Su et al. (2006) who reported that the main constituent of *E. camaldulesis* was 1,8-cineole. We assume that the discrepancy might have been caused by the differences in the chemotype of the species.

In the essential oil extracted from *E. urophylla* leaves 25 compounds were identified, corresponding to 95.78% of the total oil. Nine monoterpene hydrocarbons (13.80%), seven oxygenated monoterpenes (77.38%), five sesquiterpene hydrocarbons (3.02%), and four oxygenated sesquiterpenes (1.58%) were identified in the essential oil. The main constituents in the *E. urophylla* leaf essential oil were 1,8-cineole (58.34%), α-terpinyl acetate (14.87%), α-pinene (6.25%), *cis*-ocimene (3.55%), and α-terpineol (3.04%). Comparing

Table 1
Chemical constituents of leaf essential oils from *E. camaldulensis* and *E. urophylla*

No.	KI[a]	Constituents	Concentration (%)		Identification[b]
			E. camaldulensis	*E. urophylla*	
Monoterpene hydrocarbons			*81.41*	*13.80*	
1	939	α-Pinene	22.52	6.25	MS, KI, ST
2	978	β-Pinene	0.35	0.72	MS, KI, ST
3	991	β-Myrcene	0.45	0.33	MS, KI, ST
4	1005	α-Phellandrene	20.08	–[c]	MS, KI, ST
5	1017	α-Terpinene	1.24	–	MS, KI, ST
6	1025	*p*-Cymene	21.69	0.40	MS, KI, ST
7	1030	Limonene	4.56	–	MS, KI, ST
8	1039	*cis*-Ocimene	–	3.55	MS, KI
9	1050	*trans*-Ocimene	–	0.64	MS, KI
10	1060	γ-Terpinene	9.36	0.50	MS, KI, ST
11	1085	Terpinolene	1.16	0.87	MS, KI, ST
12	1129	*allo*-Ocimene	–	0.54	MS, KI
Oxygenated monoterpenes			*12.55*	*77.38*	
13	1034	1,8-Cineole	9.48	58.34	MS, KI, ST
14	1099	Linalool	–	0.10	MS, KI, ST
15	1171	Borneol	0.34	0.24	MS, KI, ST
16	1180	(−)-Terpinen-4-ol	1.21	0.60	MS, KI, ST
17	1194	α-Terpineol	0.99	3.04	MS, KI, ST
18	1290	Thymol	0.53	–	MS, KI, ST
19	1347	α-Terpinyl acetate	–	14.87	MS, KI, ST
20	1379	Geranyl acetate	–	0.19	MS, KI, ST
Sesquiterpene hydrocarbons			*0.50*	*3.02*	
21	1373	α-Copaene	–	0.18	MS, KI
22	1414	β-Caryophyllene	–	1.24	MS, KI, ST
23	1434	β-Gurjunene	0.50	0.19	MS, KI
24	1518	γ-Cadinene	–	1.02	MS, KI
25	1521	δ-Cadinene	–	0.39	MS, KI
Oxygenated sesquiterpenes			*3.12*	*1.58*	
26	1566	*E*-Nerolidol	0.11	0.33	MS, KI, ST
27	1577	Spathulenol	–	0.23	MS, KI
28	1584	Globulol	0.91	0.92	MS, KI
29	1623	10-*epi*-γ-Eudesmol	0.13	0.10	MS, KI
30	1632	γ-Eudesmol	0.78	–	MS, KI, ST
31	1651	β-Eudesmol	1.19	–	MS, KI, ST
Total (%)			97.58	95.78	
Oil yield (%, w/wt)			0.57	2.19	

[a] Kovats index relative to *n*-alkanes (C_9–C_{17}) on a DB-5MS column.
[b] 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.
[c] Not detected.

our results with those obtained by Shieh (1998) and Cimanga et al. (2002) shows that all the leaf essential oils extracted are similar with 1,8-cineole predominating. However, Singh et al. (1988) obtained the leaf oil of *E. urophylla* from India with *p*-cymene (75.0%), α-pinene (7.0%), and α-terpinene (4.0%) being the dominant components. We assume that such discrepancy might also be attributed to the differences in the chemotype of the species.

The composition analysis of the two eucalyptus leaf oils revealed that monoterpenes predominated. *E. camaldulensis* leaf oils had greater amounts of monoterpene hydrocarbons, while *E. urophylla* had more oxygenated monoterpenes. These two species had relatively low sesquiterpene contents, as also noted by several studies (Dellacassa et al., 1990; Bignell et al., 1997; Su et al., 2006).

3.2. Mosquito larvicidal activity of essential oil

Fig. 1 shows the results of the larval susceptibility to the leaf essential oils of *E. urophylla* and *E. camaldulensis* against fourth-instar larvae of *A. aegypti*. There was no significant difference between the two eucalyptus leaf essential oils. At the dosage of 200 μg/ml, both *E. urophylla* and *E. camaldulensis* induced 100% larval mortality of *A. aegypti* in 24 h. When the dosage was decreased to 100 μg/ml, the larval mortality of *E. urophylla* and *E. camaldulensis* leaf essential oils were 60 and 100%, respectively. Moreover,

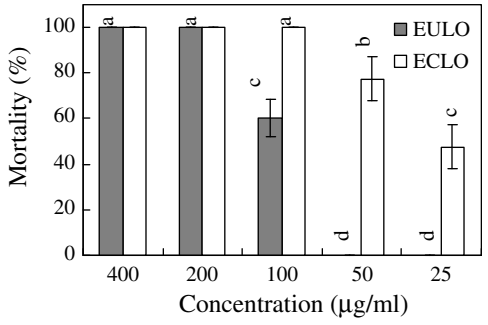

Fig. 1. Mosquito larvicidal activities of leaf essential oils from *E. urophylla* (EULO) and *E. camaldulensis* (ECLO) against fourth-instar larvae of *A. aegypti* in 24 h. Means ($n = 4$) using 10 fourth-instars mosquito larvae per replicate. Numbers followed by different letters (a–d) are significantly different at the level of $P < 0.05$ according to the Scheffe's test.

Fig. 2 shows the mosquito larvicidal activity for two eucalyptus leaf essential oils against *A. albopictus*. The results showed that *E. camaldulensis* leaf essential oil induced 100% larval mortality of *A. albopictus* in 24 h with a dosage of 400 μg/ml, while *E. urophylla* leaf essential oil induced 75.0% larval mortality. *E. camaldulensis* leaf

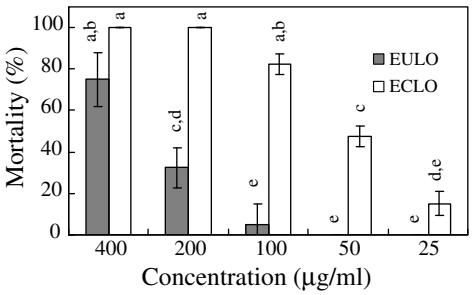

Fig. 2. Mosquito larvicidal activities of leaf essential oils from *E. urophylla* (EULO) and *E. camaldulensis* (ECLO) against fourth-instar larvae of *A. albopictus* in 24 h. Means ($n = 4$) using 10 fourth-instars mosquito larvae per replicate. Numbers followed by different letters (a–e) are significantly different at the level of $P < 0.05$ according to the Scheffe's test.

essential oil was found to be the more toxic since it still induced 82.5% larval mortality when dosage was decreased to 100 μg/ml.

From a comparison of the LC_{50} and LC_{90} values of *E. urophylla* and *E. camaldulensis* leaf essential oils against fourth-instar larvae of *A. aegypti* and *A. albopictus* in 24 h, *E. camaldulensis* leaf essential oil showed an excellent toxicity against *A. aegypti* and *A. albopictus* larvae (Table 2). The LC_{50} values are 31.0 and 55.3 μg/ml with corresponding LC_{90} values of 71.8 and 192.4 μg/ml against *A. aegypti* and *A. albopictus* larvae, respectively. Control treatments (DMSO solutions) had no effect on the larvae. The results indicated that *E. camaldulensis* leaf essential oil presented higher inhibitory performance against *A. aegypti* than *A. albopictus*. Cheng et al. (2003, 2004) examined plant essential oils against *A. aegypti* larvae with LC_{50} values ranging from 36.0 to 86.8 μg/ml. In other investigation, Cavalcanti et al. (2004) reported that the larvicidal activity of essential oils from Brazilian plants with LC_{50} values ranging from 60 to 69 μg/ml against *A. aegypti* larvae. Therefore, the present study revealed that the essential oil from the leaf of *E. camaldulensis* with a concentration as low as 31.0 μg/ml could also induce 50% mortality. It was also observed that the essential oils and the isolated compounds in the study were relatively more toxic to the larvae of *A. aegypti* and *A. albopictus*.

3.3. Mosquito larvicidal activity of constituents in essential oils

To understand the relationship between the constituents of *E. camaldulensis* leaf essential oil and larvicidal activity, twelve pure compounds in *E. camaldulensis* leaf essential oil were tested for mosquito larvicidal activity against fourth-instar larvae of *A. aegypti* and *A. albopictus*. As shown in Table 3, among the 12 compounds tested in 24 h, 1,8-cineole, α-pinene, α-terpinyl acetate, α-terpineol, terpinen-4-ol, and β-eudesmol showed a $LC_{50} > 50.0$ μg/ml against *A. aegypti* and *A. albopictus* larvae, and consequently they were considered to be inactive. On the other hand, α-phellandrene, limonene, *p*-cymene, γ-terpinene, terpinolene, and α-terpinene exhibited strong activities against *A. aegypti* and *A. albopictus* larvae ($LC_{50} < 50.0$ μg/ml). Among these constituents, α-terpinene demonstrated the best *A. aegypti* larvicidal activity in 24 h and its LC_{50} value was 14.7 μg/ml ($LC_{90} = 39.3$ μg/ml), followed by α-phellandrene ($LC_{50} = 16.6$ μg/ml, $LC_{90} = 36.9$ μg/ml), limonene ($LC_{50} = 18.1$ μg/ml, $LC_{90} = 41.0$ μg/ml), *p*-cymene ($LC_{50} = 19.2$ μg/ml, $LC_{90} = 41.3$ μg/ml), terpinolene ($LC_{50} = 28.4$ μg/ml, $LC_{90} = 46.0$ μg/ml), and γ-terpinene ($LC_{50} = 30.7$ μg/ml, $LC_{90} > 50.0$ μg/ml), respectively. As for *A. albopictus* larvae, the order of LC_{50} value was α-terpinene (25.2 μg/ml) < γ-terpinene (29.8 μg/ml) < limonene (32.7 μg/ml) < terpinolene (35.6 μg/ml) < α-phellandrene (39.9 μg/ml) < *p*-cymene (46.7 μg/ml). It is clear that α-terpinene has the best *A. albopictus* larvicidal activity. As discussed above, larvicidal activity of *E. camaldulensis* leaf essential oil against both *A. aegypti* and *A. albopictus* larvae is attributed mainly to these volatile compounds.

Table 2
Lethal concentrations (μg/ml) of leaf essential oils from *E. urophylla* and *E. camaldulensis* against fourth-instar larvae of *A. aegypti* and *A. albopictus* in 24 h

Specimens	*A. aegypti*		*A. albopictus*	
	LC_{50}	LC_{90}	LC_{50}	LC_{90}
E. urophylla	95.5	166.3	285.8	>400.0
E. camaldulensis	31.0	71.8	55.3	192.4
Chlorpyrifos[a]	1.1	2.4	1.4	3.6

[a] Positive control.

Table 3
Lethal concentrations (μg/ml) of 12 compounds from *E. urophylla* and *E. camaldulensis* leaf essential oils against fourth-instar larvae of *A. aegypti* and *A. albopictus* in 24 h

Compounds	*A. aegypti*		*A. albopictus*	
	LC_{50}	LC_{90}	LC_{50}	LC_{90}
α-Phellandrene	16.6	36.9	39.9	>50.0
1,8-Cineole	>50.0	>50.0	>50.0	>50.0
Limonene	18.1	41.0	32.7	50.0
p-Cymene	19.2	41.3	46.7	>50.0
γ-Terpinene	30.7	>50.0	29.8	47.5
Terpinolene	28.4	46.0	35.6	>50.0
α-Terpinyl acetate	>50.0	>50.0	>50.0	>50.0
α-Terpinene	14.7	39.3	25.2	>50.0
α-Pinene	>50.0	>50.0	>50.0	>50.0
α-Terpineol	>50.0	>50.0	>50.0	>50.0
(−)-Terpinen-4-ol	>50.0	>50.0	>50.0	>50.0
β-Eudesmol	>50.0	>50.0	>50.0	>50.0
Chlorpyrifos[a]	1.1	2.4	1.4	3.6

[a] Positive control.

Rahuman et al. (2000) also found that *n*-hexadecanoic acid in *Feronia limonia* dried leaves was effective against fourth-instar larvae of *Culex quinquefasciatus*, *Anopheles stephensi* and *A. aegypti* with LC_{50} values of 129.24, 79.58, and 57.23 μg/ml, respectively. In another 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. In our previous study, cinnamaldehyde, cinnamyl acetate, and eugenol all had an excellent larvicidal effect against *A. aegypti* larvae in 24 h with LC_{50} values of 29, 33, and 33 μg/ml, respectively (Cheng et al., 2004). Comparisons of these data revealed that α-phellandrene, limonene, *p*-cymene, γ-terpinene, terpinolene, and α-terpinene examined in this study exhibited great larvicidal performance.

4. Conclusions

According to GC–MS analyses the major constituents of the leaf essential oils were α-pinene, *p*-cymene, and α-phellandrene from *E. camaldulensis* and 1,8-cineole from *E. urophylla*. Results obtained from the larvicidal tests, using the leaf essential oil from *E. camaldulensis* had excellent inhibitory effects against both *A. aegypti* and *A. albopictus* larvae. In addition, we found that α-phellandrene, limonene, *p*-cymene, γ-terpinene, terpinolene, and α-terpinene show strong larvicidal effects against the two larvae tested. In brief, our findings suggested that the essential oil from *E. camaldulensis* leaves and its effective constituents may be explored as a potential environmental-benign 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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