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Phytochemical Screening and Larvicidal Activity of *Millingtonia hortensis* L.f. Flower Extract Against *Aedes aegypti* Linn.

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ABSTRACT

The phytochemical composition and larvicidal efficacy of essential oils extracted from *Millingtonia hortensis* flowers against laboratory-reared *Aedes aegypti* mosquitoes were investigated. The essential oils of fresh *M. hortensis* flowers were extracted by maceration in petroleum ether at room temperature for 12 hr and provided yields of oils of about 0.02% (volume per weight) fresh weight. The aromatic volatile components of the essential oils were analyzed using gas chromatography coupled to mass spectrometry. Out of 36 compounds, 27 were identified and accounted for 85.84% of the chromatographable components. These included: solanesol (25.72%), *trans*–farnesol (19.71%), nerolidol (8.54%), *n*-hexadecanoic acid (6.77%), vanillin (6.20%), oleic acid (4.54%), linoleic acid (3.87%), L–linalool (3.37%), 1–octen–3–ol (1.67%), α–farnesene (1.22%) and methyl salicylate (1.03%). For the larvicidal bioassay, *M. hortensis* flower extract was added to the third to fourth *Aedes aegiypti* instars in various concentrations (control, 25, 50, 100, 250 and 500 parts per million). These larvae exhibited median lethal concentrations to kill 50% of the treated larvae in 24 hr of approximately 208.5 parts per million (ppm; $Y = 20.089X + 4.935$). Based on the mortality rate, the concentration at 500 ppm of extract showed the highest effectiveness in controlling the larvae with 98% mortality after exposure for 24 hr. This study suggests that this plant extract can be used for controlling mosquito larvae.

Keywords: Phytochemistry, essential oil, mosquitocidal activity, *Millingtonia hortensis*

INTRODUCTION

Mosquitoes are prominent hematophagous parasites or bloodsuckers that annoy man, birds and many other animals and they are most efficient vectors which transmit pathogens causing human malaria, dengue, yellow fever, filariasis, viral encephalitis and other fatal diseases (Service, 1983; Becker *et al.,* 2003; Rueda, 2008). *Aedes aegypti* (Family Culicidae) is involved in dengue transmission, which is a serious problem in several tropical and subtropical countries (Tanaka *et al.,* 1979; Guzman and Kouri, 2002). Generally, *Ae. aegypti* breeds in man-made water-storage containers located in household dwellings and preferentially feeds indoors, particularly in the morning hours and in the late afternoon (Christophers, 1960; Ponlawat and Harrington,

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2005). In Thailand, *Aedes* mosquitoes are a major vector and there is presently no effective vaccine against dengue. The prevention and control of dengue virus transmission depends on mosquito eradication through two principal measures—larviciding and using insecticides (Yaicharoen *et al*., 2005; Polsomboon *et al.,* 2008). Most vector surveillance strategies rely merely or only on indicators that have been designed to detect the presence or absence of mosquito larvae or pupae. Elimination of *Ae. aegypti* through source reductions has been proposed but this approach is rather costly, needs full community participation and is invariably unsuccessful (Kongmee *et al*., 2004). Ultra-low-volume (ULV) and thermal fogging applications of synthetic pyrethroids are usually used, especially during the peak period of adult populations. In addition, numerous synthetic pyrethroids are commonly used by home owners to control household mosquitoes.

Chemical application could be a crucial cause of insecticide resistance for the house mosquito, *Ae. aegypti*. The evolution of pyrethroid resistance in this mosquito indicates the limitation of new pyrethroid candidates for *Aedes* mosquito control programs. The development of biologically active materials for mosquitocides that do not confer cross-resistance to present insecticides is constantly needed. Natural products such as plant-derived insecticides that include a multitude of active ingredients, with distinct modes of action which lessen the chance of resistance in mosquito population (Okuma *et al.,* 2007), are attractive alternatives.

Plant extracts, especially essential oils, have been used as a minor natural source of insecticides as they constitute a good source of bioactive compounds that are biodegradable into nontoxic products and this minimizes the accumulation of harmful residues and makes them more environmentally friendly compared to synthetic compounds (Sharma *et al.,* 2006). Botanical insecticides used for their larvicidal properties against *Anopheles stephensi*, *Culex quinquefasciatus* and *Aedes aegypti* are ecofriendly (Kaushik and Saini, 2008). Botanical-based larvicides that are effective and easily available at low cost and do not confer cross-resistance to current insecticides, possess great promise for controlling dengue, especially in cases where vector susceptibility is declining (Warikoo *et al*., 2011).

Millingtonia hortensis (Family Bignoniaceae) is a native deciduous tree that ranges through India, Myanmar, Thailand and south China and is often cultivated as an ornamental tree in yards, gardens and avenues (Kumari and Sharma, 2013). This plant is colloquially known as "cork tree" or "peep" or "Gaa Sa Long" (Thai). The flowers of *M. hortensis* have a very rich and pleasant scent and have been used as a traditional medicine by Indians for the treatment of a variety of conditions (Ramasubramaniaraja, 2010). The dried flowers of this plant have been used for cigarette ingredients to give a sweet aroma and scent for relaxation in Thailand. The plant has been used in Thai folklore for the treatment of asthma, sinusitis, cholagogue, tuberculosis and as a tonic (Takeshi *et al*., 1995; Chulasiri, 1998; Sittiwet, 2009). Alkaloids, tannins, flavonoids and phenolic compounds are the most important chemically active constituents of this tree (Kumari and Sharma, 2013). Some flavonoids from this plant have been isolated and characterized, including two main flavones—hispidulin (6–methoxy–5,7,4'–trihydroxyflavone) (Chulasiri *et al*., 1992) and hortensin 3,4'–dihydroxy–6,7–dimethoxyflavone) (Chulasiri, 1998). The leaf extracts of *M. hortensis* showed good antifungal activity (Sharma *et al*., 2007), bacterial activity (Jetty and Iyengar, 2000; Sittiwet, 2009; Nagaraja and Padmaa, 2011a,b), larvicidal activity (Kaushik and Saini, 2008), antiproliferation activity (Tansuwanwong *et al*., 2006) and antioxidant activity (Leelapornpisid *et al*., 2008; Babitha *et al*., 2012).

The current study investigated the composition of essential oils using chromatography/mass-spectrometry (GC–MS) and the efficacy of

essential oils and petroleum ether extract from *M. hortensis* flowers against larval stages of *Ae. aegypti.*

MATERIALS AND METHODS

Mosquito rearing

The third to fourth instar larvae of laboratory-reared *Ae. aegypti* obtained from Nakhon Si Thammarat (third generation) were established in the insectarium of the Program Biology, Faculty of Science and Technology, Pibulsongkram Rajabhat University (PSRU), Phitsanulok province, Thailand and maintained under controlled insectary conditions at 25 ± 5 ° C, 80 ± 10% relative humidity, with a 12:12 hour light:dark photophase regime. *Ae. aegypti* was used as a test species because of its easy collection as well as the expedience in rearing and maintaining the life cycle. Its sensitivity to larvicides makes *Ae. aegypti* larvae a good indicator of biocidal activity.

Plant material

Fresh flowers of *M. hortensis* were gathered from PSRU, in November 2012. Plant specimens (PSRU-Bign-001) were identified by comparison with reference material at the herbarium, Faculty of Science, Chiang Mai University, Chiang Mai province, Thailand.

Extraction of essential oil

Ten kilograms of whole fresh flowers of *M. hortensis* were harvested at one time and then macerated with petroleum ether (5 L) for 12 hr, followed by filtration and then the removal of all solvents under reduced pressure to yield a semi-solid crude petroleum. The crude was re-dissolved in ethanol (25 mL) followed by filtration and evaporation of all solvents to give a light yellow, semi-solid substance with an absolute yield of 0.02%. Some of this was used for further identification of the chemical compounds with GC-MS analysis and the remainder was used for mosquito larvicidal bioassay. The water extracts were used directly for the bioassay.

Analysis using gas chromatography coupled to mass spectrometry

The volatile constituents of *M. hortensis* were analyzed using an HP model 6890 gas chromatograph equipped with an HP-5MS (5% phenyl-polymethylsiloxane) capillary column (30 m × 0.25 mm internal dimater, film thickness 0.25 µm; Agilent Technologies, Santa Clara, CA, USA) interfaced to an HP model 5973 mass-selective detector. The oven temperature was initially held at 60 °C and then increased by 2 °C.min^{-1} to 250 °C. The injector and detector temperatures were 250 and 280 °C, respectively. Purified helium was used as the carrier gas at a flow rate of 1 mL.min^{-1}. Electron ionization mass spectra were collected at 70 eV ionization voltages over the range 29–300 m.z^{-1}. The electron multiplier voltage was 1150 V. The ion source and quadrupole temperatures were set at 230 and 150 °C, respectively.

Identification of the compounds

Identification of the volatile components was performed using a comparison of their Kovat retention indices, relative to C_8-C_{22} *n*-alkanes, and a comparison of the mass spectra of individual components with the reference mass spectra in the Wiley 275 and NIST 98 databases (Adams, 1995).

Larvicidal bioassay

According to the procedure of World Health Organization (1981), third to fourth instar larvae of *Ae. aegypti* were exposed to extracted oils at various concentrations. Five different extract concentrations ranging from 500 to 25 parts per million (ppm; 500, 250, 100, 50 and 25) were prepared. Twenty five healthy mosquito larvae were placed in each plastic cup containing 150 mL of water and the test concentration. Four replications for each concentration and a control

(with water) were tested for larval bioefficacy. The larval mortality at different concentrations and in the control was counted after exposure for 24 hr.

Statistical analysis

The mortality data were subjected to log probit regression analysis (Finney, 1971) to determine the median lethal concentrations to kill 50% of the treated larvae (LC_{50}). The percentage of larval mortality was calculated and when the control mortality ranged from 5 to 20%, it was corrected using the formula of Abbott (1925). To determine the difference in larval mortality between concentrations, analysis of variance followed by least significant difference tests were performed using the SPSS software (version 16.0; Statistical Package for Social Sciences, 2007). Results with $P < 0.05$ were considered to be statistically significant.

RESULTS AND DISCUSSION

Chemical composition of essential oils

The essential oils were extracted from a maceration of fresh flowers in petroleum ether. Afterwards, filtration and removal of the solvent yielded volatile crude. The crude was re-dissolved in ethanol, followed by filtration and evaporation to give a semi-solid substance absolute in a yield of 0.02% (volume per weight; v/w) of fresh weight. This extraction procedure showed the scent of the obtained absolute was the closest to the fresh flower of *M. hortensis*. Identification of the aromatic volatile components was undertaken by a comparison of mass spectra with data in the literature (Wiley 275 and NIST 98; see Adams, 1995) and by comparison of their retention indices with those reported in the literature (Chung *et al*., 1993; Leffingwell and Alford, 2005; Pino *et al*., 2005). The 26 extracted compounds are shown in Table 1, each being identified according to its elution time on a capillary column. A typical GC–MS total ion current profile of the aromatic volatiles from the flower of *M. hortensis* is shown in Figure 1. The most abundant compounds found were: solanesol (25.72%), *trans*-farnesol (19.71%), nerolidol (8.54%), *n*-hexadecanoic acid (6.77%), vanillin (6.20%), oleic acid (4.54%), linoleic acid (3.87%), L–linalool (3.37%), 1–octen–3–ol (1.67%), *α*–farnesene (1.22%) and methyl salicylate (1.03%). Integrator raw peak areas were expressed as a percentage of the total chromatographable components of the volatiles. These compounds accounted for approximately 85.84% of the total volatile components. A number of components could not be identified due to the lack of reference spectra and/or their low abundance.

The average yield of *M. hortensis* flower oil obtained by petroleum ether maceration in this study (0.02%) was much lower than the yield (v/w) of 0.87% produced by Kietthanakorn *et al*. (2012), which was prepared using hexane maceration. The current yield was much lower than those of Sittiwet (2009) (0.5–2%), Kietthanakorn *et al*. (2012) (8.57–14.40%) and Babitha *et al*. (2012) (14.05%), which were prepared using vapor distillation, supercritical carbon dioxide fluid extraction and ethanolic extract, respectively. The differences in oil extractions may have resulted from the extraction being obtained from different parts of the tree such as stem, bark and leaves (Jetty and Iyengar, 2000; Kaushik and Saini, 2008; Nagaraja and Padmaa, 2011b). Nagaraja and Padmaa (2011b) reported that extract obtained from bark using soxhlation involved different solvents in increasing order of polarity—petroleum ether, benzene, chloroform, methanol and distilled water—with a yield (weight per weight) of 1.44, 0.52, 0.61, 15.91 and 2.33 %, respectively.

The yield of essential oils from the same species is different depending on the method of extraction and source, genetic characteristics of the plant, climatic and geographic conditions (Martins *et al*., 1997; Vieira and Simon, 2000; Tawatsin *et al*., 2001; Wandscheer *et al*., 2004; Jalal *et al*., 2009).

Table 1 Chemical composition of *M. hortensis* flower extract as determined by gas chromatography coupled to mass spectrometry analysis

ID No.	Compound	RA (%)	RI (Exp)	RI (Lit)	MW	Identification*
1	1–Octen–3–ol	1.67	974	974[T]	128	1,2
2	3-Octanol	0.13	993	988 [T]	130	1,2
3	*cis*–Linalool oxide (furanoid)	0.09	1039	1067 [T]	170	1,2
4	*L*–Linalool	3.37	1043	1095 [T]	154	1,2
5	Nonanal	0.13	1104	1100 [T]	142	1,2
6	Phenylethyl alcohol	0.07	1112	1106 [T]	122	1,2
7	*cis*–Linalool oxide (pyranoid)	0.09	1172	1170 [T]	170	1,2
8	Methyl salicylate	1.03	1195	1090 [T]	152	1,2
9	Geraniol	0.09	1252	1249 [T]	154	1,2
10	2–Methyl naphthalene	tr	1291	1295 [T]	142	1,2,3
11	1–Methyl naphthalene	tr	1308	1312 [T]	142	1,2,3
12	Unidentified	tr	1391			1,2
13	Vanillin	6.20	1395	1393 [T]	152	1,2
14	1,6–Dimethylnaphthalene	0.12	1414		156	1,2
15	Isoeugenol	0.21	1447	1448 [T]	164	1,2
16	Unidentified	0.16	1451			
17	Unidentified	0.08	1469			
18	Unidentified	0.72	1478			
19	*α*–Farnesene	1.22	1507	1505 [T]	204	1,2
20	Nerolidol	8.54	1563	1561 [T]	222	1,2
21	*cis*–Farnesol	0.17	1694	1698 [T]	222	1,2
22	*trans*–Farnesol	19.71	1740	1742 [T]	222	1,2
23	Unidentified	0.10	1840			
24	Unidentified	0.19	1917			
25	*n*-Hexadecanoic acid	6.77	1967	1959 [T]	256	1,2
26	Hexadecanal	0.18	2017		240	1,2
27	Methyl linoleate	0.55	2091	2095 [T]	294	1,2
28	Heneicosane	0.32	2097	2100 [T]	296	1,2
29	Phytol	0.75	2110	2111 [T]	296	1,2,4
30	Linoleic acid	3.87	2133	2132 [T]	280	1,2
31	Oleic acid	4.54	2141	2141 [T]	282	1,2
32	Stearic acid	0.30	2162	2172 [T]	284	1,2,5
33	Solanesol	25.72	2191		631	1
34	Unidentified	4.75	2197			
35	Unidentified	0.47	2210			
36	Unidentified	1.85	2215			

ID No. = Identification number used in Figure 1.

RA = % Relative peak area.

RI (Exp) = Program temperature retention indices as determined on HP–5MS column using a homologous series of n-alkanes (C8–C22) as internal standard and He as the carrier gas.

RI (Lit) = Value from Adams (1995).

T = Program temperature values; MW : Molecular weight.

* = 1, Based on retention index; 2, Based on comparison of mass spectra with literature data (Wiley 275, NIST 98, see Adams, 1995); 3, Leffingwell and Alford (2005); 4, Chung *et al*. (1993); 5, Pino *et al*. (2005).

tr = Trace present only.

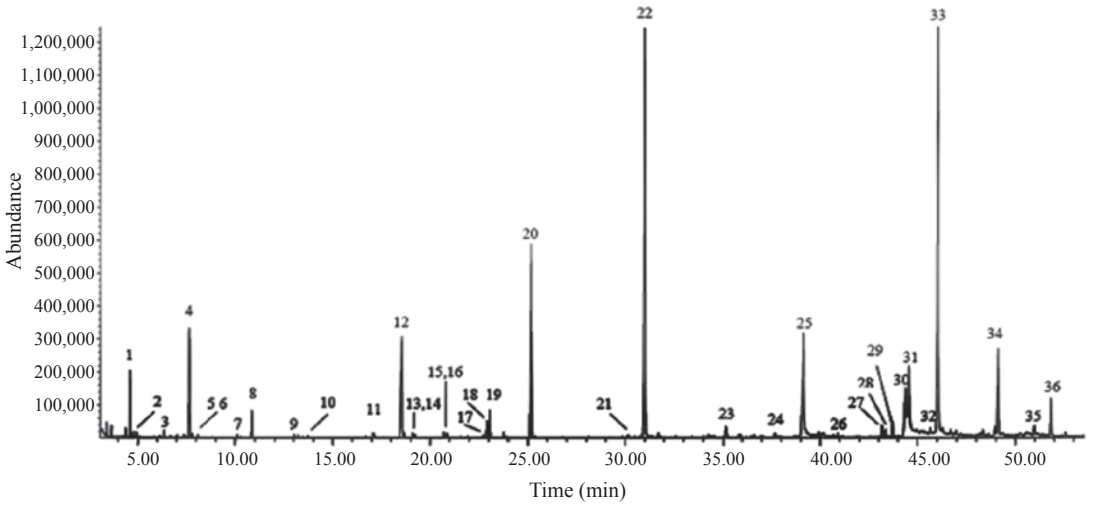

Figure 1 Gas chromatography mass spectrometry (GC–MS) total ion current profile of the aromatic volatile oil from fresh flowers of *M. hortensis.* See GC–MS analysis conditions and Table 1 for peak number identification.

Larvicidal activity of essential oils

The oil extracted had a promising larvicidal efficacy on *Ae. aegypti* after 24 hr exposure with an LC_{50} value of 208.5 ppm (Table 2). No larval mortality was observed in the control and untreated groups, while dose-dependent larval mortalities were substantial in the extract-treated groups. Increasing concentrations from 25 to 500 ppm were evident (Table 2). The highest mortality percentage (98%) of larval mosquito was at 500 ppm, followed by 250, 100, 50 and 25 ppm (46, 13, 4 and 2% larval mortalities), respectively.

The analysis of variance F-test showed that there was a significant difference among the concentration groups ($P < 0.05$). Differences between means using the least significant difference tests were also considered significant at the 95% level of confidence as shown in Table 2.

The larvicidal bioassay exhibited a promising efficacy of plant oils extracted from *M. hortenis* flowers against *Ae. aegypti.* The results conformed with Kaushik and Saini (2008), who used concentrations between 25 and 500 ppm, prepared by soxhlation using acetone as the solvent. The flower extract was significantly less effective (LC_{50} = 208.5 ppm) when compared to its leaf extract with ethtanolic extraction (maceration) (LC_{50} = 123 ppm) (Kaushik and Saini, 2009).

The susceptibility of *Ae. aegypti* larvae to a graded series of extracted essential oils under laboratory conditions was dose dependent. Mortality increased when exposed to a higher concentration. According to Kaushik and Saini (2008), the toxic effect of this plant oil was probably on the neuromuscular system resulting in abnormal behavior of the treated larvae (restlessness, sluggishness and coiling movement).

These is great medical importance in controlling a main vector of viral diseases including dengue fever, dengue hemorrhagic fever and Chikungunya fever, which are serious health problems in Thailand and other developing countries (Guzman and Kouri, 2002; Jansen *et al*., 2008). There is the possibility of developing new types of mosquito larvicides from essential oils for application in mosquito control programs. *M. hortensis* has potential usefulness for other arthropod vector control and should be selected for further study, particularly in controlling dengue and other mosquito-borne diseases.

Table 2 Larvicidal activity of extracted essential oils against third to fourth instar larvae of *Ae. aegypti*.

Concentration (parts per million)	Number tested	Mortality (%)	LC_{50} (parts per million)	Regression#	r	R^2
Control	100	0[d]				
25	100	2[d]				
50	100	4[cd]				
100	100	13[c]				
250	100	46[b]	208.5	Y = 20.089X + 4.935	0.998	0.996
500	100	98[a]				
F–test		*				
$LSD_{0.05}$		10.50				
Coefficient of Variation (%)		25.91				

[a-d] = Mean values with the same superscript lowercase letter are not significantly different at $P < 0.05$.

[#] = Regression equation (Y); plant extract concentration (X) at 24 hr.

LC_{50} = Median lethal concentrations to kill 50% of the treated larvae in 24 hr.

$LSD_{0.05}$ = Least significant difference

r = Correlation coefficient of mosquito larvae mortality and plant extract concentration.

R^2 = Regression coefficient.

* = Significant difference ($P < 0.05$).

CONCLUSION

The aromatic volatile components from fresh flowers of *M. hortensis* were extracted using maceration and a non-polar organic solvent (petroleum ether), followed by the polar organic solvent ethanol. The volatile absolute yield of the maceration was 0.02% based on the dried weight of the plant. Of 36 compounds extracted, 27 could be identified using the GC–MS technique. The three most abundant components were solanesol (25.72%), *trans*-farnesol (19.71%) and nerolidol (8.54%). A larvicidal bioassay was made to evaluate the larvicidal activity of plant extract against *Ae. aegypti*. The essential oils displayed good larvicidal properties with an LC_{50} value of 208.5 ppm. Based upon the mortality rate, the concentration of 500 ppm had the highest insecticidal efficacy on mosquito larvae (98% after 24 hr exposure). Larvicidal activity increased with increased dosage in all trials. The results reported here open the possibility of further investigations of the efficacy of the larvicidal property of natural product extract. The most suitable procedures should be determined, including the conditions for preparation and extraction in order to achieve the best yield. The quality and quantity of extracts, especially active larvicidal ingredients, need to be initiated.

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