Phytochemical and Mosquito Larvicidal Properties of *Millingtonia hortensis* L.f.

Chalermporn Thongpoon, and Pisit Poolprasert

Abstract**—**The phytochemical composition and larvicidal efficacy of essential oils extracted from *Millingtonia hortensis* flowers against *Aedes aegypti* were investigated. The essential oils of fresh *M. hortensis* flowers were extracted by maceration in petroleum ether, provided yields of oils about 0.02% (v/w) of dry weight. The aromatic volatile components of the essential oils were analyzed by gas chromatography coupled to mass spectrometry (GC-MS). Out of 37 compounds, twenty seven have been identified and accounted for 85.84% of the chromatographable components. These included solanesol (25.72%), *trans*-farnesol (19.71%), nerolidol (8.54%), palmetic 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%). The larvae (3^{rd}-4^{th}) exhibited LC_{50} (24 hours) ca. 208.5 ppm. The concentration at 500 ppm of extract showed the highest effectiveness in controlling the larvae with 98% mortality after 24 hours exposure.

Keywords**—**Phytochemistry, essential oil, mosquitocidal activity, *Millingtonia hortensis*.

I. Introduction

MOSQUITOES are prominent hematophagous parasites or bloodsuckers that annoy man, birds and many other animals. They are the most efficient vectors which transmit pathogens causing human malaria, dengue, yellow fever, filariasis, viral encephalitis and other mortal diseases [1]–[3]. Interestingly, *Aedes aegypti* Linn. (F. Culicidae) is involved in dengue transmission, which is a seriously problem in several tropical and subtropical countries [4], [5]. Generally, *Ae. aegypti* breeds in household man-made water-storage containers and preferentially feeds indoors, particularly in the morning hours and in the late afternoon [6], [7]. In Thailand, *Aedes* mosquitoes are the major vectors since there is presently no effective vaccine against dengue. The prevention and control of dengue virus transmission depends on their eradication through two principal measures larviciding and using insecticides [8], [9]. 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 [10]. 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 *Ae. aegypti*. The evolution of pytethriod resistance in this mosquito indicates the limitation of new pyrathorid 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 [11], are attractive alternative.

Plant extracts, especially essential oils have been used as minor natural resource of insecticides. They constitute a good source of bioactive compounds that are biodegradable into nontoxic products. This minimizes the accumulation of harmful residues and making them more environmentally friendly compared to synthetic compounds [12]. Botanical insecticides used for their larvicidal properties against *Anopheles stephensi*, *Culex quinquefasciatus* and *Aedes aegypti* are ecofriendly [13]. 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.

Millingtonia hortensis L.f. (F. Bignoniaceae), a native deciduous tree ranges from India, Myanmar, Thailand and south China, is often cultivated as an ornamental tree in yards, gardens and avenues [14]. 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 has been used as traditional medicine by Indians for treatment of a variety of conditions [15]. Dried flowers of this plant have been used for cigarette ingredients to give sweet aroma and scent for relaxation in Thailand. It has been used in Thai folklore for the treatment of asthma, sinusitis, cholagogue, tuberculosis and a tonic [16]–[18]. Alkaloids, tannins, flavonoids and phenolic compounds are the most important chemically active constituents of this tree [14]. Some flavonoids from this plant have been isolated and characterized, including two main flavones; hispidulin (6-methoxy-5,7,4’-trihydroxyflavone) and hortensin 3,4’-

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dihydroxy-6,7-dimethoxyflavone) [17], [19]. The leave extracts of *M. hortensis* showed good antifungal activity [21], bacterial activity [18], [21]–[23], larvicidal activity [13], antiproliferation activity [24], and antioxidant activity [25], [26].

In this study, we investigated the composition of essential oils using a chromatography/mass-spectrometer (GC-MS) and the efficacy of essential oils and petroleum ether extract from *M. hortensis* flowers against larval stages of *Ae. aegypti*.

II. Materials And Methods

A. Mosquito rearing

The 3rd-4th instar larvae of laboratory-reared *Aedes aegypti* were established in the insectarium of the Program Biology, Faculty of Science and Technology, Pibulsongkram Rajabhat University, Pitsanilok, Thailand and maintained under controlled insectary condition 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 expedience in rearing and maintaining the life cycle. Its sensitivity to larvicides makes *Ae. aegypti* larvae a good indicator of biocidal activity.

B. Plant material

Fresh flowers of *Millingtonia hortensis* were gathered from Pibulsongkram Rajabhat University, Phitsanulok province, Thailand in Noverber 2012. Plant specimen was identified by comparison with reference material at the CMU herbarium, Faculty of Science, Chiang Mai University, Chiang Mai province, Thailand.

C. Extraction of essential oil

Fresh flowers of *Millingtonia hortensis* (10 kg) were macerated with petroleum ether (5 L) as solvent for 12 hours, followed by filtration and then removing 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 absolute in a yield of 0.02%. Some of this was used for identification of the chemical compounds and the others were used for the bioassay. The water extracts were used directly for the bioassay.

D. GC-MS analysis

The volatile constituents of Millingtonia hortensis were analysed using an HP model 6890 gas chromatograph equipped with an HP-5MS (5% phenyl-polymethylsiloxane) capillary column (30 m × 0.25 mm i.d., film thickness 0.25 μm; Agilent Technologies, 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 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 1 mL/min. EI mass spectra were collected at 70 eV ionisation voltages over the range of m/z 29-300. The electron multiplier voltage was 1150 V. The ion source and quadrupole temperatures were set at 230 and 150 °C, respectively.

E. Identification of the compounds

Identification of the volatile components was performed by comparison of their Kovat retention indices, relative to C_8-C_{22} n-alkanes, and comparison of the mass spectra of individual components with the reference mass spectra in the Wiley 275, NIST 98 databases [27].

F. Larvicidal bioassy

According to WHO recommendations [28], 3rd-4th instar larvae of Aedes aegypty were exposed to extracted oils at various concentrations. Five different extract solution each with concentration ranging from 500 to 25 ppm (500, 250, 100, 50 and 25) were prepared. Twenty five healthy mosquito larvae were placed in each plastic cups containing 150 ml of water and the test concentration. Four replication for each concentration and control (with water) were tested for larval bioefficacy. The larval mortality at different concentrations and in the control was counted after 24 hours exposure.

G. Statistical analysis

The mortality data were subjected to log probit regression analysis [29] to determine the median lethal concentrations (LC_{50}) to kill 50 percent of the treated larvae. The percentage of larval mortality was calculated and when control mortality ranged from 5-20% it was corrected using Abbott's [30] formula. To determine the difference in larval mortality between concentrations, ANOVA followed by LSD tests were performed by using SPSS for windows version 16.0 [31]. Results with $P < 0.05$ were also considered to be statistically significant.

III. Results And Discussion

A. Chemical composition of the essential oils

The essential oils were extracted by 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 semi-solid substance absolute in a yield of 0.02% (v/w) of dry 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 under taken by a comparison of mass spectra with literature data (Wiley 275, NIST 98) and by comparison of their retention indices (RI) with those reported in the literature [32]–[34]. The extracted compounds are shown in Table I, each being identified according to their elution time on a capillary column. A typical GC-MS TIC profile of the aromatic volatiles from *M. hortensis* flower is shown in Fig. 1. The most abundant compounds found were solanesol (25.72%), *trans*-farnesol (19.71%), nerolidol (8.54%), palmetic 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 total volatile components. A number of components could not be identified due to the lack of reference spectra and/or their low abundance.

TABLE I
CHEMICAL COMPOSITION OF *M. HORTENSIS* FLOWER EXTRACT AS DETERMINED BY GC-MS ANLYSIS.

No	Compound	RA[a] (%)	RI[b] (exp)	RI[c] (Lit)	MW	Id[d]
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			

[a]RA, % Relative peak area
[b]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.
[c]RI (lit): Value from Adams [27].

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*. [35], which was prepared by hexane maceration. Our yield was much lower than those of Sittiwet [18] (0.5-2%), Kietthanakorn *et al*. [35] (8.57-14.40%) and Babitha *et al*. [26] (14.05%), which were prepared by vapor distillation, supercritical carbon dioxide fluid ($scCO_2$) extraction and ethanolic extract, respectively. The differences in oil extractions obtained from different parts of this tree such as stem, bark and leaves [13], [21], [23]. Nagaraja and Padmaa [23] reported that the extract was obtained from bark by soxhlation, which was done with different solvents in increasing orders of polarity, i.e. petroleum ether, benzene, chloroform, methanol and distilled water with a yield (w/w) of 1.44, 0.52, 0.61, 15.91, and 2.33 %, respectively.

The yield of essential oils in the same species is different depending on the method of extraction and source, genetic characteristics of the plant, climatic and geographic conditions [36]– [40].

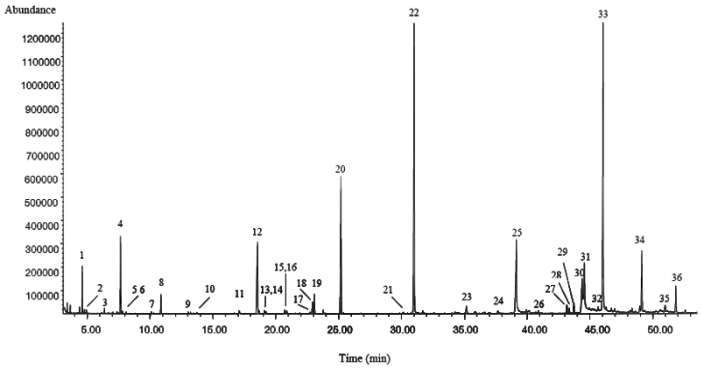

Fig. 1 GC-MS TIC profile of the aromatic volatile oil from fresh flowers of *M. hortensis.* See GC-MS analysis condition and Table I for peak identifications.

B. Larvicidal activity of the essential oils

The oil extracted examined in our study had a promising larvicidal efficacy on *Ae. aegypti* after 24 hours exposure with an LC_{50} of 208.5 ppm (Table II). No larval mortality was observed in the control and untreated groups, whereas dose dependent larval mortalities were substantial in the extract treated groups. Increasing concentrations from 25 to 500 ppm were evident (Table II). 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).

The analysis of variance (ANOVA) F-test shows that there was significant difference in means between the concentration groups ($P < 0.05$). Differences between means using the least significant difference (LSD) tests were also considered significant at the 95% level of confidence. The results indicated that means with the same letter were not significantly different as shown in Table II.

The larvicidal bioassay exhibited a promising efficacy of plant oils extracted from *M. hortenis* flowers against *Ae.*

aegypti. Our results conformed with Kaushik and Saini [13], who used concentrations between 25 to 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) [41].

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 concentrations. According to Kaushik and Saini [13], the toxic effect of this plant oil was probably on the neuromuscular system resulting in abnormal behavior of the treated larvae i.e. restlessness, sluggishness and coiling movement.

TABLE II
LARVICIDAL ACTIVITY OF EXTRACTED ESSENTIAL OILS AGAINST 3RD-4TH INSTAR LARVAE OF *AE.AGYPTI*. MEANS WITH THE SAME LETTER WERE NOT SIGNIFICANTLY DIFFERENT.

Conc. (ppm)	Mortality (%)	LC_{50} (ppm)	Regression[1/]	r[2/]	R^{2}[3/]
Control	0[d]				
25	2[d]				
50	4[cd]				
100	13[c]				
250	46[b]	208.5	Y = 20.089X + 4.935	0.998	0.996
500	98[a]				
F-test	*				
$LSD_{0.05}$	10.50				
C.V. (%)	25.91				

[1/] = Regression equation (Y): plant extract concentration (X) at 24 hours
[2/] r = Correlation Coefficient of mosquito larvae mortality and plant extract concentration
[3/] R^2 = Regression Coefficient
* = Significant difference ($P < 0.05$)

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 [5], [42]. There is 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.

IV. CONCLUSION

The aromatic volatile components from fresh flower of *M. hortensis* were extracted by maceration using 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 dried weight of the plant. Of 36 compounds extracted, 27 could be distinctly presumed by 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 property with the LC_{50} of 208.5 ppm. Based upon the mortality rate, the concentration of 500 ppm had the highest insecticidal efficacy on mosquito larvae with 98% after 24 hours of exposure. Larvicidal activity increased with increased dosage in all trials. The results reported here open the possibility of further investigations of efficacy on its larvicidal property of natural product extract. The most suitable procedure, conditions in preparation and extraction in order to achieve the best yield should be determined. The quality and quantity of extracts, especially active larvicidal ingredients, need to be studied.

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