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Research Article

Chemical Constituents and Combined Larvicidal Effects of Selected Essential Oils against *Anopheles cracens* (Diptera: Culicidae)

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A preliminary study on larvicidal activity against laboratory-colonized *Anopheles cracens* mosquitos revealed that five of ten plant oils at concentration of 100 ppm showed 95–100% larval mortality. The essential oils of five plants, including *Piper sarmentosum, Foeniculum vulgare, Curcuma longa, Myristica fragrans*, and *Zanthoxylum piperitum,* were then selected for chemical analysis, dose-response larvicidal experiments, and combination-based bioassays. Chemical compositions analyzed by gas chromatography coupled to mass spectrometry demonstrated that the main component in the oil derived from *P. sarmentosum, F. vulgare, C. longa, M. fragrans, and Z. piperitum* was croweacin (71.01%), anethole (63.00%), ar-turmerone (30.19%), safrole (46.60%), and 1,8-cineole (21.27%), respectively. For larvicidal bioassay, all five essential oils exerted promising efficacy in a dose-dependent manner and different performances on *A. cracens* after 24 hours of exposure. The strongest larvicidal potential was established from *P. sarmentosum*, followed by *F. vulgare, C. longa, M. fragrans*, and *Z. piperitum*, with LC_{50} values of 16.03, 32.77, 33.61, 40.00, and 63.17 ppm, respectively. Binary mixtures between *P. sarmentosum*, the most effective oil, and the others at the highest ratio were proved to be highly efficacious with a cotoxicity coefficient value greater than 100, indicating synergistic activity. Results of mixed formulations of different essential oils generating synergistic effects may prove helpful in developing effective, economical, and ecofriendly larvicides, as favorable alternatives for mosquito management.

1. Introduction

Presently, the risk of contracting arthropod-borne diseases has increased due to the climate change and intensifying globalization [1]. Malaria, a life-threatening disease transmitted by mosquitoes, is continuing to be a major public health problem causing death and illness in children and adults around the world, especially in tropical countries. About 3.3 billion people—half of the world's population—are at risk of malaria. Every year, this leads to about 250 million malaria cases and nearly one million deaths [2]. Malaria control requires an integrated approach, including prompt treatment with effective antimalarials and prevention, primarily based on vector control. However, an inappropriate use of antimalarial drugs in the past century contributed to the increasing and widespread drug-resistant malarial parasites in the endemic areas, leading to rising rates of sickness and death. Therefore, mosquito management has played an essential role in the substantial reduction of malaria. The control of mosquito at the larval stage is necessary and efficient in the integrated approach to mosquito management. Mosquito adulticides, although effective, are often

applied only as a temporary solution to disease outbreaks for transiently minimizing adult populations. Furthermore, in recent years, control of adult mosquitoes has become increasingly difficult because of insecticide resistance and behavioral changes such as the avoidance of mosquito vectors to residual insecticides [3–5]. It is easier and more efficient to control the delicate larvae that are relatively immobile and more concentrated, having not yet left their aquatic breeding sites [6, 7]. Moreover, there has been increasing documentation of resistance of larval populations of anopheline mosquitoes, malaria vectors, to one or more of the main groups of conventional synthetic insecticides, that is, organochlorines, organophosphates, carbamates, and pyrethroids [8–14]. One of the most promising ways of minimizing development of insecticide resistance and reducing negative impacts to human and other living organisms and the environment is applying nonchemical materials, that is, biopesticides that do not confer cross-resistance to current insecticides and are naturally biodegradable into nontoxic [15–18].

Insecticides of botanical origin are attractive alternatives because they contained rich sources and various bioactive compounds, many of which are selective and have little or no harmful effect on nontarget organisms and the environment [19, 20]. Furthermore, the complex and variable mixtures of bioactive constituents with different modes of action may lessen the chance of resistance in mosquito populations [21]. Recently, essential oils have received considerable attention as a potentially useful bioactive insecticide, with their low mammalian toxicity and rapid degradability in the environment [22]. Larvicidal activities have been demonstrated in many plant oils such as neem, basil, cinnamon, citronella, camphor, eucalyptus, lemon, and pine [15, 23–26]. Combined formulations of different essential oils, which have more active substances than individuals, have also been investigated as larvicides, and some mixtures were found to be more effective than neem (*Azadirachta indica*) extract [27, 28]. Neem and neem-based products have been widely acknowledged and currently available as the prominent biopesticides because of their pesticidal potential with larvicidal and growth regulating activity. Nevertheless, if they are used indiscriminately, they may induce resistance in the pests and can be rendered ineffective within a few years [29]. Thus, the finding of new botanical pesticides, particular combinations of two or more toxicants with different mechanisms of action, is the need of the hour. However, a lot more work has been done on the coupled effects of synthetic-synthetic pesticides than plant-synthetic and plant-plant pesticide combinations [30]. Furthermore, most studies on the combined insecticidal efficacy of phytochemical-mixed formulations have been conducted on agricultural pests rather than pests of medical importance [31]. The present study was undertaken, therefore, to investigate the chemical composition and larvicidal efficacy of indigenous plant-derived essential oils and their combinations against *A. cracens*; a potential vector of malaria, with the aim of developing essential oil-mixed larvicides as supplementary and complementary measures for the management of malaria vectors.

2. Materials and Methods

2.1. Plant Materials. Ten plant species belonging to six families, Cyperaceae, Myristicaceae, Piperaceae, Rutacea, Umbelliferae, and Zingiberaceae, which mostly consist of botanicals with promising bioactivity against mosquitoes [31, 32], were selected for screening larvicidal activity against *A. cracens*. The plant materials (Table 1) were collected from natural habitats or commercially obtained from medicinal herb suppliers in Chiang Mai province. The herbarium specimen of each plant was identified and authenticated by botanists and plant taxonomists from the Department of Biology, Faculty of Science and the Pharmaceutical Sciences, Faculty of Pharmacy, Chiang Mai University, Thailand. The voucher specimens were numbered and deposited at the Department of Parasitology, Faculty of Medicine, Chiang Mai University.

2.2. Extraction of the Essential Oils. The plant materials utilized for extracting the essential oil were shade-dried at the environmental temperature (27–36°C) and then separately ground by an electrical blender. Dried and coarsely ground plants were extracted individually by steam distillation at 100°C for at least 3 hours to obtain the ethereal oil. The oil layer was separated from the aqueous phase, filtrated and dried over anhydrous sodium sulfate (Na_2So_4) to remove traces of moisture. Physical characteristics of the oil were recorded and the percentage yield was averaged over three experiments and calculated according to dry weight of the plant materials. The resulting essential oils were subsequently stored in an amber-colored bottle under refrigeration (4°C) until analysis for chemical compositions and larvicidal activity.

2.3. Mosquito Colony Handling. The colony of *A. cracens* [33], formerly *A. dirus* (species B), was obtained originally from the Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok, Thailand. The free-mating populations of this mosquito had been established for more than 2 decades in the insectary of Department of Parasitology, Faculty of Medicine, Chiang Mai University [34]. The mosquito colony was maintained continually without exposure to any pathogens and insecticides under a constant laboratory condition at temperature of 27 ± 2°C and 70–80% relative humidity under a photoperiod of 12 : 12 hours (light/dark). Adults were incessantly provided with 10% sucrose and 5% multivitamin syrup solution in a small bottle with a cotton wick. Rats were supplied as a blood source for egg production of adult females. Eggs were collected and kept in plastic cups lining with moistened filter paper. Larvae were reared in plastic trays on the meal of powdered fish food. Freshly molted larvae (L_4) of *A. cracens* taken from the mass culture were available continuously for the mosquito larvicidal experiments.

2.4. Preliminary Screening for Larvicidal Activity of Essential Oils. Preliminary screening of essential oils derived from various parts of ten plants was carried out at the high concentration, 100 ppm, to check for larvicidal activity. Essential

Table 1: Physical characteristics and percentage yields (% Yield) of essential oils derived from ten plant species.

Family and botanical name (reference number)	English name	Part used	Physical characteristics			% Yield
			Color	Odor	Density (g/mL)	
Cyperaceae						
Cyperus rotundus Linn. (PARA-CY-001/1)	Nut grass	Tuber	Golden yellow	Nut grass-like	0.95	0.42
Myristicaceae						
Myristica fragrans Houtt. (PARA-MY-001/1)	Nutmeg	Mace	Light yellow	Nutmeg-like	0.96	3.41
Piperaceae						
Piper nigrum Linn. (PARA-PI-004/1)	Black pepper	Fruit	Clear	Pepper-like	0.90	0.39
Piper longum Linn. (PARA-PI-001/5)	Long pepper	Fruit	Light yellow	Pepper-like	0.87	0.64
Piper sarmentosum Roxb. (PARA-PI-003/2)	Wild betel	Leaf and stem	Brown	Pepper-like	0.91	0.31
Rutacea						
Zanthoxylum piperitum DC. (PARA-ZA-002/4)	Japanese Prickly Ash	Fruit	Pale yellow	Orange-like	0.74	0.34
Umbelliferae						
Coriandrum sativum Linn. (PARA-CO-002/2)	Coriander	Fruit	Pale yellow	Bug-like	0.86	0.97
Foeniculum vulgare Mill. (PARA-FO-001/3)	Fennel	Fruit	Pale yellow	Anise-like	0.89	0.57
Zingiberaceae						
Amomum uliginosum Koenig (PARA-AM-002/2)	Cardamom	Rhizome	Light yellow	Camphor-like	0.92	0.95
Curcuma longa Linn. (PARA-CU-005/1)	Turmeric	Rhizome	Pale yellow	Ginger-like	0.81	0.56

oil was individually dissolved in a nontoxic emulsifying agent, dimethylsulphoxide (DMSO). Groups of 25 early 4th instar larvae of *A. cracens* were selected and then exposed to the test concentration containing 249 mL of distilled water and 1 mL of essential oil-DMSO solution. Bioassays were set up according to a slightly modified version of the standard WHO larval susceptibility test methods [35] under the similar conditions used for rearing. Four replicates were maintained for the individual oil along with the concurrent control and untreated groups. A control group received DMSO-distilled water, while the untreated one was maintained in distilled water only. Mortalities of treated larvae were determined after an exposure period of 24 hours. The larvae were considered dead if they were unable to move or respond when stimulated by probing with a blunt dissecting needle. Moribund larvae were those incapable of rising to the surface of the water or showing a characteristic diving reaction when the water was disturbed. The moribund and dead larvae in each test were combined and expressed as percentage mortalities, which were corrected for control mortality using Abbott's formula [36].

2.5. Dose-Response Bioassay. Based on the initially larvicidal screening results, the promising oils, which produced 95–100% larval mortalities, were subjected to a dose-mortality response bioassay. Plant oil-DMSO solutions were prepared into different concentrations with distilled water in the range of 10 to 80 ppm, depending on the plant species. The dose response bioassays were carried out as in the screening protocol previously described. Tests were conducted using four batches of 25 larvae with the final total number of 100 larvae for each concentration. Every bioassay was replicated four times with mosquitoes from different rearing batches. The percentage mortality was reported from the average of four replicates.

2.6. Essential Oil-Mixed Formulation Experiment. Combinations comprising various mixing ratios of pairs of the most effective and the other oils established from the dose-response experiments were evaluated against *A. cracens*, as previously done, to determine whether these mixtures increase larvicidal efficacy compared with the constituted oil

Table 2: Chemical constituents of essential oils derived from five plants.

No.	Constituent	RT	Percentage composition (%)				
			P. sarmentosum	*F. vulgare*	*C. longa*	*M. fragrans*	*Z. piperitum*
1	α-Thujene	7.12				0.67	
2	α-Pinene	7.27				0.98	1.40
3	Sabinene	8.13				14.25	6.13
4	β-Pinene	8.20				0.52	
5	β-Myrcene	8.48				1.07	3.08
6	Phellandrene	8.76				0.81	
7	α-Terpinene	8.99				1.11	
8	p-Cymene	9.15			0.87	1.52	4.42
9	α-Limonene	9.23		2.07			12.03
10	β-Terpinene	9.24				16.13	
11	1,8-Cineole	9.29			0.91	0.66	21.27
12	γ-Terpinene	9.79				2.66	
13	p-Mentha-1,4-diene	9.97				0.72	
14	α-Terpinolene	10.33				0.65	
15	Fenchone	10.35		8.90			
16	Linalool	10.52				0.67	6.10
17	Thujene	10.92					0.83
18	1-Terpinen-4-ol	11.85				6.56	4.74
19	2-Allyltoluene	11.97					0.86
20	Cryptone	12.01					3.15
21	α-Terpineol	12.06				0.57	5.48
22	Estragole	12.16		5.70			1.54
23	Cuminal	12.83					0.68
24	3-Carene	12.98					2.96
25	4-Anisaldehyde	13.04		16.29			
26	Piperitone	13.05					7.31
27	Anethole	13.50		63.00			
28	Safrole	13.56				46.60	
29	Limonene	14.36					8.50
30	Geraniol	14.76					1.21
31	α-Copaene	14.78	3.77				
32	p-Acetonylanisole	14.84		1.16			
33	β-Elemene	14.98	0.70				
34	Methyleugenol	15.06				2.80	
35	β-Caryophyllene	15.40	7.38		1.58		
36	α-Humulene	15.84	0.80				
37	γ-Muurolene	16.09	0.48				
38	α-Curcumene	16.12			9.53		
39	d-Germacrene	16.19	1.22				
40	β-Selinene	16.26	1.56				
41	Zingiberene	16.27			3.93		
42	α-Selinene	16.37	1.56				
43	β-Bisabolene	16.44			2.25		
44	α-Amorphene	16.58					0.70
45	β-Sesquiphellandrene	16.64			8.55		
46	Croweacin	16.67	71.01				
47	Elemicin	16.96	2.47			1.03	
48	Farnesol	17.07	0.44				

Table 2: Continued.

No.	Constituent	RT	Percentage composition (%)				
			P. sarmentosum	*F. vulgare*	*C. longa*	*M. fragrans*	*Z. piperitum*
49	Caryophyllene oxide	17.46					1.45
50	Aromadendrene	17.47	0.77				
51	α-Cedrene	17.71			0.75		
52	γ-Gurjunene	18.24	0.61				
53	β-Maaliene	18.26	0.52				
54	ar-Turmerone	18.32			30.19		
55	Tumerone	18.36			19.02		
56	Brevifolin	18.42					6.15
57	Curlone	18.73			13.30		
	Total identified		93.29	97.12	90.88	99.98	99.99

RT: Retention time (min).

alone. The combined action of essential oils individually in the oil-mixed formulation was decided on the basis of LC_{50} value of each oil and cotoxicity coefficient (CTC) of mixtures.

2.7. GC/MS Analysis of the Effective Plant Oils. GC/MS analysis was carried out to identify the chemical constituents of the effective plant oils. Essential oils demonstrating highly larvicidal activity against *A. cracens* were subjected to analysis by using an Agilent 7890 GC system 5975 MSD, performing under the following conditions: carrier gas helium (1.0 mL/min), diluter dichloromethane (1/10, v/v), and injector temperatures 250°C using a capillary column (HP5MS 30 m × 0.25 mm, ID × 0.25 μm film thickness). The sample (0.5 μL) was injected neat with a split ratio of 250 : 1. The initial oven temperature was 50°C (hold 4 min) with a 10°C/min dynamic ramp to 250°C. Identification of oil constituents was made by comparison of mass spectra of each peak with those of authentic samples in a mass spectra Wiley 8N08 GC/MS library. Relative percentage amount of the identified compound was computed from a total ion chromatogram (TIC).

2.8. Data Management and Statistical Analysis. In all cases where deaths had occurred in the control experiment, the mortality data was corrected by Abbott's formula [36] and then determined by computerized probit analysis (Harvard Programming; Hg1, 2). Larvicidal activity was reported as LC_{50}, LC_{95}, and LC_{99} values along with corresponding 95% confidence intervals (CI), representing the concentrations that induced 50, 95, and 99% mortality, respectively. Values were considered to be significantly different ($P \leq 0.05$) if CI were nonoverlapping. A cotoxicity coefficient (CTC) for mixed formulation experiments, which is based on the lethal concentration and the proportion of each oil component in the mixture, was used to determine their responses: similar, synergism, and antagonism. When CTC of a mixture is 100, it indicates the probability of similar (additive) action. If the mixture gives a CTC greater than 100, it indicates a synergistic action. On the other hand, when a mixture gives a CTC less than 100, it is considered antagonism [37–39]. If a mixture (M) formulation of two oils (A and B), and both components have LC_{50}, then the following formulas are used (A serving as standard):

$$\begin{aligned}
&\text{Toxicity index (TI) of A} = 100,\\
&\text{Toxicity index (TI) of B} = \frac{LC_{50}\text{ of A}}{LC_{50}\text{ of B}} \times 100,\\
&\text{Actual TI of M} = \frac{LC_{50}\text{ of A}}{LC_{50}\text{ of M}} \times 100,\\
&\text{Theoretical TI of M} = \text{TI of A} \times \%\text{ of A in M}\\
&\qquad\qquad + \text{TI of B} \times \%\text{ of B in M},\\
&\text{Cotoxicity coefficient (CTC)}\\
&\qquad\qquad = \frac{\text{Actual TI of M}}{\text{Theoretical TI of M}} \times 100.
\end{aligned} \tag{1}$$

If one component of the mixture alone (e.g., B) causes low mortality at all doses (<20%), then CTC of the mixture was calculated by the formula:

$$\text{Cotoxicity coefficient} = \frac{LC_{50}\text{ of A alone}}{LC_{50}\text{ of A in the mixture}} \times 100. \tag{2}$$

3. Results and Discussion

Steam distillation of ten medicinal plants yielded from 0.31 to 3.41% (v/w) essential oils according to dry weight (Table 1). The highest oil content was found in *M. fragrans* (3.41%), followed by *C. sativum* (0.97%), *A. uliginosum* (0.95%), *P. longum* (0.64%), *F. vulgare* (0.57%), *C. longa* (0.56%), *C. rotundus* (0.42%), *P. nigrum* (0.39%), *Z. piperitum* (0.34%), and *P. sarmentosum* (0.31%). The physical and organoleptic properties of these oils presented in Table 1 demonstrate the slight differences in appearance, color, odor, and density. These volatile oils had a characteristic smell and were clear, yellow, and brown liquids that were less dense than water.

In the larvicidal screening experiment, of the essential oils initially tested at a concentration of 100 ppm, the oils

Table 3: Larvicidal activity of plant-derived essential oils against the 4th instar larvae of *A. cracens*.

Concentration of plant oil (ppm)	% Mortality (mean ± SE)	Larvicidal activity (95% CI, ppm)			Slope values ± SE
		LC_{50}	LC_{95}	LC_{99}	
Piper sarmentosum					
12.7	9.25 ± 3.30				
14.6	23.50 ± 1.29				
16.4	53.75 ± 5.44	16.03 (15.51–16.54)	20.64 (20.01–21.86)	22.91 (22.12–24.66)	14.9920 ± 0.5669
18.2	79.50 ± 2.65				
20.0	94.50 ± 3.11				
Foeniculum vulgare					
22.3	6.50 ± 1.73				
26.7	12.00 ± 4.08				
31.2	41.00 ± 11.83	32.77 (31.44–34.11)	46.56 (44.84–49.83)	53.86 (51.67–58.61)	10.7846 ± 0.3708
35.6	64.75 ± 6.65				
40.1	82.00 ± 3.74				
44.5	94.25 ± 4.99				
Curcuma longa					
20.3	12.50 ± 2.08				
24.3	17.50 ± 3.00				
28.4	23.50 ± 2.65				
32.4	31.00 ± 4.40	33.61 (29.43–39.15)	56.49 (59.66–82.13)	70.04 (79.34–112.48)	7.2941 ± 0.2698
36.5	47.25 ± 5.44				
40.5	83.75 ± 0.96				
44.6	90.50 ± 1.29				
Myristica fragrans					
28.8	10.00 ± 1.15				
33.6	17.75 ± 2.50				
38.4	34.75 ± 4.19	40.00 (37.33–43.32)	56.56 (55.76–67.70)	65.28 (65.35–81.90)	10.9335 ± 0.4652
43.2	63.75 ± 3.86				
48.0	85.75 ± 3.09				
Zanthoxylum piperitum					
51.8	11.75 ± 1.71				
55.5	29.50 ± 5.26				
59.2	35.25 ± 9.22				
62.9	43.50 ± 2.08	63.17 (61.90–64.50)	85.01 (82.59–89.33)	96.13 (92.67–102.67)	12.7574 ± 0.5292
66.6	61.00 ± 4.32				
70.3	73.50 ± 2.08				
74.0	83.00 ± 3.77				

derived from five plants, including *P. nigrum*, *A. uliginosum*, *C. sativum*, *P. longum*, and *C. rotundus* produced no or low larval mortality of 0, 4, 8, 36, and 52%, respectively. No larval mortality was observed in the control and untreated groups. The other oils, including *P. sarmentosum*, *F. vulgare*, *C. longa*, *M. fragrans*, and *Z. piperitum* demonstrated promising efficacy with larval mortality of 100, 100, 100, 100, and 96%, respectively. These five plants were then selected for further experiments, including chemical analysis, dose-response larvicidal experiments, and combination-based bioassays for quantifying their toxicity.

Results of phytochemical analysis of the essential oils with promising larvicidal activity are displayed in Table 2. A total of 57 compounds were identified from five essential oils, including *P. sarmentosum*, *F. vulgare*, *C. longa*, *M. fragrans*, and *Z. piperitum*, representing 90.88–99.99% of the oil obtained. The oil derived from leaf and stem of *P. sarmentosum* contained 14 identified compounds, amounting to 93.29% of the whole oil with croweacin (71.01%) as the chief constituent, together with minor amounts of β-caryophyllene (7.38%), α-copaene (3.77%), and elemicin (2.47%). In the fruit oil of *F. vulgare*, 6 compounds

Table 4: Larvicidal activity and cotoxicity coefficient (CTC) of five essential oils and *P. sarmentosum*-combined oil formulations against the 4th instar larvae of *A. cracens*.

Essential oil	Combination of essential oil	LC_{50} (95% CI, ppm)	Slope values ± SE	Cotoxicity coefficient (CTC)	Effect
P. sarmentosum (P)	P 100%	16.03 (15.51–16.54)	14.9920 ± 0.5669	—	—
F. vulgare (F)	F 100%	32.77 (31.44–34.11)	10.7846 ± 0.3708	—	—
C. longa (C)	C 100%	33.61 (29.43–39.15)	7.2941 ± 0.2698	—	—
M. fragrans (M)	M 100%	40.00 (37.33–43.32)	10.9335 ± 0.4652	—	—
Z. piperitum (Z)	Z 100%	63.17 (61.90–64.50)	12.7574 ± 0.5292	—	—
P + F	P 25% : F 75%	28.60 (28.37–28.83)	17.0907 ± 0.8318	90.8595	Antagonism
	P 50% : F 50%	27.29 (26.09–28.43)	15.0440 ± 0.6262	78.8890	Antagonism
	P 75% : F 25%	18.32 (17.65–18.99)	9.1834 ± 0.4084	100.3105	Synergism
P + C	P 25% : C 75%	27.10 (25.04–28.80)	6.8012 ± 0.2937	97.3354	Antagonism
	P 50% : C 50%	22.08 (21.51–22.61)	14.9567 ± 0.5742	98.3108	Antagonism
	P 75% : C 25%	16.81 (16.59–17.03)	10.8101 ± 0.4357	109.7055	Synergism
P + M	P 25% : M 75%	35.72 (33.51–37.74)	9.7333 ± 0.4288	81.5108	Antagonism
	P 50% : M 50%	28.51 (27.48–29.67)	14.7402 ± 0.7607	80.2797	Antagonism
	P 75% : M 25%	18.18 (17.69–18.64)	12.4666 ± 0.4585	103.7110	Synergism
P + Z	P 25% : Z 75%	41.40 (40.45–42.19)	32.5982 ± 1.4760	87.9356	Antagonism
	P 50% : Z 50%	29.40 (28.33–30.59)	19.9294 ± 0.7629	86.9765	Antagonism
	P 75% : Z 25%	17.99 (16.31–20.45)	6.8213 ± 0.3053	109.5410	Synergism

were identified, representing 97.12% of the oils obtained. Compounds in this oil comprised mostly anethole (63.00%), followed by 4-anisaldehyde (16.29%), with minor contents of fenchone (8.90%), estragole (5.70%), and α-limonene (2.07%). For *C. longa* rhizome oil, 11 compounds were identified, corresponding to 90.88% of the total oil. The major components were ar-turmerone (30.19%), tumerone (19.02%), and curlone (13.30%), whereas α-curcumene (9.53%) and β-sesquiphellandrene (8.55%) were seen as minor constituents. The mace oil of *M. fragrans* demonstrated the presence of 19 compounds, accounting for 99.98% of the whole oil with safrole (46.60%) as the principal constituents, followed by β-terpinene (16.13%), sabinene (14.25%), and 1-terpinen-4-ol (6.56%). Twenty one compounds constituting 99.99% of all the volatile compositions were characterized from *Z. piperitum* fruit oil. The main chemical compounds identified were 1,8-cineole (21.27%) and α-limonene (12.03%), followed by minor quantities of limonene (8.50%), piperitone (7.31%), brevifolin (6.15%), sabinene (6.13%), and linalool (6.10%).

In the dose-response larvicidal assessment, all the oils examined exhibited a promising larvicidal efficacy on larvae of *A. cracens* with dose dependent and different performances among plant species. The strongest larvicidal potential was established from *P. sarmentosum*, followed by *F. vulgare*, *C. longa*, *M. fragrans*, and *Z. piperitum*, with LC_{50} values of 16.03, 32.77, 33.61, 40.00, and 63.17 ppm, respectively (Table 3). Although bioactivity of the essential oil results from interaction among structural components, particularly the major constituents, the other compounds, even trace elements, can also have a vital function; this is due to coupled effects, additive action between chemical classes and synergy or antagonism [40, 41]. Further investigations of comparative toxicity of chemical constituents derived from these plants, either individually or in selected blends, are necessary for identifying components contributing to the observed larvicidal action. Bekele and Hassanali [42] investigated the lethal toxicity of major components derived from essential oils of *Ocimum kilimandscharicum* (camphor, limonene, 4-terpeneol, 1,8-cineole, camphene, and *t*-caryophyllene) and *Ocimum kenyense* (methyl chavicol, ethyl isovalerate, α-humulene, 1,8-cineole, and isoeugenol) against two postharvest insect pests, *Sitophilus zeamais* and *Rhyzopertha dominica*. They discovered that a major compound of *O. kilimandscharicum* was largely responsible for the toxic effect against *R. dominica*. However, the results with the other treatments indicated that the toxic action of the essential oils was due to the combined effects of different components, either with or without significant individual toxic action of their own against the insects. Some of these compounds such as 1,8-cineole, limonene, and humulene are presented in the plant oils tested in this study and also found in other plants with biological activity against various insect species [43–45].

Generally, individual botanical insecticides are slow acting, time consuming, and active only at high concentration, which makes them impractical and uneconomical for field application [27, 46]. Phytochemical-combined formulations, which not only improve activity, but also decrease the needed dose, are therefore considered very advantageous in vector control program. The importance of proper selection of plant extracts as synergists in mixed formulations with different botanicals is being increasingly recognized in mosquito management [30]. Mixtures of more than one insecticide

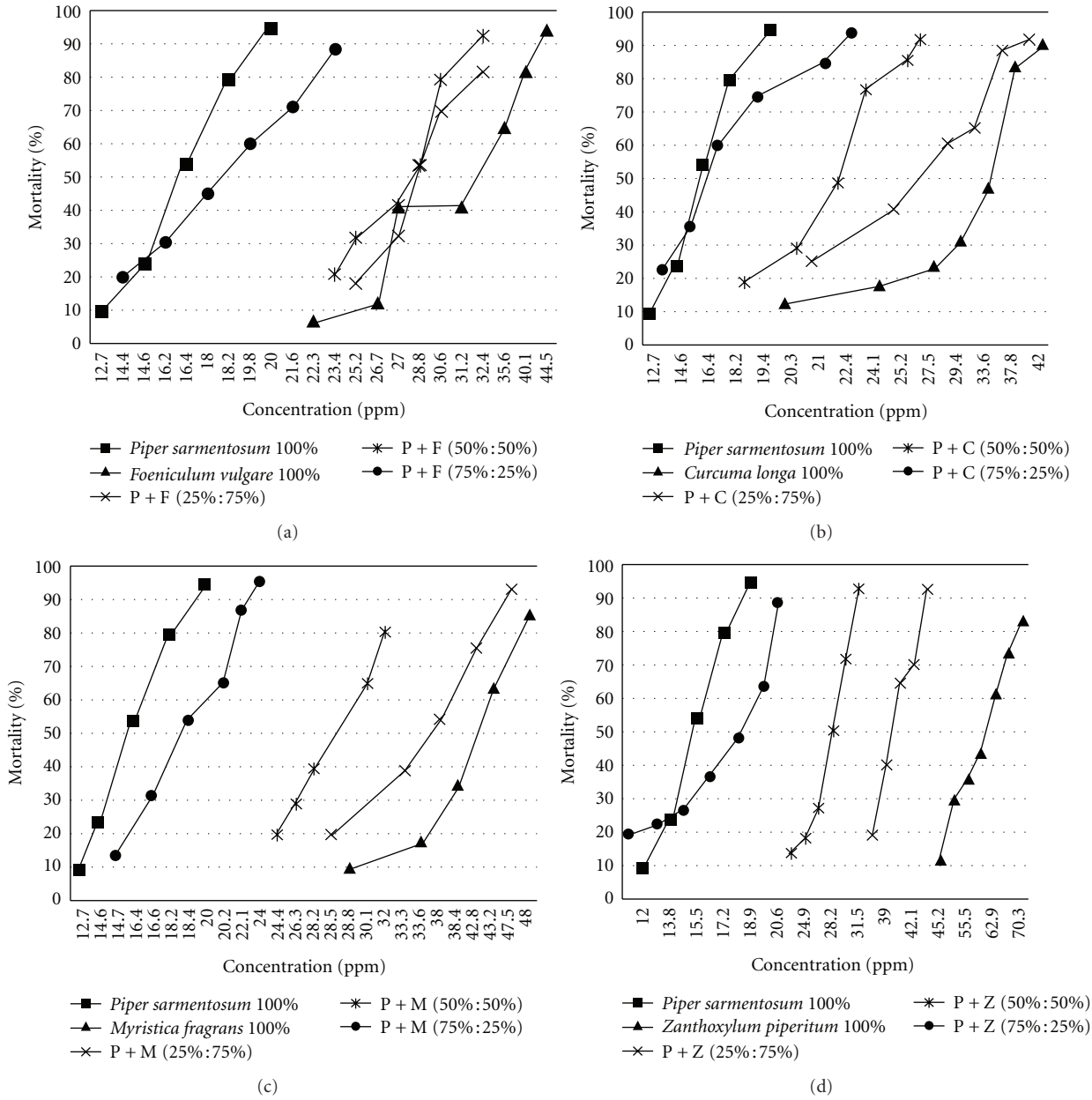

Figure 1: Larvicidal activity of combined formulations between *P. sarmentosum* (P) oil and the other plant oils: (a) *F. vulgare* (F), (b) *C. longa* (C), (c) *M. fragrans* (M), and (d) *Z. piperitum* (Z) against the 4th instar larvae of *A. cracens*.

with different modes of actions are proving to be effective and recommended for integrated resistance management in some insect pests [47–50]. In this study, comparative evaluation of the larvicidal efficacy of combinations between *P. sarmentosum*, the most efficient oil, and the others was carried out and the results are demonstrated in Figure 1 and Table 4. It was found that the addition of *P. sarmentosum* oil to the other individual oils affected the larvicidal activity, leading to increasing mortality of *A. cracens* larvae in all trials. The binary mixtures of oils of *P. sarmentosum* and the others, including *F. vulgare*, *C. longa*, *M. fragrans*, and *Z. piperitum* at the ratios of 25% : 75%, 50% : 50%, and 75% : 25% showed remarkably reduced LC_{50} values, ranging from 18.32–28.60, 16.81–27.10, 18.18–35.72, and 17.99–41.40 ppm, respectively. The cotoxicity coefficient (CTC) determined from these LC_{50} values were ranged from 78.8890–100.3105, 97.3354–109.7055, 80.2797–103.7110, and 86.9765–109.5410, respectively. The combined effect of *P. sarmentosum* and the other oils at the highest ratio (75% : 25%) possessed synergistic activity with a value CTC (relative to LC_{50}) greater than 100. However, all mixtures at the lower ratios (25% : 75% and 50% : 50%) exhibited antagonistic action with a CTC value lower than 100.

In the present study, combinations of *P. sarmentosum* and the other oils exhibited better larvicidal activity than most independent oils. Although the effect at the lower ratios

(25% : 75% and 50% : 50%) was relatively moderate, the larvicidal activity was significantly improved when the mixtures (75% : 25% ratio) contained higher amount of *P. sarmentosum*. Of special interest is in the case of *C. longa*, *Z. piperitum*, and *M. fragrans* oils, which have lower larvicidal efficacy than that of *F. vulgare*; addition of *P. sarmentosum* in these three oils at the highest ratio (75% : 25%) gave a mixture that is more active (LC_{50} = 16.81, 17.99, and 18.18 ppm, resp.) than that of *P. sarmentosum-F. vulgare* mixed formulation (LC_{50} =18.32 ppm). From these findings, it was suggested that combinations between *P. sarmentosum* and the other oils in the appropriate varieties and proportions are beneficial in enhancing larvicidal toxicity toward anopheline mosquitoes. In addition, in the case of *Z. piperitum* oil (2.71 USD/mL), which is approximately three times more expensive than *P. sarmentosum* oil (0.94 USD/mL), combined formulations of these two oils provided not only better efficacy but also lower cost. The synergistic larvicidal activity of combinations between two plant extracts, *Hyptis suaveolens* and *Lantana camara*, was previously reported by Tanprasit [28]. It was revealed that the mixture of *H. suaveolens* and *L. camara* (LC_{50} = 14.04%) possessed significantly higher larvicidal activity against *Aedes aegypti* than those of the individual substances, *H. suaveolens* (LC_{50} = 20.24%) and *L. camara* (LC_{50} = 74.44%). The individual and combined efficacy of *Annona squamosa* and *Pongamia glabra* extracts against three mosquito vectors, *Culex quinquefasciatus*, *Anopheles stephensi*, and *A. aegypti*, compared to that of *A. indica* was investigated by George and Vincent [27]. It was found that *P. glabra* has a greater larvicidal effect than that of *A. squamosa*, and all of their combined formulations exhibited significantly greater effect than those of independent extracts. Furthermore, the most effective mixture of these plant extracts (LC_{50} = 28.804 ppm) was found to be more effective than the prominent biopesticide, *A. indica* (neem) extract (LC_{50} = 45.120 ppm). Singha et al. [51] reported the synergistic effect of *Croton caudatus* (fruit) and *Tiliacora acuminate* (flower) extracts against filarial vector, *C. quinquefasciatus*. The combined formulation of *C. caudatus* and *T. acuminate* exhibited good bioactive potentiality against *C. quinquefasciatus* larvae due to synergism of plant extracts. These findings correspond to those of this study, which presents an insight into the high possibility of developing new mosquitocides from combinations of different essential oils or phytochemicals, generating synergism. Remarkably better performance of *P. sarmentosum* in the essential oil-mixed formulation experiment herein suggests that it may have good potential to be an alternative synergist in efficient mixtures of control agents. This performance may achieve satisfactory levels of efficacy, economic benefit, and ecological friendliness and minimize the development of resistance in the vector population.

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