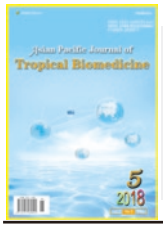

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Larvicidal efficacy of crude and fractionated extracts of *Dracaena loureiri* Gagnep against *Aedes aegypti*, *Aedes albopictus*, *Culex quinquefasciatus*, and *Anopheles minimus* mosquito vectors

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ABSTRACT

Objective: To evaluate the larvicidal efficacy of crude and fractionated extracts of *Dracaena loureiri* endocarp against *Aedes aegypti*, *Aedes albopictus*, *Culex quinquefasciatus*, and *Anopheles minimus* mosquitos. **Methods:** Larvicidal activity was tested according to World Health Organization standard protocol. The third-stage larvae of each mosquito species were exposed to various concentrations of *Dracaena loureiri* crude extract and six groups of *Dracaena loureiri* fractionated extracts (RC-DT 009–014). Larval mortality rates were observed after 24 h and 48 h of exposure. Then, a computerized probit analysis of the mortality data was performed to determine lethal concentration 50 (LC_{50}) and lethal concentration 90 values. **Results:** *Anopheles minimus* larvae (24-h LC_{50} 77.88 mg/L) had the highest susceptibility to crude extract, whereas others (*Aedes aegypti*, 24-h LC_{50} 224.73 mg/L; *Aedes albopictus*, 24-h LC_{50} 261.75 mg/L; and *Culex quinquefasciatus*, 24-h LC_{50} 282.86 mg/L) were significantly less susceptible. The most effective groups of fractionated extracts were RC-DT 012 and RC-DT 013. The mosquito species most susceptible to fractionated extracts was *Culex quinquefasciatus*, with 24-h LC_{50} values of 0.66 and 0.94 mg/L for RC-DT 012 and RC-DT 013, respectively. **Conclusions:** The larvicidal activity of fractionated extracts is more effective than that of crude extract against all tested mosquito species. For the most effective alternative larvicide, purification and a phytochemical constituent analysis must be performed.

1. Introduction

Mosquito-borne diseases remain the biggest health problem for humans worldwide. In Thailand, *Aedes aegypti* (*Ae. aegypti*) and *Aedes albopictus* (*Ae. albopictus*) are the primary vectors for transmitting dengue and dengue hemorrhagic fever[1], *Anopheles minimus* (*An. minimus*) is one of the primary vector for the seasonal outbreaks of malaria[2], and *Culex quinquefasciatus* (*Cx. quinquefasciatus*) transmits Japanese encephalitis[3]. In 2017, the Bureau of Epidemiology, Department of Disease Control Ministry of Public Health in Thailand reported that more than 30 000 Thai

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were infected by those mosquitoes-borne diseases.

Insecticides have traditionally been the first option for controlling outbreaks of vector-borne diseases, owing to their outstanding efficacy[4]. Temephos, the most well-known larvicide, is widely used for controlling the mosquito larvae population[5]. However, continuous use of temephos has led to negative effects on humans. Moreover, reports of temephos-resistant mosquitoes are continuously being published[6–8]. Therefore, plant biosubstances have been the focus of replacement insecticides.

Plant extracts have been a challenging subject with regard to vector control because of the abundance of plant species and human safety issues. One potentially safer alternative is *Dracaena loureiri* Gagnep (*D. loureiri*), commonly known as "*Chan Pha*", "*Chan Daeng*", and "*Lukka Chan*". *D. loureiri* is a folkloric medical plant with antipyretic and analgesic properties that is used in Thailand for the treatment of colds, fever, cough, inflammation, and gastrointestinal disturbances[9,10]. We previously reported on the larvicidal efficacy of crude extract from the endocarp of *D. loureiri* against third-stage larvae of *Ae. aegypti*, in which the 24-h and 48-h lethal concentration 50 (LC_{50}) values were 84.00 mg/L and < 50.00 mg/L, respectively[11]. Thus, we aimed to assess the larvicidal efficacy of crude and fractionated extracts of *D. loureiri* against *Ae. aegypti* and other mosquito species (*i.e.*, *Ae. albopictus*, *Cx. quinquefasciatus*, and *An. minimus*).

2. Materials and methods

2.1. Crude extracts

Crude extracts of *D. loureiri* (voucher number: DTNU008) endocarp were prepared according to the method outlined in the previous study[11]. Briefly, the fruits were collected from naturally growing trees and cleaned with tap water. Their endocarps (2.36 kg) were completely dried in a hot air oven at 45 ℃. The dried endocarps (586.33 g) were ground with an electric blender at 22 000 r/min, and the resulting dried powder was macerated with absolute ethanol at a ratio of 1:10 (powder:solvent, w/v) with 24 h of continuous shaking (180 r/min) on a rotary shaker. The suspension was then filtered through a WhatmanTM No.1 filter paper (GE Healthcare UK Limited, UK) via a Büchner funnel. Afterward, the extracts were evaporated to dryness under reduced pressure to yield crude extract (26.29 g), which was stored in a desiccator.

2.2. Column chromatographic fractionation

The crude extract was fractionated by column chromatography (Merck silica gel 60 PF_{254}, 250 g) using a gradient solvent system of CH_2Cl_2, CH_2Cl_2–MeOH, and MeOH, with increasing amounts of the more polar solvent (mobile phase: 10% MeOH in dichloromethane). After heating at 90–110 ℃ for 4 min, the developing reagent (anisaldehyde reagent, consisting of 3 mL *p*-methoxybenzaldehyde, 2 mL concentrated sulfuric acid, 2 mL water, and 90 mL absolute ethanol) caused organic compounds to emit specific colors, which were examined by thin-layer chromatography. From there, six groups of fractionated extracts were obtained: RC-DT 009 (1.23 g), RC-DT 010 (0.59 g), RC-DT 011 (0.75 g), RC-DT 012 (0.70 g), RC-DT 013 (3.80 g) and RC-DT 014 (1.31 g).

2.3. Mosquito colonization

Ae. aegypti and *Ae. albopictus* colonies were obtained from laboratory strains from the Department of Microbiology and Parasitology, Faculty of Medical Science, Naresuan University, Thailand. *Cx. quinquefasciatus* and *An. minimus* were obtained from laboratory colonies from the Department of Parasitology, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand. The larvae were reared in tap water under laboratory conditions: (25±2) ℃, 70%–80% relative humidity, and 10:14 (light:dark) photoperiod. Larval food consisted of powdery dog biscuits (for *Aedes* and *Culex*) and fish food (for *Anopheles*). After pupation, the larvae were transferred into plastic cups filled with tap water that were placed in mosquito cages (30 cm × 30 cm × 30 cm). After emergence, the adults were provided solutions of 5% sugar mixed with 5% multivitamin syrup. After 5 d, the females were provided blood meal through an artificial membrane feeding method. After blood-feeding, female *Aedes* and *Culex* were reared until gravid and permitted to lay eggs. Meanwhile, blood-fed female *Anopheles* were mated though an artificial mating method[12], after which they were permitted to lay eggs. After the eggs hatched, the larvae were reared according to the above conditions until they were required for bioassays.

2.4. Larvicidal bioassay

The protocol for testing larvicidal activity followed that of our previous study[11]. Briefly, a stock solution of crude and fractionated extracts (1%, w/v) were prepared with dimethyl sulfoxide as the diluent. From the stock solutions, a series of crude and fractionated extract concentrations were prepared (30–190 mg/L and 2–110 mg/L, respectively). Afterward, 200 mL of each concentration of extract was placed into plastic bowls. Twenty-five of the late third-stage larvae were transferred into the extract solutions. Mortality rates were determined after 24 h and 48 h of exposure. Larvae confirmed dead when they were pricked by a needle and not moved. This experiment was performed in quadruplicate (total of 100 larvae for each concentration). Dimethyl sulfoxide in distilled water was used as the control.

2.5. Data analysis

Larval mortality data from the larvicidal bioassays were analyzed using a computerized probit analysis for determination of LC_{50} and lethal concentration 90 (LC_{90})[13]. The *chi*-square values and 95% fiducial confidence intervals [lower confidence limit (LCL) and upper confidence limit (UCL)] were calculated. A commercial LdP Line® software (Plant Protection Research Institute, Egypt) was used.

3. Results

The larvicidal activities of *D. loureiri* crude endocarp extract against *Ae. aegypti*, *Ae. albopictus*, *Cx. quinquefasciatus*, and *An. minimus*

mosquitoes were presented in Table 1. At 24 h, *An. minimus* larvae had the highest susceptibility to crude extract (LC_{50} 77.88 mg/L). Its 24-h LC_{50} was significantly lower than that of *Ae. aegypti* (224.73 mg/L), *Ae. albopictus* (261.75 mg/L), and *Cx. quinquefasciatus* (282.86 mg/L). At 48 h, *An. minimus* was so highly susceptible to crude extract (> 90% mortality rate at 30 mg/L) that we did not calculate the 48-h LC_{50} value, although it was estimated to be < 30 mg/L.

Fractionated extraction by column chromatography produced 188 eluted fractions from the crude extract. The fractions were classified into six groups: RC-DT 009 to RC-DT 014 (Figure 1). All groups were preliminarily screened for larvicidal ability. One concentration (110 mg/L) from each group was tested against the third-stage *Ae. aegypti* larvae. After 24 h of exposure, the RC-DT 012 and RC-DT 013 fractions produced > 90% mortality rates, while the remaining fractions produced 0%–3% mortality rates. For that reason, RC-DT 012 and RC-DT 013 were selected for the bioassays.

The results of larvicidal activity experiments on RC-DT 012 and RC-DT 013 were presented in Tables 2 and 3, respectively. In contrast to results from crude extract, *Cx. quinquefasciatus* (as opposed to *An. minimus*) was extremely susceptible to both fractions. For RC-DT 012, the 24-h LC_{50} and LC_{90} values were 0.66 and 3.29 mg/L, respectively. For RC-DT 013, those values were 0.94 and 2.77 mg/L, respectively. *An. minimus*, *Ae. aegypti*, and *Ae. albopictus* larvae had minor susceptibility to the fractions. However, the mortality rates of all mosquito species were significantly higher for those exposed to fractionated extracts than for those exposed to crude extract.

The LC_{50} and LC_{90} values of the crude and fractionated extracts for each mosquito species were compared and statistically analyzed. Results showed that the larvicidal activities of fractionated extracts were statistically greater than that of the crude extract for all mosquito species. In fact, the only values that were not statistically significant were the 48-h LC_{90} values for *Ae. albopictus* (crude

Table 1

Larvicidal activities of crude ethanolic *D. loureiri* extracts against the third-stage larvae of 4 mosquito vectors.

Mosquito	Concentration (mg/L)	24-hour exposure time				48-hour exposure time			
		Mortality rate (%)	LC_{50} (mg/L) (LCL-UCL)	LC_{90} (mg/L) (LCL-UCL)	χ^2	Mortality rate (%)	LC_{50} (mg/L) (LCL-UCL)	LC_{90} (mg/L) (LCL-UCL)	χ^2
Ae. aegypti	Control	0	224.73 (204.19-267.17)	367.97 (299.11-545.42)	1.532	0	93.37 (89.03-97.58)	156.52 (147.59-167.88)	4.770
	50	0				5.00±3.83			
	70	0				26.00±7.66			
	90	0				45.00±6.00			
	110	2.00±2.31				64.00±3.27			
	130	10.00±4.00				83.00±5.03			
	150	15.00±3.83				90.00±7.66			
	170	21.00±2.83				89.00±6.00			
	190	34.00±6.93				97.00±3.83			
Ae. albopictus	Control	0	261.75 (220.28-369.95)	648.75 (434.17-1 502.71)	1.582	0	134.40 (127.15-142.73)	279.89 (247.85-328.88)	7.404
	50	0				1.00±2.00			
	70	0				14.00±5.16			
	90	5.00±2.00				28.00±8.64			
	110	13.00±2.00				42.00±6.93			
	130	15.00±3.83				48.00±8.64			
	150	24.00±3.27				50.00±8.33			
	170	28.00±3.27				65.00±3.83			
	190	30.00±5.16				74.00±2.31			
Cx. quinquefasciatus	Control	0	282.86 (228.79-426.04)	974.88 (583.32-2 741.42)	3.800	0	82.55 (58.94-97.65)	541.33 (342.47-1 633.50)	9.929
	70	7.00±5.03				49.00±10.00			
	90	10.00±5.16				41.00±8.87			
	110	21.00±5.03				63.00±7.57			
	130	17.00±5.03				70.00±7.66			
	150	28.00±7.30				63.00±10.52			
	170	32.00±5.66				67.00±11.94			
	190	31.00±6.00				71.00±8.87			
An. minimus	Control	0	77.88 (67.84-87.73)	462.98 (344.62-720.30)	8.050	0	-*	-*	-*
	30	31.00±2.00				93.00±5.03			
	50	36.00±3.27				95.00±5.03			
	70	42.00±5.16				92.00±4.62			
	90	50.00±8.33				95.00±3.83			
	110	56.00±3.27				94.00±2.31			
	130	63.00±6.00				96.00±2.00			
	150	69.00±6.83				98.00±2.31			
	170	71.00±8.25				96.00±3.27			
	190	82.00±8.35				97.00±2.00			

Values of mortality rate were expressed as mean±SD. χ^2 *chi*-square test, $P<0.05$ represented significant difference.

*The mortality rates were very high, so the parameters could not be calculated.

Table 2

Larvicidal activities of RC-DT 012 fractionated *D. loureiri* extract against the third-stage larvae of 4 mosquito vectors.

Mosquito	Concentration (mg/L)	24-hour exposure time				48-hour exposure time			
		Mortality rate (%)	LC_{50} (mg/L) (LCL-UCL)	LC_{90} (mg/L) (LCL-UCL)	χ^2	Mortality rate(%)	LC_{50} (mg/L) (LCL-UCL)	LC_{90} (mg/L) (LCL-UCL)	χ^2
Ae. aegypti	Control	0	26.45 (21.39-30.58)	60.87 (54.78-69.45)	4.999	0	18.43 (16.65-20.28)	31.17 (27.96-35.57)	3.002
	10	0				6.00 ± 2.31			
	30	61.00±10.52				91.00 ± 6.83			
	50	77.00±10.00				98.00 ± 4.00			
	70	96.00±3.27				100			
	90	97.00±2.00				100			
	110	99.00±2.00				100			
Ae. albopictus	Control	0	65.98 (56.55-82.81)	234.53 (160.97-431.73)	7.862	0	29.54 (24.86-34.91)	224.29 (141.96-495.02)	3.634
	10	5.00±2.00				27.00±5.03			
	20	11.00±3.83				41.00±8.23			
	30	12.00±3.27				42.00±5.16			
	40	33.00±6.83				58.00±7.66			
	50	43.00±10.52				65.00±11.94			
	60	47.00±8.87				70.00±6.93			
Cx. quinquefasciatus	Control	1.00±2.00	0.66 (0.09-1.19)	3.29 (2.50-4.62)	0.606	1.00±2.00	-*	-*	-*
	2	81.82±9.52				97.98±2.31			
	4	90.91±3.83				96.97±2.00			
	6	96.97±3.83				100			
	8	100				100			
	10	100				100			
An. minimus	Control	2.00±2.31	24.57 (21.41-29.48)	109.72 (75.38-199.29)	3.609	6.00±4.00	6.13 (5.35-6.83)	13.49 (12.08-15.51)	0.454
	5	10.20±3.27				37.23±8.87			
	10	23.47±2.00				77.66±10.52			
	15	26.53±5.66				96.81±2.00			
	20	40.82±7.66				96.81±3.83			
	25	54.08±8.87				98.94±2.00			
	30	59.18±9.80				100			

Values of mortality rate were expressed as mean±SD. χ^2 *chi*-square test, $P<0.05$ represented significant difference.

*The mortality rates were very high, so the parameters could not be calculated.

extract: 279.89 mg/L; and RC-DT 012: 224.29 mg/L). According to the results in this study, fractionated extracts were more effective than crude extract against all tested mosquito species.

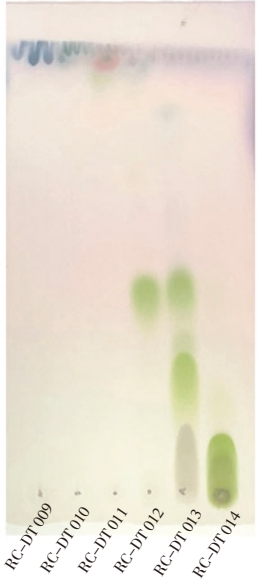

Figure 1. Thin-layer chromatography spots of organic compounds from isolated fractions (RC-DT 009-014) of *D. loureiri*.

4. Discussion

Surprisingly, the crude ethanol endocarp extract of *D. loureiri* had lower activity against *Ae. aegypti* at 24 h (LC_{50} 224.73 mg/L) and 48 h (LC_{50} 93.37 mg/L) than in the previous study (24-h LC_{50} 84.00 mg/L and 48-h LC_{50} < 50 mg/L)[11]. Both studies utilized the same protocol for producing crude extract, so the differences in larvicidal efficacy could be attributed to climate and seasonal difference. That is, the previous study used plants harvested in October 2013[11]; this study used the same plants, but the plants were harvested in September 2016.

Of all mosquito species tested, *An. minimus* showed the greatest susceptibility to *D. loureiri* crude extract. Other species (*Ae. aegypti*, *Ae. albopictus*, and *Cx. quinquefasciatus*) demonstrated a significant, threefold greater tolerance than that of *An. minimus*. Similarly, other studies have found that *Anopheles* larvae are more susceptible to plant extracts than other mosquitoes. For example, Govindarajan *et al.* discovered that *Anopheles stephensi* is more susceptible (LC_{50} 61.65 μg/mL) to *Origanum scabrum* essential oil than *Ae. aegypti* (LC_{50} 67.13 μg/mL), *Cx. quinquefasciatus* (LC_{50} 72.45 μg/mL), and *Culex tritaeniorhynchus* (LC_{50} 78.87 μg/mL)[14]. In addition, *Anopheles stephensi* is more susceptible to *Terminalia chebula* extract than *Ae. aegypti* and *Cx. quinquefasciatus*, with LC_{50} values of 87.13, 93.24, and 111.98 ppm, respectively[15].

Table 3

Larvicidal activities of RC-DT 013 fractionated *D. loureiri* extract against the third-stage larvae of 4 mosquito vectors.

Mosquito	Concentration (mg/L)	24-hour exposure time				48-hour exposure time			
		Mortality rate (%)	LC_{50} (mg/L) (LCL-UCL)	LC_{90} (mg/L) (LCL-UCL)	χ^2	Mortality rate (%)	LC_{50} (mg/L) (LCL-UCL)	LC_{90} (mg/L) (LCL-UCL)	χ^2
Ae. aegypti	Control	0	16.53 (14.20-18.85)	43.62 (37.83-51.88)	6.735	0	-*	-*	-*
	10	23.00±3.83				89.00±8.25			
	30	85.00±6.83				99.00±2.00			
	50	97.00±3.83				100			
	70	98.00±2.31				100			
	90	99.00±2.00				100			
	110	99.00±2.00				100			
Ae. albopictus	Control	0	34.62 (30.77-39.23)	143.85 (108.29-220.16)	8.940	0	14.52 (12.73-16.16)	35.56 (31.64-41.21)	4.940
	10	9.00±3.83				26.00±2.31			
	20	35.00±11.49				76.00±11.78			
	30	48.00±11.31				82.00±6.93			
	40	64.00±5.66				93.00±6.00			
	50	55.00±9.45				95.00±6.00			
	60	66.00±9.52				100			
Cx. quinquefasciatus	Control	1.00±2.00	0.94 (0.37-1.36)	2.77 (2.25-3.43)	0.235	2.00±2.31	-*	-*	-*
	2	81.82±8.33				90.82±3.83			
	4	94.95±5.03				100			
	6	98.99±5.03				100			
	8	100				100			
	10	100				100			
An. minimus	Control	1.00±2.00	20.99 (19.72-22.42)	40.74 (36.10-48.20)	8.274	6.00±4.00	7.73 (7.03-8.39)	14.95 (13.63-16.71)	1.593
	5	1.01±2.31				18.09±9.45			
	10	10.10±3.83				72.34±8.33			
	15	21.21±9.52				90.43±5.03			
	20	38.38±7.57				96.81±6.00			
	25	65.66±10.07				97.87±2.31			
	30	80.81±3.83				100			

Values of mortality rate were expressed as mean±SD. χ^2 *chi*-square test, $P<0.05$ represented significant difference.

*The mortality rates were very high, so the parameters could not be calculated.

While *An. minimus* was the species most susceptible to crude extract, this did not hold true for fractionated extracts. On the contrary, the mosquitoes most susceptible to RC-DT 012 (LC_{50} 0.66 mg/L) and RC-DT 013 (LC_{50} 0.94 mg/L) were *Cx. quinquefasciatus*, which had the lowest LC_{50} values. Furthermore, *Cx. quinquefasciatus* had the highest tolerance (LC_{50} 282.86 mg/L) to crude extract compared to other species: *Ae. aegypti* (LC_{50} 224.73 mg/L), *Ae. albopictus* (LC_{50} 261.75 mg/L), and *An. minimus* (LC_{50} 77.88 mg/L). This outcome could not be explained because of the data limitations of this study. However, we hypothesize that both fractions (RC-DT 012 and RC-DT 013) must contain compounds that are highly toxic only to *Culex* larvae.

The fractionated extracts of *D. loureiri* provided much better larvicidal efficacy against mosquito vectors than crude extract, which concurs with studies on *Sphaeranthus indicus* Linn. (Asteraceae) extracts. In those studies, steam-distilled crude extract of leaves were compared with the most effective fractionated ethyl acetate extract of the whole plant[16,17], revealing that fractionated extract is more effective than crude extract against *Ae. aegypti* (24-h LC_{50} 36.76 ppm *vs* 140 ppm, respectively) and *Cx. quinquefasciatus* (24-h LC_{50} 32.60 ppm *vs* 130 ppm, respectively).

Our findings suggest that the larvicidal activity of crude extract was not a synergistic action of all compounds in the extract, echoing another recent study that reported the same[18]. In that study, only two of seven groups of fractionated extracts of *Acacia pennata* (L.) Willd. subsp. *insuavis* shoot tips contained compounds active against *Ae. aegypti* larvae. The LC_{50} values of the Fr-G2 and Fr-G3 fractions were 50.75 and 39.45 mg/L, respectively, while the LC_{50} values of the other fractions (Fr-G1 and Fr-G4-Fr-G7) were > 100 mg/L. Similarly, our study found that the active substances in *D. loureiri* extract were contained only in RC-DT 012 and RC-DT 013, which had the lowest LC_{50} and LC_{90} values for all tested mosquito species.

Phytochemical studies have revealed several flavonoids isolated from stems of *D. loureiri*, including homoisoflavans[9], dihydrochalcone[19], and stilbene[20]. Of those, (2*S*)-pinocembrin, (3*S*)-7,4′-dihydroxy-3-(4-hydroxybenzyl)-chromane, and loureirin D have antibacterial activity against *Staphylococcus aureus* and *Bacillus subtilis*; and 7,4′-dihydroxyflavan is fungitoxic against *Botrytis cinerea* and *Cladosporium herbarum*[9]. Studies by Meksuriyen and Cordell and Ichikawa *et al.* have reported that retrodihydrochalcones and homoisoflavones isolated from stem wood are estrogen agonists[19,21]. In addition, stilbenoids, isolated from stem wood are potent inhibitors of cyclooxygenase (COX)-1 and COX-2 enzymes[20]. Although some phytochemical constituents and their activities have been studied, the phytochemical compounds in the fruit endocarp of *D. loureiri* have never been investigated. Moreover, until our previous study of crude extract[11], the larvicidal activity of *D. loureiri* has never been elucidated. Thus, the results of this study could not be compared to

the results of other studies. Further studies on the larvicidal activity of *D. loureiri* extract, phytochemical constituent analysis (*e.g.*, gas chromatography-mass spectroscopy)[22], purification, and mosquito larvicide evaluation of substances purified from the RC-DT 012 and RC-DT 013 groups must be performed.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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