LARVICIDAL AND PUPICIDAL ACTIVITIES OF CRUDE AND FRACTIONATED EXTRACTS OF *ACACIA PENNATA* (L.) WILLD. SUBSP *INSUAVIS* SHOOT TIPS AGAINST *AEDES AEGYPTI* (L.) (DIPTERA: CULICIDAE)

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Abstract. *Acacia pennata* subsp *insuavis*, or *Cha-om* in Thai, is a common vegetable found in Thailand. It has been used as a medicinal herb for a long time. From the literature, antinociceptive, anti-inflammatory, antimicrobial, and anti-helminthic activities were reported. In this study, we investigated two new actions of this plant: larvicide and pupicide. The crude ethanolic and fractionated extracts of *A. pennata* shoot tips were tested against aquatic stages of the dengue virus vector, *Aedes aegypti* mosquito. The 1st-4th instar larvae and pupae of *Ae. aegypti* were subjected for bioassays by following the standard protocol of WHO. The larval and pupal mortalities were observed after 24- and 48-hour exposure times. The bioassays demonstrated that stronger efficacy was found from the fractionated extracts than the crude extracts. The LC_{50} values against the 3rd instar larvae were 39.45-50.75 mg/l (fractionated extracts) and 244.50 mg/l (crude extracts). It also effects the pupae with the LC_{50} values of 44.10-53.73 mg/l and 87.27 mg/l for the fractionnated and the crude extracts, respectively. The bioassays demonstrated the effective mosquito larvicide and pupicide of *A. pennata* extracts. It could be an alternative candidate for the development of phytotoxin for controlling mosquito vectors.

Keywords: *Aedes aegypti*, *Acacia pennata*, crude extract, larvicide, pupicide

INTRODUCTION

For vector control strategy, insecticides are the most effective substance. However, it causes some adverse effects, *eg*, environmental pollution and toxic endangerment to non-target organisms (Colin *et al*, 2004; Diepens *et al*, 2014; Ogbeide *et al*, 2015). One of leading chemical larvicides, temephos, not only causes the above-mentioned effects, but also leads to insecticide resistance after indiscriminate use (Chareonviriyaphap, 1999; Sornpeng *et al*, 2009; Tikar *et al*, 2009). In order to reduce those problems, a bio-insecticide has been intensively researched and developed for substitution of insecticides.

Currently, plant extracts, some of the bio-substances, have been considered for controlling the population of insect vectors, including mosquitoes. A number of

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plant species: *Callistemon rigidus* (Pierre *et al*, 2014), *Chloroxylon swietenia* (Jayaraman *et al*, 2015), and *Leucas aspera* (Elumalai *et al*, 2016) were found to have larvicidal activity against *Aedes aegypti* mosquito, the most important vector of the dengue virus (WHO, 2011). A great quantity of such plant species in Thailand has led to a continuing search to find a novel larvicide-contained plant.

Acacia pennata (L.) Willd. subsp *insuavis* (Lace) I.C. Nielsen, Thorny tree (common name) or *Cha-om* (Thai name), is one of thirteen *Acacia* species native to Thailand. The young leaf (shoot tip) of *A. pennata* is a common and an important food source for Thai people. For the traditional medicine aspect, *A. pennata* is used for the treatment of indigestion in infants, urine scald, and the treatment of bleeding gums. It was also used for treatment of cholera, digestive complaints, headache, body pain, snake bites, and fish poisoning (Bhumibhamon, 2002). The literature suggests that the leaves extracts of *A. pennata* have antinociceptive and anti-inflammatory activities (Dongmo *et al*, 2005), antimicrobial activity (*Bacillus cereus* and *Lactobacillus plantarum*) (Nanasombat and Teckchuen, 2009), and anticancer (Rifai *et al*, 2010) activities. Recently, the root bark extract of the plant showed killing efficacy against *Raillietina echinobothrida*, the avian parasitic helminth, with profound structural damages of the worm (Lalchhandama, 2013).

For our study, the potential larvicidal and pupicidal activities of *A. pennata* shoot tips extract, against aquatic stages of the *Ae. aegypti* mosquito were studied.

MATERIALS AND METHODS

Acacia pennata crude extract preparation

Fresh shoot tips of *A. pennata* were purchased from a food market in Mueang District, Phitsanulok Province, Thailand. The shoot tips were cleaned with tap water and air dried. After weighing, the *A. pennata* shoot tips (5 kg) were completely dried in a hot air oven at 45°C. They were then ground to powder using an electric blender at 22,000 rpm. The dried powder (677.72 g) was extracted with absolute ethanol in a ratio of 1:10 (powder:solvent).

Twenty-five grams of the plant powder were suspended in 250 ml of the solvent (absolute ethanol) in a 500-ml Erlenmeyer flask, which was continuously shaken at 180 rpm on a rotary shaker for 24 hours at room temperature. The suspension was then suction filtered through a Whatman No. 1 filter paper via a Buchner funnel. The filtrates were evaporated to dryness by using a rotary evaporator (Buchi Rotavapor® R-205 with a Buchi Vac® V-500; BUCHI, Flawil, Switzerland).

Yields for the *A. pennata* shoot tips crude extract were 36.45 g. The crude extract was retained in a desiccator until required for a further fractionated extraction and bioassays. A voucher specimen of the *A. pennata* shoot tips (DTNU009) was deposited at the Department of Microbiology and Parasitology, Faculty of Medical Science, Naresuan University, Thailand.

Column chromatography fractionated extraction

The *A. pennata* shoot tips ethanolic crude extract (15.00 g) was fractionated by a column chromatography (Silica gel 60, less than 0.063 mm, 200 g, P/N 1.07729.5000; Merck, Frankfurt, Germany), using a gradient solvent system of *n*-hexane, *n*-hexane-EtOAc, EtOAc, EtOAc-MeOH, and MeOH with increasing amounts of the more polar solvent.

The eluates were examined using a Thin Layer Chromatography (TLC Silica gel 60 F_{254}, P/N 1.05554.0001; Merck,

Frankfurt, Germany). They were then fractionated into seven groups of eluting fractions: Gr1, fractions 1-15 (4.20 g); Gr2, fractions 16-33 (1.05 g); Gr3, fractions 34-55 (0.64 g); Gr4, fractions 56-150 (0.10 g); Gr5, fractions 151-230 (0.20 g); Gr6, fractions 231-425 (0.51 g) and Gr7, fractions 426-505 (5.00 g).

Mosquito colonization

The laboratory strain of *Ae. aegypti* mosquito, originally collected from Mueang District, Phitsanulok Province, Thailand was colonized as previously described (Thongwat *et al*, 2014). In brief, larvae were reared in tap water under laboratory condition [25±2°C, 70-80% relative humidity, and 10:14 (L:D) photoperiod]. Powdery dog biscuits (Adult Complete Nutrition, Pedigree®, Mars Petcare, Franklin, TN) were fed to the larvae. After pupation, they were transferred into a mosquito cage until they became adults. The adults were provided with a 5% sugar solution containing 5% multivitamin syrup (Seven Seas®, Feltham, Feltham, UK).

After 5-7 days, the females were allowed to feed on a blood meal by using an artificial membrane feeding method (Rutledge *et al*, 1964). After a further 2-4 days, the gravid females were permitted to lay eggs on a wet filter paper (Whatman No. 1). The eggs were air-dried (3 days at room temperature) and maintained in a humidity-controlling glass jar until required.

Larvicidal and pupicidal bioassays

The bioassays of *A. pennata* extracts were conducted on *Ae. aegypti* larvae and pupae by following the protocol of WHO (2005). Briefly, a stock solution of the extracts (1%w/v) was prepared by adding 200 mg of the extract into 20 ml of dimethylsulphoxide (DMSO). The stock solutions were kept at 4°C. A series of concentrations were prepared for the bioassay testing. Two hundred ml of various concentrations was then put into plastic bowls. Twenty-five of the 1st, 2nd, 3rd, and 4th instar larvae or pupae were transferred into the plant extract solutions. The concentrations of 50-400 mg/l were prepared for crude extract testing, while lower concentration series were prepared for the fractionated one. After 24 and 48 hours, mortality rates were assessed. The larvae were considered dead when they were unable to normally move after gentle touching with a needle. The experiments were performed in four replicates. The control group, containing 2 ml of DMSO in 198 ml distilled water was also included.

Data analysis

The larvicidal and pupicidal data were analyzed using a computerized probit analysis for the 50% lethal concentration (LC_{50}) determination (Finney, 1971). The chi-square values (χ^2) and 95% confidence intervals (CI) of upper and lower fiducial limits (UCL and LCL) were also calculated [LdP Line® (Plant Protection Research Institute), Cairo, Egypt].

RESULTS

The 24- and 48-hour bioassay results of the crude ethanolic extract of *A. pennata* shoot tips against aquatic stages, including the 1st to 4th instars larva and the pupa, of *Ae. aegypti* mosquito are presented in Table 1, Fig 1, and Fig 2. Among all five stages, the pupa displayed the highest susceptibility to the extract with LC_{50} values of 82.27 and 56.41 mg/l for the 24- and 48-hour exposure times, respectively. For all larval stages, the 4th instar showed the highest tolerance with 467.46 mg/l LC_{50} value from the 24-hour exposure time, while the lowest response (175.11 mg/l) was found from the 48-hour bioassay.

Table 1
24- and 48-hour larvicidal and pupicidal activities of *A. pennata* crude extract against aquatic stages of *Ae. aegypti*.

Mosquito stage[a]	24-hour			48-hour		
	LC_{50} with fiducial limit (mg/l)	Parameter		LC_{50} with fiducial limit (mg/l)	Parameter	
		χ^2	Slope (± SE)		χ^2	Slope (± SE)
1st instar larva	169.78 (149.13 - 225.62)	11.85	3.8020 ± 0.4209	162.36 (142.03 - 206.92)	11.78	3.9547 ± 0.4190
2nd instar larva	237.26 (216.85 - 269.71)	13.47	4.8137 ± 0.3724	169.332 (162.79 - 176.14)	7.44	6.1560 ± 0.3667
3rd instar larva	244.50 (220.25 - 278.56)	6.54	2.6785 ± 0.2794	109.73 (83.20 - 132.96)	12.69	3.2611 ± 0.2484
4th instar larva	467.46 (390.47 - 628.99)	10.66	1.9151 ± 0.2829	175.11 (157.65 - 191.20)	3.29	2.7400 ± 0.2638
Pupa	87.27 (79.60 - 99.26)	2.18	4.2304 ± 0.5377	56.41 (39.32 - 87.81)	11.36	2.9164 ± 0.2902

[a]Zero mortality rates were observed from all control groups.

For the fractionated extracts, seven fractions (Fr-G1 to Fr-G7) were obtained from the crude. It was found that only Fr-G2 and Fr-G3 demonstrated larvicidal activity against the 3rd stage larvae with superior efficacies than the crude extract. After statistical analysis, the Fr-G3 showed significantly greater activity than the Fr-G2, with LC_{50} values of 39.45 mg/l (24h), 37.45 mg/l (48h); and 50.75 mg/l (24h), 45.28 mg/l (48h), for the Fr-G3 and Fr-G2, respectively (Table 2, Fig 3).

Unexpectedly, fractions (Fr-G2 and Fr-G3), which contained larvicide bioactive compound, did not show the pupicidal activity (with nil pupal mortality after 100 mg/l testing for 48 hours). It was then found from Fr-G4 and Fr-G5 fraction groups with lower LC_{50} values. Fr-G4 showed LC_{50} values of 53.73 mg/l (24 hrs) and 49.15 mg/l (48 hrs). Fr-G5 showed a slightly higher activity with 44.10 mg/l (24 hrs) and 40.64 mg/l (48 hrs) LC_{50} values (Table 3). Compared to the crude extract, both the Fr-G4 and Fr-G5 fractions showed significant pupicidal activity greater than the crude extracts (Fig 4).

In this study, improvement of both larvicidal and pupicidal, with lower LC_{50} values were found after extending the exposure bioassay time to 48 hours.

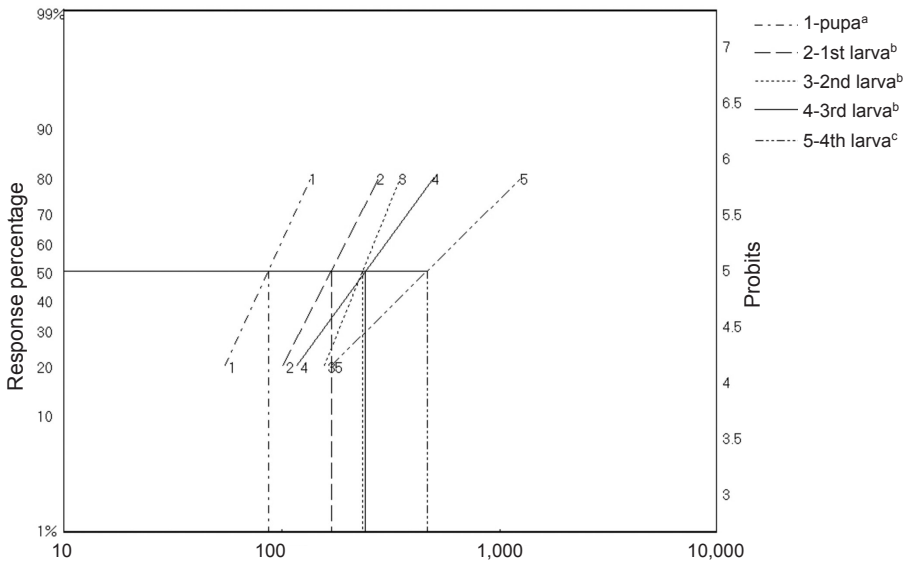

Fig 1–The comparison of 24-hour LC_{50} values of the *A. pennata* crude extracts on the aquatic stages of *Ae. aegypti*. Statistically significant differences are indicated by different letter on the mosquito stage categories (upper right).

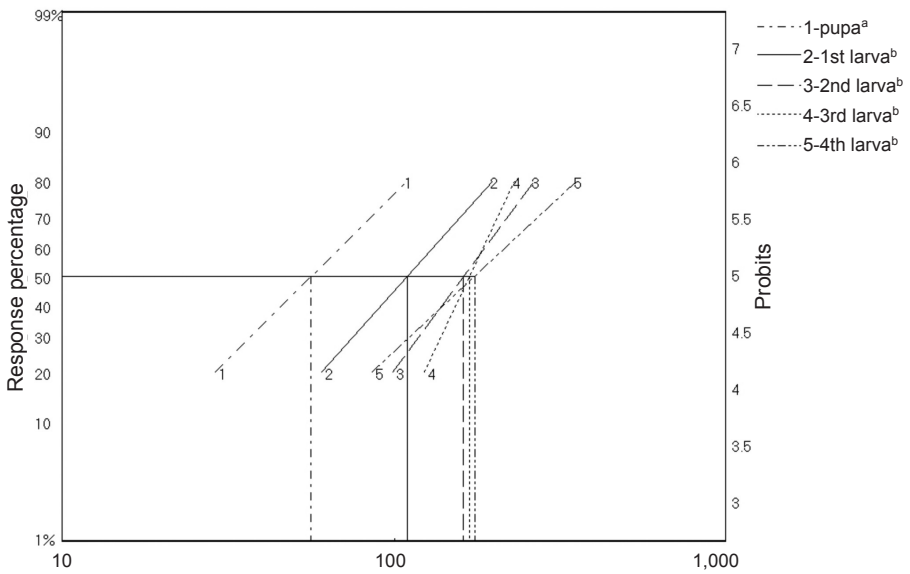

Fig 2–The comparison of 48-hour LC_{50} values of the *A. pennata* crude extracts on the aquatic stages of *Ae. aegypti*. Statistically significant differences are indicated by different letter on the mosquito stage categories (upper right).

DISCUSSION

Although *A. pennata* is one of the most common ingredients for traditional Thai foods, it has never been reported for other activities, such as the medical aspect, for example, antinociceptive, anti-inflammatory, or antimicrobial activities (Dongmo *et al*, 2005; Nanasombat and Teckchuen, 2009). In 2013, the killing efficacy against the avian parasitic helminth, *R. echinobothrida*, was reported (Lalchhandama, 2013). In our study, we discovered and reported some novel properties—larvicidal and pupicidal activities—of the *A. pennata* shoot tips against the dengue vector mosquito. Both ethanolic crude and fractionated extracts contained a promising insecticide against the 1^{st}-3^{th} instars larvae (39.45-244.50 mg/l) and pupae (44.10-87.27 mg/l) of *Ae. aegypti*.

Table 2
24- and 48-hour larvicidal activities of *A. pennata* fractionated extracts against the 3rd instar larvae of *Ae. aegypti*.

Fractionated group[a]	24-hour			48-hour		
	LC_{50} with fiducial limit (mg/l)	Parameter		LC_{50} with fiducial limit (mg/l)	Parameter	
		χ^2	Slope (± SE)		χ^2	Slope (± SE)
Fr-G1	>100[b]	-	-	>100[b]	-	-
Fr-G2	50.75 (48.25 - 53.14)	1.35	9.0023 ± 0.7633	45.28 (43.09 - 47.41)	1.66	10.4594 ± 0.9719
Fr-G3	39.45 (36.34 - 42.32)	3.94	5.3264 ± 0.4812	37.45 (34.99 - 39.77)	1.24	6.9271 ± 0.6188
Fr-G4	>100[b]	-	-	>100[b]	-	-
Fr-G5	>100[b]	-	-	>100[b]	-	-
Fr-G6	>100[b]	-	-	>100[b]	-	-
Fr-G7	>100[b]	-	-	>100[b]	-	-

[a]Zero mortality rates were observed from all control groups; [b]The mortality rates were very low at 100 mg/l, so the LC_{50} value is estimated to over 100 mg/l and the parameters could not be calculated.

Only the 4th instar larva slightly tolerated the crude extract, with a LC_{50} value of 467.46 mg/l. However, stronger efficacy was found (175.11 mg/l) after extending the exposure time to 48 hours. It indicated that the extension of the exposure period would result in improvements. Similar findings have been found from the *Aedes* larvicide studies of the other plant extracts, for example, the ether extract of *Coriandrum sativum*, *Nigella sativa*, and *Syzygium aromaticum* (Bilal *et al*, 2012), the ethanolic extract of *Ricinus communis*, *Coutarea hexandra* and *Cnidoscolus phyllacanthus* (Candido *et al*, 2013), and, recently, the ethanolic extract of *Pereskia bleo* (Thongwat *et al*, 2014).

From our fractionated extracts study, the larvicide was found only from fractions Fr-G2 and Fr-G3, while the pupicide was from Fr-G4 and Fr-G5. This can be concluded that the larvicide and pupicide activities are contained in different substances. Therefore, it is interesting to investigate which active substances contain the larvicidal or pupicidal activities.

To pursue that study, the isolation and identification of the active substances have to undergo further investigation. For example, from the literature, n-hexadecanoic isolated from *Feronia limonia* leaves can kill the larvae of *Ae. aegypti*, *Culex quinquefasciatus*, and *Anopheles stephensi* (Rahuman *et al*, 2000). *Hyptis martiusii* leaf oil, 1,8-cineole,

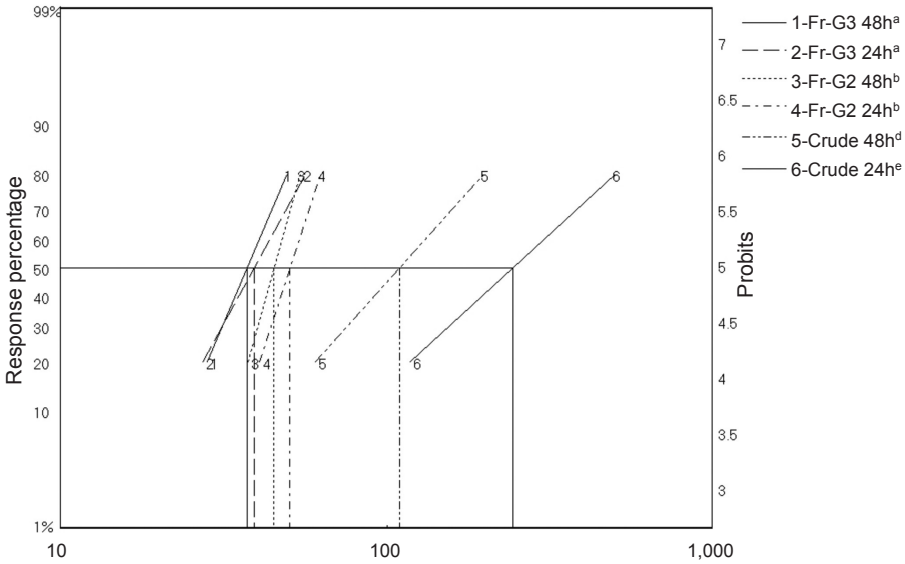

Fig 3–The comparison of 24- and 48-hour LC_{50} values of the *A. pennata* crude and fractionated extracts on the 3rd instar larvae of *Ae. aegypti*. Statistically significant differences are indicated by different letter on the crude or fractionated group categories (upper right).

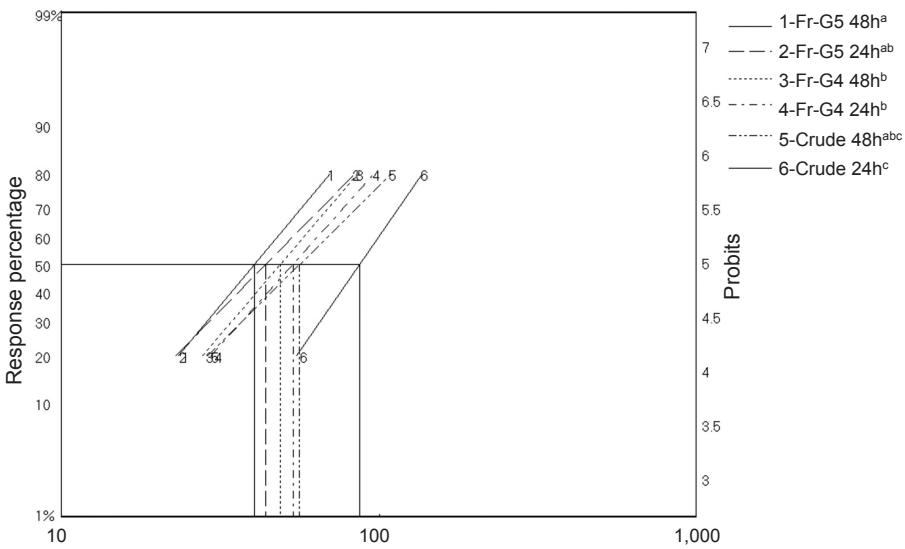

Fig 4–The comparison of 24- and 48-hour LC_{50} values of the *A. pennata* crude and fractionated extracts on the pupae of *Ae. aegypti*. Statistically significant differences are indicated by different letter on the crude or fractionated group categories (upper right).

have shown larvicidal activity against *Ae. aegypti* (Araujo *et al*, 2003).

In addition, D-pinitol from the aerial parts of *Acacia nilotica* was also found effective against *Ae. aegypti* and *Cx. quinquefasciatus* larvae (Chaubal *et al*, 2005). However, although the finding of known active compound that contained the mosquito larvicidal activity was extensively reported, the pupicidal activity has never been mentioned. This could be further investigated.

For the other *Acacia* plants, very few references have been cited for their mosquito larvicidal activity. Only *Acacia nilotica* extracts were reported by Chaubal *et al* (2005) who extracted the aerial parts of *A. nilotica* into 6 fractions with the LC_{50} values of 60-148 mg/l against *Ae. aegypti* larvae, and of 59-126 mg/l against *Cx. quinquefasciatus*. In that

Table 3
24- and 48-hour pupicidal activities of *A. pennata* fractionated extracts against the pupae of *Ae. aegypti*.

Fractionated group[a]	24-hour			48-hour		
	LC_{50} with fiducial limit (mg/l)	Parameter		LC_{50} with fiducial limit (mg/l)	Parameter	
		χ^2	Slope (± SE)		χ^2	Slope (± SE)
Fr-G1	>100[b]	-	-	>100[b]	-	-
Fr-G2	>100[b]	-	-	>100[b]	-	-
Fr-G3	>100[b]	-	-	>100[b]	-	-
Fr-G4	53.73 (48.73 - 58.94)	0.55	3.2716 ± 0.3903	49.15 (44.47 - 53.70)	0.64	3.4403 ± 0.3923
Fr-G5	44.10 (38.70 - 48.93)	0.47	2.9848 ± 0.3822	40.64 (36.85 - 44.39)	1.03	3.5146 ± 0.2976
Fr-G6	>100b	-	-	>100b	-	-
Fr-G7	>100 b	-	-	>100b	-	-

[a]Zero mortality rates were observed from all control groups; [b]The mortality rates were very low at 100 mg/l, so the LC_{50} value is estimated to over 100 mg/l and the parameters could not be calculated.

study, D-pinitol (=3-*O*-methyl-D-*chiro*-inositol; 1) was isolated and found that it was comparatively less active than the fractions were. They concluded that the larvicidal activity of the *A. nolitica* is not due to the D-pinitol alone but due to the presence of another compound along with the D-pinitol.

A similar study of Sakthivadivel and Daniel (2008) reported the LC_{50} values of the *A. nolitica* leave extract with 70.42, 58.16 and 55.72 mg/l against *Ae. aegypti*, *Cx. quinquefasciatus*, and *An. stephensi*, respectively. In 2012, various solvent extracts (water, ethanol, and petroleum ether) of *A. nolitica* leaves were found to display 41.1-65.8% mortality rates of the *Anopheles arabiensis* larvae (Edriss *et al*, 2012).

When *A. nolitica* extracts were compared with those studies, *A. pennata* crude extract had lower efficiency with 244.50 mg/l LC_{50} value against the *Ae. aegypti* mosquito at the same larval stage (3rd) and exposure time (24-hour). However, the stronger activities, LC_{50} values of 39.45 and 50.75 mg/l, were found from the fractionated extracts. For the insecticidal activity of the other *Acacia* species, there was only a report of *Acacia auriculiformis* bark extracts that displayed some biological effects on *Bactrocera cucurbitae*, a melon fruit fly. The biological effects include the prolongation of larval and total developmental period, the abnormality of pupation and emergence percentage, and the decreasing oviposition

and egg hatching, on the fly were found (Kaur *et al*, 2010).

In conclusion, the bioassay results of this work introduced new and promising properties—larvicidal and pupicidal activities—against the *Ae. aegypti* mosquito of the *A. pennata* shoot tips extracts, especially the fractionated extracts. The isolation of the active substances is proposed to be further studied. Toxicology, cost effectiveness, and impact on non-target organisms of this edible plant extract also need to be studied.

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