# LARVICIDAL ACTIVITY OF LINOLEIC ACID ISOLATED FROM *ACACIA PENNATA* (L.) WILLD. SUBSP *INSUAVIS* AGAINST *AEDES AEGYPTI* (L.) MOSQUITO

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**Abstract.** The use of bio-insecticides to control mosquitoes is gaining popularity and is the subject of substantial research; nevertheless, some of these chemicals may be hazardous to non-target organisms, including humans. Thus, it is preferable to concentrate on identifying bio-insecticides from edible plants. In this study, the larvicidal efficacy of linoleic acid extracted from *Acacia pennata* (L.) Willd. Subsp *insuavis*, a typical vegetable in Thai cuisine, was assessed against 3rd-instar larvae of *Aedes aegypti*. Linoleic acid was extracted and demonstrated to have mosquito larvicidal activity; this was verified by bioassay-guided fractionation of *A. pennata* ethanolic extract. According to the WHO guidelines, bioassays revealed that all *A. pennata* extracts had mild larvicidal effects against *Ae. aegypti* third-instar larvae, with $LC_{50}$ value of crude extract, fractionated extract, and purified linoleic acid of 197.78, 79.51 and 108.49 mg/l, respectively. As linoleic acid is an essential fatty acid of the human diet and is commonly found in edible plants, it is of particular interest to explore the development of linoleic acid as a possible environmental-friendly mosquito larvicide.

**Keywords:** *Acacia pennata*, *Aedes aegypti*, edible plant, linoleic acid, mosquito larvicide, plant extract

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## INTRODUCTION

Nowadays, bio-insecticides have attracted wide interest in mosquito control programs and are being studied extensively, not only because it is environment friendly, but also help reduce the growing appearance of insecticide resistance worldwide (Chareonviriyaphap *et al*, 2013; Guedes *et al*, 2020; Zulfa *et al*, 2022). Insecticidal activities of plants are from secondary metabolites, which are used as a plant defense mechanism against herbivore predators and other environmental menaces (Abirami *et al*, 2017). Many plant species have been tested for larvicidal properties against various species of mosquitoes. For instance, essential oils and organic/aqueous extracts from more than 100 plant species have been examined for their larvicidal effects against *Aedes aegypti*, an important mosquito vector of human arboviral diseases (Muangmoon *et al*, 2018; Luz *et al*, 2020; Silverio *et al*, 2020), including adulticidal and/or ovicidal activities (Chansang *et al*, 2018; Muangmoon *et al*, 2019). Although many species of plant extracts were demonstrated to have promising mosquitocidal activity against various stages of the vectors, several extracts were toxic to non-target organisms, including humans (Jiang *et al*, 2018; Farzaei *et al*, 2020; Sripriya *et al*, 2021). Thus, toxicity towards non-target organisms should be taken into consideration when plant extracts are applied as mosquito larvicides. It is preferable to focus on edible plants as sources of bio-insecticides (Sharma *et al*, 2014; Mbatchou *et al*, 2017; Murthy *et al*, 2020).

*Acacia pennata* (L.) Willd. Subsp *insuavis* (Lace) I. C. Nielsen, a thorny tree, is native to South and Southeast Asia. Young leaves from the upper part of the branch can be eaten year-round and is a common vegetable ("cha-om") in Thai cuisine. Because of its thorns, *Acacia pennata* is frequently planted along the fence of a house. *A. pennata* is also considered a herb. Thai people use *A. pennata* for the treatment of digestive complaints, body pain, headache, cholera, fish poisoning, and snake bite (Bhumibhamon, 2002). Its leaf extracts have anti-nociceptive, -inflammatory, -microbial, -cancer, and -cyclooxygenase activities (Dongmo *et al*, 2005; Dongmo *et al*, 2007; Nanasombat and Teckchuen, 2009; Rifai *et al*, 2010). The extract of root bark has inhibitory activity against avian helminth *Raillietina echinobothrida* (Lalchhandama, 2013). Consumption of *A. pennata* leaves/extracts may be beneficial in preventing Alzheimer's disease (Lomarat *et al*, 2015). Recently, *A. pennata* extract was reported to control metabolic derangement, and pancreatic and hepatorenal dysfunctions in diabetes-induced rats (Shao *et al*, 2022). "Cha-om" is safe for human consumption, and it would be of interest if it could be shown to have mosquito larvicide activity as well.

A previous report indicated that crude and fractionated ethanolic extracts of *A. pennata* demonstrate larvicidal and pupicidal activities against *Ae. aegypti* (Thongwat *et al*, 2017). The fractionated extracts show higher activities than the crude extract. However, the chemical composition of the extracts from that study was not determined. Thus, this study aimed to isolate and characterize the major bioactive compound(s) present in *A. pennata* ethanol extract and test larvicidal activity against *Ae. aegypti* 3rd instar larvae. The information should be useful for mosquito vector management by providing bioactive compound(s) safe for humans but toxic to mosquitoes.

## MATERIALS AND METHODS

### Crude extract preparation

*A. pennata* was collected from Mueang District, Phitsanulok Province, Thailand and a voucher specimen (DTNU011) was deposited at the Department of Microbiology and Parasitology, Faculty of Medical Science, Naresuan University, Thailand. The specimen was taxonomic identified by Assistant Professor of Biology, Dr Unchalee Nilsuwan, Department of Biology, Faculty of Science and Technology, Phranakhon Rajabhat University, Bangkok, Thailand. Young leaves (9.05 kg) were washed with tap water, air dried and placed in a hot air oven at 45°C for 4 days. The dried leaves (1.39 kg) were ground to powder in an electric blender at 22,000 rpm (800G instrument; MRC Laboratory-Instruments, Essex, UK), and then macerated with absolute ethanol (1 g:100 ml) in a rotary shaker (Platform Shaker, New Brunswick™ Innova® 2300, Eppendorf, Hamburg, Germany) at 180 rpm for 24 hours. The suspension was then suction filtered through a Whatman™ No. 1 filter paper (GE Healthcare Ltd, Buckinghamshire, UK), and the filtrate was evaporated to dryness using a rotary evaporator (Buchi Rotavapor® R-205 equipped with a Buchi Vac® V-500; Buchi, Flawil, Switzerland) and placed in the hot air oven (45°C) until completely dry. The dried crude extract (78.22 g) was kept in a desiccator at ambient temperature.

### Isolation and purification of bioactive components

Fractionation of crude ethanol extract was performed using column chromatography with a Silica gel 60 column (63 μm bead diameter, 200 g; Merck, Frankfurt, Germany) eluted with a linear gradient of 5-50 % (v/v) ethyl acetate (RCI Labscan, Bangkok, Thailand) (EtOAc)/50-95% (v/v) hexane, 1-5% (v/v) methanol (RCI Labscan, Bangkok, Thailand) MeOH/99-95% (v/v) EtOAc in 100% MeOH. The eluates were monitored using thin layer chromatography employing Silica gel 60 F254 (Merck,

Frankfurt, Germany), and the eluted fractions were then pooled into six fractions (RC-DT 027 - RC-DT 032). Each RC-DT fraction (100 mg/l) was tested for larvicidal activity as described below and the fraction having the highest larvicidal activity was separated by column (Silica gel 60) chromatography using 100% $CH_2Cl_2$ as elution solvent to yield a purified compound.

**Compound characterization**

The structure of the isolated compound was characterized using high-resolution mass spectrometry (MS) (PE SCIEX API 4000 mass spectrometer; Applied Biosystem, Fostercity, CA) and 1D- and 2D-nuclear magnetic resonance (NMR) analyses (Bruker AVANCE 400 FT-NMR spectrometer; Bruker BioSpin AG, Fällanden, Switzerland).

**Mosquito rearing and maintenance**

A laboratory strain *Ae aegypti*, obtained from the Department of Microbiology and Parasitology, Faculty of Medical Science, Naresuan University, Phitsanulok, Thailand was reared at 25±2°C, 70-80% relative humidity and 10:14 light:dark 24-hour period. Larvae were kept in a white plastic tray containing tap water and fed with powdered dog biscuits (Adult Complete Nutrition, Pedigree®; Mars Petcare, Franklin, TN). Pupae were moved into a mosquito cage (30 x 30 x 30 cm) until they became adults, which were fed a solution of 5% (w/v) sugar and 5% (v/v) multivitamin syrup (Seven Seas®, Feltham, UK). After 5 days, the females were fed blood meal through an artificial membrane feeding apparatus (Rutledge *et al*, 1964). After becoming gravid, the female mosquitoes were facilitated to lay eggs. Plastic cups, which were half-filled with tap water and lined with filter paper, were placed in the mosquito cage. After 2 days, the cups were left, and then the filter papers with mosquito eggs attached to them were air-dried. The air-dried filter papers were kept in a humidity-controlled glass jar until required. Then, the eggs were allowed to hatch and larvae reared as described above.

**Larvicidal bioassay**

The bioassay of *A. pennata* ethanolic samples (crude extract, fractions and pure compound) was conducted on 3rd instar larvae following WHO (2005) protocols. A stock solution of each sample (2% w/v) was prepared in dimethyl sulfoxide (DMSO) and kept at 4°C until used. Each stock solution was serially diluted in 100 ml of tap water (crude extract, 100-300 mg/l; fractionated extract, 40-100 mg/l; and purified compound, 20-100 mg/l) in food-grade plastic bowls (7 cm diameter x 4.5 cm height). Third instar larvae ($n$ = 25) were placed into the assay solutions and mortality rates determined at 24- and 48-hour post-exposure, during which no food was provided to the larvae.

Larvae were considered dead when they were unable to move after being gently touched with a brush. Each diluted sample was assayed in three independent experiments, each experiment carried out in quadruplicate. DMSO (1% v/v) in tap water was used as a control.

**Data analysis**

The larvicidal data were analyzed using probit analysis for the determination of 50% ($LC_{50}$) lethal value (Finney, 1971). Fiducial confidence intervals (95%) [lower confidence limit (LCL) and upper confidence limit (UCL)] are reported, with significantly different $LC_{50}$ values accepted when the LCL-UCL ranges do not overlap. Calculations were conducted using Ldp-line software (Plant Protection Research Institute, Agricultural Research Center, Giza, Egypt).

## RESULTS

**Isolation of larvicidal compound from *A. pennata* leaf ethanol extract**

The yield of crude ethanol extract of *A. pennata* leaf was 5.63% (w/w) (78.22 g from 1.39 kg). Silica gel 60 column chromatography of the crude extract (50.0 g) generated 58 eluted fractions, which were pooled into six fractions, namely, RC-DT 027 (fractions 1-4) containing 1.25 g of extract, RC-DT 028 (fractions 5-12) 1.45 g, RC-DT 029 (fractions 13-22) 1.49 g, RC-DT 030 (fractions 23-34) 0.36 g, RC-DT 031 (fractions 35-51) 1.24 g, and RC-DT 032 (fractions 52-58) 1.53 g. Each RC-DT fraction (100 mg/l) was tested against 3$^{rd}$ instar *Ae. aegypti* larvae for 24 hours, resulting in 43, 97, 3, 0, 22, and 0% larval mortality for each fraction, respectively. RC-DT 028, having the highest larvicidal activity was subjected to further column (Silica gel 60) chromatography resulting in 44.2 mg of a purified compound, the crystallization of which yielded an amorphous solid.

Analysis of this compound by electrospray ionization high-resolution mass spectrometry (HRMS [$ESI^+$]) indicated a major peak with a mass-to-charge ratio (m/z+) of 281.2486, indicative of a compound with a molecular formula $C_{18}H_{33}O_2$ (Fig 1). Analysis by proton ($^1$H NMR) and 13C ($^{13}$C NMR) nuclear magnetic resonance spectrometry indicated the presence of linoleic acid (Fig 2).

**Mosquito larvicidal activity of *A. pennata* extracts**

Comparison of the *A. pennata* leaf crude ethanol extract, fraction RC-DT 028 and isolated linoleic acid larvicidal activities against 3$^{rd}$ instar *Ae. aegypti* larvae was evaluated after 24- and 48-hour exposure. Only the $LC_{50}$ value of the crude ethanol extract is significantly lower at 48 hours (80%) compared to 24 hours of treatment, indicating that the lethality of

RC-DT028 fraction, and isolated linoleic acid and a commercial sample (TCI, Tokyo, Japan) occurred within 24 hours (Fig 3). Surprisingly, $LC_{50}$ values of RC-DT 028 fraction are significantly lower than those of the isolated linoleic acid and equal to those of the commercial sample.

## DISCUSSION

The current study revealed that the putative major larvicidal compound against 3rd instar *Ae. aegypti* present in the ethanol extract of *A. pennata* leaf was linoleic acid. The observation that the most bioactive fraction, RC-DT 028, was as active as a commercial linoleic acid sample, and was more active than the isolated compound, could be due to several factors. The presence of smaller amounts of other larvicidal compounds and/or synergism among them with the predominant larvicidal compound

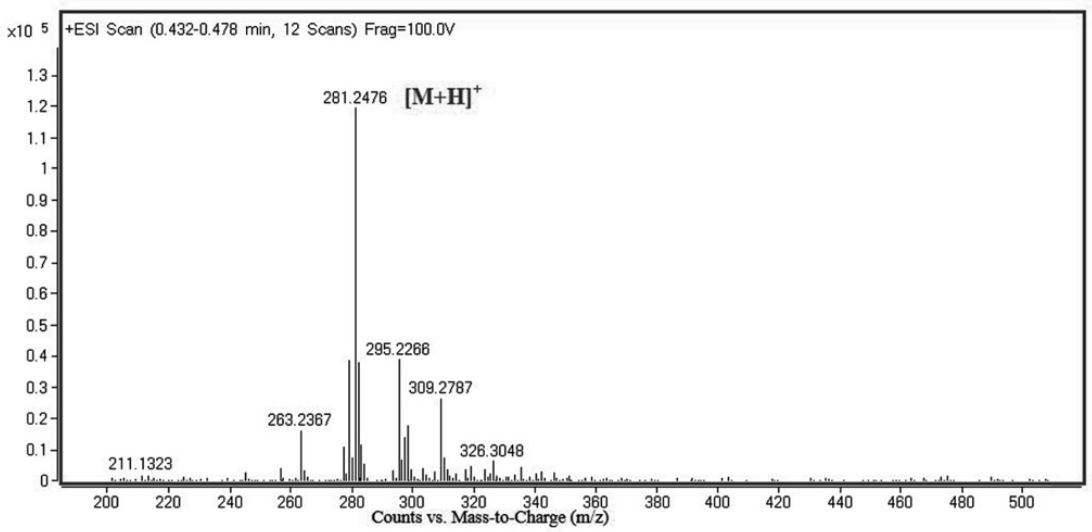


Fig 1 - High-resolution mass spectrometry electrospray ionization (HRMS [$ESI^+$]) analysis of linoleic acid from ethanolic extract of *Acacia pennata* young leaves

Note: Positive ionization mode was used to analyze the sample in a mass range of 100-1000 amu using electrospray ionization mass spectrometry. The ESI-MS conditions included a capillary voltage of +3500V, a drying gas flow rate of 7 l/min, dry gas temperature of 350°C, and nebulizer pressure of 30 psig.

amu: atomic mass unit; ESI-MS: electrospray ionization mass spectrometry; l/min: liters per minute; psig: pounds per square inch gauge; V: volt

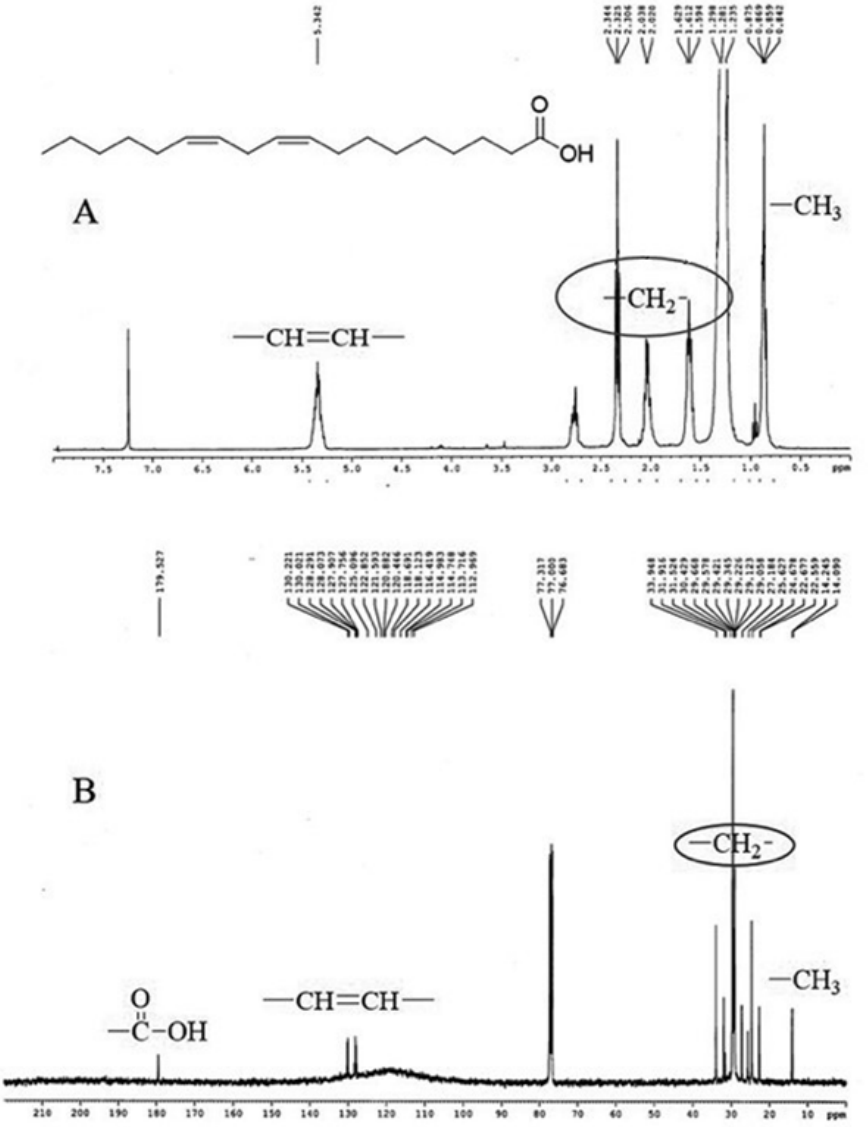


Fig 2 - (A) Proton nuclear magnetic resonance [$^{1}$H NMR] and (B) carbon-13 nuclear magnetic resonance [$^{13}$C NMR] spectra of linoleic acid from ethanolic extract of *Acacia pennata* young leaves

Note: The $^{1}$H and $^{13}$C NMR spectra were acquired on a NMR spectrometer operating at 400 ($^{1}$H) and 100 ($^{13}$C) MHz. Chemical shifts were reported in ppm using $CDCl_3$ as the solvent with reference to its signals at $\delta_H$ 7.24 and $\delta_C$ 77.0.

$CDCl_3$: deuterated chloroform; MHz: megahertz; $\delta_C$: chemical shift of a carbon signal; $\delta_H$: chemical shift of a proton signal

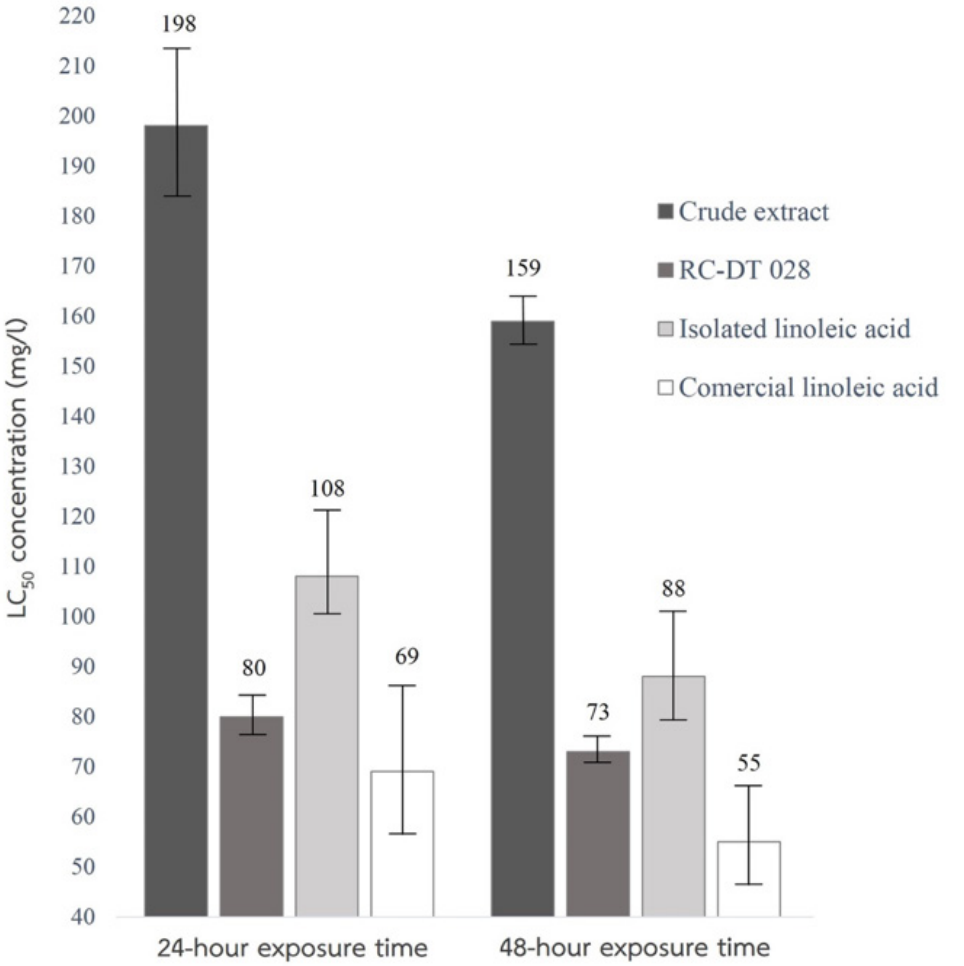


Fig 3 - Fifty % lethal concentrations ($LC_{50}$s) of *Acacia pennata* ethanol extract samples on third instar *Aedes aegypti* larvae at 24- and 48-hours post-exposure

Third instar larvae ($n$ = 25) were placed into the assay solutions and mortality rates determined at 24- and 48-hours post-exposure, during which no food was provided to the larvae. Larvae were considered dead when they were unable to move after being gently touched with a brush. Each sample was assayed in three independent experiments, each experiment carried out in quadruplicate.

Number above each bar indicates mean value and whisker the upper confidence (UCL) and lower confidence (LCL) limits. Between two extracts in a test assay period, if the LCL-UCL range of each extract overlaps, there is no significant difference in $LC_{50}$ values. RC-DT 028, chromatography fraction with highest larvicidal activity. Commercial linoleic acid from TCI (Tokyo, Japan)

$LC_{50}$: 50% lethal concentration; LCL: lower confidence limit; mg/l: milligrams per liter; UCL: upper confidence limit

(linoleic acid) could explain the former phenomenon (Rasoanaivo *et al*, 2011; Sharma *et al*, 2014; Pezzani *et al*, 2019), and impurities in the isolated linoleic acid sample, as evidenced by the mass spectrometry and NMR spectra, could adulterate its larvicidal activity.

Perumalsamy *et al* (2015) reported that linoleic acid extracted from *Millettia pinnata* has a higher *Ae. aegypti* larvicidal activity ($LC_{50}$ = 21.28 mg/l) than the crude extract ($LC_{50}$ = 27.70 mg/l), but not as active as a chloroform-soluble fraction ($LC_{50}$ = 14.51 mg/l). Earlier, Rahuman *et al* (2008) reported that linoleic acid isolated from *Citrullus colocynthis* shows $LC_{50}$ value of 18.20, 11.49 and 27.24 mg/l against *Ae. aegypti*, *Anopheles stephensi* and *Culex quinquefasciatus*, respectively. Larvicidal activity against *Ae. aegypti* of *C. colocynthis* linoleic acid was approximately 5-fold higher than that of *A. pennata* linoleic acid and 2-fold higher activity than the commercial compound (80%, pure; TCI, Tokyo, Japan). Recently, Njoroge and Berenbaum (2019), in a study of the larvicidal activity of 13 plant-derived edible oils, found that oils with a higher linoleic acid content, *eg*, hempseed oil ($LC_{50}$ = 348.25 mg/l), sesame oil ($LC_{50}$ = 670.44 mg/l) and pumpkin seed oil ($LC_{50}$ = 826.91 mg/l), are more lethal to larvae than those with low linoleic acid content; interestingly, the larvicidal activity of a commercial linoleic acid (99%, pure; Sigma-Aldrich, St Louis, MO) is lower ($LC_{50}$ = 94.23 mg/l) than that the commercial product used in the current study.

Similarly, a study of *Anopheles gambiae* mosquito larvicidal activity of *Cassia tora* seed extract reported that fractionated extracts are 2-4 times less bioactive than the crude extract, while a pure compound (azadirachtin) exhibits the same activity as the crude extract (Mbatchou *et al*, 2017). Sharma *et al* (2014) observed that *An. stephensi* larvicides from *Artemisia annua* (also known as sweet wormwood) leaf extract, *viz* arteannuin B, artemisinic acid and artemisinin (a potent antimalarial in clinical use), show lower killing activity than a chloroform crude extract.

The mode of insecticidal action of linoleic acid is unclear. Perumalsamy *et al* (2015) reported that linoleic acid moderately inhibits acetylcholinesterase (AChE) activity and causes a considerable increase in cyclic adenosine monophosphate (cAMP) levels in mosquito larvae, indicating linoleic acid might act on both AChE and octopaminergic receptors.

In conclusion, the study reveals that the putative major larvicidal compound against 3$^{rd}$ instar *Ae. aegypti* present in the ethanol extract of *A. pennata* leaf was linoleic acid.

Further studies on the mode of insecticidal action of the linoleic acid should be performed to enable appropriate dosage and means of application for each species of mosquito vectors.

## ACKNOWLEDGEMENTS

The research was funded by the National Science, Research and Innovation Fund (NSRF), grant no. R2565B052. The authors thank Naresuan University and Thailand Science Research and Innovation (TSRI) for their support. The authors would like to express their acknowledgment to Assistant Professor Dr Unchalee Nilsuwan, Department of Biology, Faculty of Science and Technology, Phranakhon Rajabhat University, Bangkok, for providing valuable assistance with taxonomic identification of the plant specimens.

## CONFLICT OF INTEREST DISCLOSURE

The authors declare no conflict of interest.

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