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Original Article

Novel Point Mutations of the *ace-1* Gene of *Aedes aegypti* Larva Treated with Methanolic Extract of *Citrus hystrix*

Hebert Adrianto[1,2], Heny Arwati[3,*], Sri Subekti[4,5], Etik Ainun Rohmah[4], Reviany Vibrianita Nidom[6], Setyarina Indrasari[6]

ABSTRACT

Introduction: The mosquito species of *Ae. aegypti* is a vector of arthropod-borne diseases such as dengue haemorrhagic fever. Acetylcholinesterase (AChE) enzyme in *Ae. aegypti* that encoded by the *ace-1* gene. Damage in the *ace-1* gene as target of insecticide lead to the loss of the normal structure and function of AChE. However, damage in the *ace-1* gene remains uncharacterised. The main aim of this study was to find out the point mutations of *ace-1* gene in *Ae. aegypti* larvae treated with methanolic extract of *Citrus hystrix* leaves. **Method:** This experiment using a completely randomized design with two treatment groups. A container containing lethal concentration 50 of methanolic extract of *C. hystrix leaves*, and a control group containing only water with 0.5% Tween-20. Each group contained 50 third instar larvae of *Ae. aegypti*, and each group was repeated four times. Observation was performed for 24 h for the number of survived and dead larvae. Survived and dead larvae were collected prior to the DNA extraction, PCR, electrophoresis, and sequencing. The sequences of those two groups were then compared to determine the point mutations using genetyx ver 12. **Results:** The PCR products of both groups showed clear bands of 500-600 bp long. Furthermore, the presence of the mutation was confirmed by sequencing the PCR product of ace-1 between each treatment group. The survived larva in the extract-treated group showed more point mutation compared with that of dead larvae. **Conclusions:** This first report indicated that many mutations in the form of deletions and insertions in nitrogenous bases and different amino acid variations of the *ace-1* gene of third instar larvae of *Ae. aegypti* after 24 h treated with methanolic extract of *C. hystrix* leaves than those in control group.

Key words: *ace-1* gene, *Aedes aegypti*, *Citrus hystrix*, Sequence, Point mutation.

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INTRODUCTION

Dengue fever is a major public health issue in tropical and subtropical regions.[1] Dengue Hemorrhagic Fever (DHF) also occurs frequently in Indonesia throughout the year, both in urban and semi-urban areas.[2] This disease is caused by any of the four serotypes of dengue virus (DENV), which is transmitted by mosquitos, most notably the *Aedes aegypti* and *Aedes albopictus* species. Dengue infection cases were estimated to number 390 million, with a high prevalence in over 128 countries. According to the WHO, 500,000 people are hospitalized each year with severe dengue infection, and 2.5% of those infected died. Dengue fever is endemic in the South-East Asia (SEA) region. In 2015, this region accounted for approximately 451,422 of the total number of dengue cases worldwide (14.11%).[3] Dengue infection threatens an estimated 1.8 billion people in South-East Asia. After Brazil, Indonesia is the second hyperendemic country for dengue in the last decade, with an increasing number of cases.[4]

The synthetic chemical insecticides such as larvicides and adulticides is commonly used to control the mosquito vectors due to their cost-effectiveness, faster, and more effective in killing mosquito populations.[5,6] However, the resistance of mosquitoes have been reported worldwide after a long period of using chemical insecticides, and resulting in a failure of reducing the mosquito population.[7,8] The synthetic chemical compounds have disadvantages in that they are not biodegradable and are toxic to nontarget organisms.[9]

A promising alternative is the use of plants secondary metabolites, which can act naturally as antifeedant, attractant, nematicide, fungicide, repellent, insecticide, insect growth regulator, and allelopathic agents. The potential plants secondary metabolites are promising source for novel pest control agents or biopesticides.[10,11] These substances generally do not harm the environment, and exhibit low toxicity to off-target organisms.[12,13] One of the mechanisms by which plants against *Aedes* larvae is by inhibiting the acetylcholinesterase enzyme (AChE).[14,15] Acetylcholinesterase, encoded by the *ace-1* gene, catalyses the hydrolysis of the neurotransmitter acetylcholine to terminate nerve impulses at cholinergic synapses in the central nervous system of insects.[16] The damage in *ace-1* gene is predicted to cause failure of AChE enzyme synthesis and cause the inability to hydrolyse the neurotransmitter acetylcholine at cholinergic synapses in the central nervous system. Accumulation of AChE causes hyper excitableness, paralysis, and eventual death in mosquito larvae.[14] Some plants have been reported to have potential inhibition properties of AChE enzymes in *Aedes* larvae, such as *Melaleuca cajuputi,*[17] *Salvia officinalis,*[18] *Gallesia integrifolia,*[19] *Cassia fistula,*[20] *Lumnitzera racemose,*[21] *Excoecaria agallocha,*[21] *Artemisia absinthium,*[22] *Cynadon dactylon,*[23] *Spilanthes acmella.*[23] However, the available scientific data regarding the mechanisms by which secondary metabolites from plants cause

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damage to *ace-1* gene, is very limited. The possibility of moderating the response of cells in mosquito larvae to a particular mutagen by natural secondary metabolites from plants opens new horizons and surprises in the discovery of new larvicide in vector control.

Citrus is a member of the Rutaceae family, which contains approximately 162 species that are found all over the world. *Citrus* plants have long been used for vitamins and medicinal purposes.[24] Previous study has reported that methanolic extract of *C. hystrix* leaves was the most effective bio larvicidal against *Ae. aegypti* larvae compared with *Citrus amblycarpa* and *Citrus maxima*.[25]

Despite the mortality effects, mutagenic potential in dead and survived larvae remains unknown. Few studies on the effect of plant extracts on gene mutations in mosquito larvae have been reported. Field trials of insecticides from *Citrus* leaves should be carried out together with their detailed modes of action for more comfortable results.[26] Therefore, the genotoxic effect of the methanolic extract of *C. hystrix* leaves on the *ace-1* gene simultaneously with PCR and sequencing of the gene is needed to be evaluated. The main aim of this study was to find out the point mutation of *ace-1* gene in *Ae. aegypti* larvae treated with methanolic extract of *C. hystrix* leaves.

MATERIALS AND METHODS

Plant materials

C. hystrix leaves were collected from Taman Geluran Sub District, Sidoarjo City, East Java Province, Indonesia during 2022. Determination of scientific name and species based on the morphology of plant was carried out at the Department of Pharmacy, Universitas Katolik Widya Mandala, Surabaya, Indonesia (No.76/LJ-FF/I/2021).

Extraction of *C. hystrix* leaves

The leaves of *C. hystrix* were thinly sliced and sterilized with 70% alcohol. The leaves were dried in the open air for one month. The dried leaves were then crushed into powder using a blender. The leaf powder was macerated in methanol for one week. The maceration results were filtered using filter paper. The solvent was then removed using a rotary evaporator to produce a viscous extract. This extract was used for the larvicidal bioassay.

Rearing and colonization of larvae

The eggs of *Ae. aegypti* were provided by and maintained to third instar larvae in the Laboratory of Entomology, Institute of Tropical Diseases, Universitas Airlangga, Surabaya, Indonesia. The eggs were then kept to hatch in a tray containing mineral water under optimum conditions including room humidity of 65-80%, water temperature at 28-30°C and further reared into third instar larvae. The larvae were fed with fish pellet. The colony of third instar larvae were then harvested for larvicidal bioassay.

Larvicidal bioassay

The larvicidal assay of the extract was evaluated according to the WHO standard protocol for larval bioassay.[27] This experiment used a complete randomized design with two groups. Group 1 was a container containing 10.000 ppm of methanolic extract of *C. hystrix* leaves as LC50 (lethal concentration 50), and Group 2 a control group containing only water (aquadest) with Tween-20. The LC50 is the concentration of extract required to kill 50% of larvae during observation. This LC50 was determined obtained based on a preliminary tests with various concentrations of extract,[28] where 1000 ppm of concentration was obtained as LC50: The LC50 was used in this bioassay to obtain both the dead and survived larvae due to the exposure to the extract. Further, bioassay was performed using 50 third instar larvae of *Ae. aegypti* in each group, and each group was repeated three times. The observation was performed after 24 h of exposure for the number survived and dead larvae. The death of larvae was characterized by no movement on the surface or bottom of the container but sank on bottom of the container and no response to the flashlight. They were then sent to the Laboratory of Professor Nidom Foundation, Surabaya City, Indonesia for DNA extraction, PCR, electrophoresis, and sequencing.

DNA extraction from *Aedes aegypti* larvae samples

Genomic DNA was isolated from larvae (5 survived and 5 dead) from each group using DNA extraction kit of ZymoBIOMICSTM DNA Miniprep Kit (Zymo Research, CA, USA) according to manufacturer's instructions. The DNA were then used as template in PCR reaction.

PCR and electrophoresis

PCR amplifications were carried out based on Hasmiwati *et al.* (2018) using Applied Biosystems SimpliAmpTM Thermal Cycler (Thermofisher Scientific, MA, USA).[29] The PCR amplification products (5 µl) were then electrophoresed pre-stained with ethidium bromide and visualized under ultraviolet illumination. The expected PCR product size was 581 bp.

Sequencing and analysis of mutations

The PCR products were then purified according to the BigDyeH Terminator v3.1 Sequencing Kit (Thermo Fisher Scientific, Inc. MA USA). Forward and reverse sequencing reactions were done using the forward and reverse PCR primers as mentioned above. Double-sequencing were performed using an ABI PRISM 377 Genetic Analyzer. Sequence results were compared between groups to determine point mutations using genetyx ver 12.

Ethical approval

The proposal of the study has been approved by The Ethical Committee of the School of Medicine of Universitas Ciputra, Surabaya, Indonesia, as described on the Ethical Clearance No. 141/EC/KEPK- FKUC/II/2022.

RESULTS

A short treatment of the third instar of *Ae. aegypti* larvae with methanolic extract of *C. hystrix* leaves resulted in the average number of survived larvae from 3 replications was 25.33 (50.66%), and the average number of dead larvae from 3 replications was 24.67 (49.34%). Interestingly, no dead larvae in the negative control group (100% survived). The region of the *ace-1* gene was amplified from 15 *Ae. aegypti* larvae consisted of 5 from negative control group (survived larvae), 10 from extract of *C. hystrix* leaves (5 dead larvae and 5 survived larvae). The results of electrophoresis of *ace-1* DNA from third instar larvae of *Ae. aegypti*, is shown in Figure 1. The bands were 500-600bp long (Figure 1).

The resulting 500-600 bp amplicons were purified and sequenced to determine genetic profile. Sequence analyses of both survived and dead larvae treated with methanolic extract of *C. hystrix* leaves confirmed that the sequences of PCR products were sequence of the *ace-1* gene. This current study showed that the point mutations in the sequences have indicated the damage of DNA. Sequence comparison showed that the survived larvae treated with the methanolic extract showed more point mutations than the dead larvae. The mutations were including transitions, transversion, deletions and insertions in nitrogenous bases and different amino acid variations in the *ace-1* gene of third instar larvae of *Ae. aegypti* after 24 hours treated with methanolic extract of *C. hystrix* leaves (Figure 2 and Figure 3). Interestingly, no mutation was found in the survived larvae in negative control group.

Table 1 shows that the most frequently mutation occurrences were transitions (36.3%), followed by insertion (31.8%), and transversion

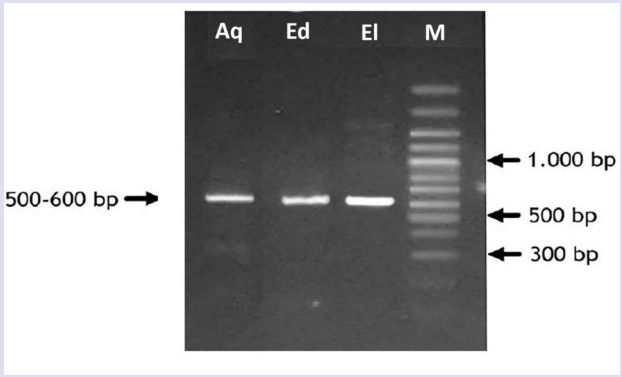

Figure 1: Electrophoresis of PCR products in 1% agarose gel. The sizes of bands are between 500-600bp. M, DNA ladder marker; Aq, control group; Ed, extract- dead larvae; El, extract- survived larvae.

ALIGNMENT :
Nukleotida

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                    10        20        30        40        50        60        70
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|
Aquadest            -TTCGATAACGAATGGGGAACGAACGCTACCGAGGCTATGCCGCAGACATTCATGACAGCGCCTCGCACG
Extract-dead        -TTCGATAACGAATGGGGAACGAACGCTACCGAGGCTATGCCGCAGACATTCATGACAGCGCCTCGCACG
Extract-lived       ----------------------------------------CGCAGAC-TTCATGACAGCGCCTCGCACG

                    80        90        100       110       120       130       140
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|
Aquadest            CGCTTGCTGTCATCGGCGCGATGTTGCGGCCCGAAGGACACGGCGAAGGATCGGCGCGCACGCCGGACGC
Extract-dead        CGCTTGCTGTCATCGGCGCGATGTTGCGGCCCGAAGGACACGGCGAAGGATCGGCGCGCACGCCGGACGC
Extract-lived       CGCTTGCTGTCATCGGCGCGATGTTGCGGCCCGAAGGACACGGCGAAGGATCGGCGCGCACGCCGGACGC

                    150       160       170       180       190       200       210
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|
Aquadest            GATCGACCTTAACGCACTCGCGGAGCGTGTCGTATCAACCATGCAGCCGCTTGCGGCGGAACGGCAGTTG
Extract-dead        GATCGACCTTAACGCACTCGCGGAGCGTGTCGTATCAACCATGCAGCCGCTTGCGGCGGAACGGCAGTTG
Extract-lived       GATCGACCTTAACGCACTCGCGGAGCGTGTCGTATCAACCATGCAGCCGCTTGCGGCGGAACGGCAGTTG

                    220       230       240       250       260       270       280
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|
Aquadest            CGTCTCGCACTCAATCTCGCCAAGGCGCGGCCGAACGTTGTCGTCAACGCGACCTCGTTACGGCAAATCC
Extract-dead        CGTCTCGCACTCAATCTCGCCAAGGCGCGGCCGAACGTTGTCGTCAACGCGACCTCGTTACGGCAAATCC
Extract-lived       CGTCTCGCACTCAATCTCGCCAAGGCGCGGCCGAACGTTGTCGTCAACGCGACCTCGTTACGGCAAATCC

                    290       300       310       320       330       340       350
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|
Aquadest            TCCTCAACCTGCTTACCAACGCCATCAAGTTCACCGAAGCGGGCGGCGATGTACGCGTTGCAACCGGTTA
Extract-dead        TCCTCAACCTGCTTACCAACGCCATCAAGTTCACCGAAGCGGGCGGCGANGTACGCGTTGCAACCGGTTA
Extract-lived       TCCTCAACCTGCTTACCAACGCCATCAAGTTCACCGAAGCGGGCGGCGATGTACGCGTTGCAACCGGTTA

                    360       370       380       390       400       410       420
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|
Aquadest            TTTGGACGACGGACGTGTGTTCCTCGTGGTGCGCGATACCGGGAACGGCTTTAGCATCGACACGGAAAGC
Extract-dead        TTTGGACGACGGACGTGTGTTCCTCGTGGTGCGCGATACCGGGAACGGCTTTAGCATCGACACGGAAAGC
Extract-lived       TTTGGACGACGGACGTGTGTTCCTCGTGGTGCGCGATACCGGGAACGGCTTTAGCATCGACACGGAAAGC

                    430       440       450       460       470       480       490
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|
Aquadest            GACAGTGCGCGCAGCGCGCCGGCATCACGTCTCGGGAAGGGCCACGGCTTGGGCCTGCCGCTTGTCCGAC
Extract-dead        GACAGTGCGCGCAGCGCGCCGGCATCACGTCTCGGGAAGGGCCACGGCTTGGGCCTGCCGCTTGTCCGAC
Extract-lived       GACAGTGCGCGCAGCGCGCCGGCATCACGTCTCGGGAAGGGCCACGGCTTGGGCCTGCCGCTTGTCCGAC

                    500       510       520       530       540       550       560
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|
Aquadest            GCCTTGCGGAAGACATC-GGCGCCACTATC-GAAATCGACAGCGCTGAGGGTAAGGGAACGGTTGTCCTC
Extract-dead        GCCTTGCGGAAGACATCCGGCGCCACTATCTGAAATCGACAGCGCTGAGGGTAAGGGAACGGTTGTCCTC
Extract-lived       GCCTTGCGGAAGACATC-GGCGCCACTATC-GAAATCGACAGCGCTGAGGGTAAGGGAACGGTTGTCCTC

                    570       580
                    ....|....|....|...
Aquadest            GTCGTGTTCGGTGAGCCTCTGAA
Extract-dead        GTCGTGTTCGGTGAGCCTCTGAA
Extract-lived       GTCGTGTTCGGTGAGCCTCTGAA
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Figure 2: A representative sequence of the PCR products of the *ace-1* gene from *Ae. aegypti* larvae treated with methanolic extract of *C. hystrix* leaves extract. Changes in nitrogenous bases are indicated by arrows and gray color.

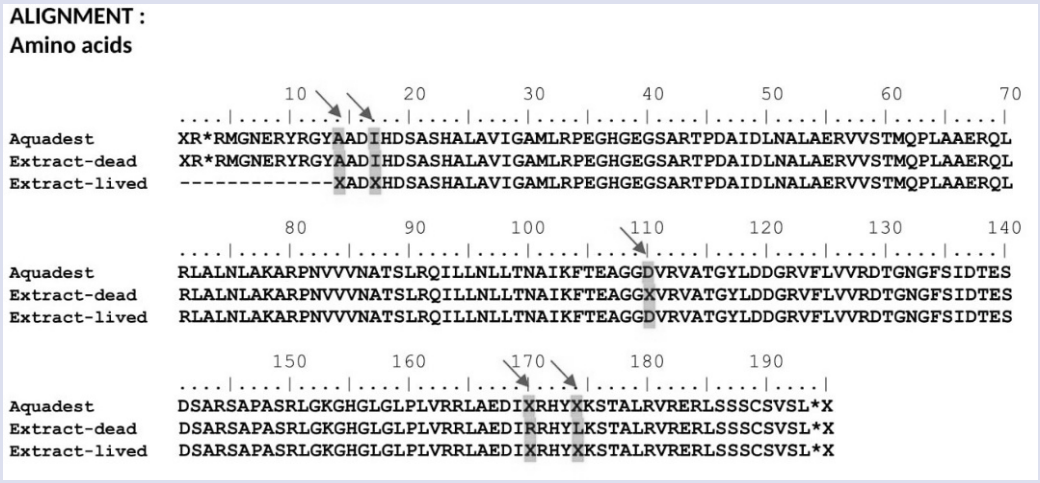

Figure 3: Representative sequence amino acid of the *ace-1* gene from *Ae. aegypti* larvae treated methanolic extract of *C. hystrix* leaves. Changes in amino acid are indicated by arrows and gray color.

Table 1: Variations in base and amino acid changes in the treatment group.

Samples	Replication	Base nitrogen mutations	Amino acid mutations
Dead larvae	1	-507C (insertion)	X173L (any amino acid → leucine)
		-520T (insertion)	X169R (any amino acid → arginine)
		T554C (transition)	X32R (any amino acid → arginine)
		C562G (transversion)	-190R (arginine)
	2	T567C (transition)	I194T (isoleucine → threonine)
		G571T (transition)	
		T580C (transition)	
	3	A11T (transversion)	n/a
	1	A8- (deletion)	A1X (alanine → any amino acid)
			I4X (isoleucine → any amino acid)
	2	G530A (transition)	X41R (any amino acid → arginine)
		T583C (transition)	R182Q (arginine → glutamine)
		C591G (transversion)	-199R (arginine)
		T596C (transition)	I203T (isoleucine → threonine)
		G600A (transition)	
Survived larvae		T2C (transition)	A14S (alanine → serine)
		C10A (transversion)	X88H (any amino acid → histidine)
		G40T (transversion)	X126Q (any amino acid → glutamine)
	3	-262C (insertion)	X161Q (any amino acid → glutamine)
		-377A (insertion)	X165A (any amino acid → alanine)
		-482A (insertion)	X194P (any amino acid → proline)
		-493G (insertion)	
		-581C (insertion)	

(27.3%). Only one deletion occurs (4.5%). Changes in nitrogenous bases cause changes in the amino acid products resulting from the translation process. The exposure of *C. hystrix* extracts to the *ace-1* gene in third instar of *Ae. aegypti* larvae led the finding of 17 new amino acids in its sequence. The insertion of arginine (R) and transition of any amino acid with arginine (X → R) often occurred in the larvae treated with methanolic extract of *C. hystrix* leaves.

DISCUSSION

Encouraged by interesting results observed in the reduction of *Aedes* larvae populations in countries where larvicide from plants has been intensively developed during the last decade. Several other countries are planning to start using this strategy to control the *Aedes* population and overcome the problem of resistance from temephos. This research is the first report of the analysis of point mutations in plant extracts-treated mosquito larvae by PCR and sequencing. This research has focused on the biological control of *Ae. aegypti* (Diptera: Culicidae) mosquitoes using natural active products from *C. hystrix* leaves. Point mutations in bases and amino acids were found in both survived and dead *Ae. aegypti* larvae exposed to LC50 of methanolic extract of *C. hystrix* leaves extract. The chemical contents of the methanolic extract of *C. hystrix* leaves might damage the DNA that led to the variety of point mutations in DNA sequences.

Culex quinquefasciatus larvae treated with acetone and chloroform extracts of *Curcuma longa* and *Melia azedarach* caused greater changes in the RAPD (Random Amplified Polymorphic DNA) patterns.[30] Now, the PCR test can detect particular mutations in *A. gambiae, C. pipiens,* and *A. albimanus*. PCR test is probably of broad applicability within the Culicidae family.[31] AChE, which is encoded by the *ace-1* gene, is a key enzyme in both cholinergic system and other non-

neuronal tissues.[32] The AChE is an enzyme becoming the target of organophosphate insecticide. The occurrence of target site alteration is due to the gene mutation. Three mutations in *ace-1* gene have been associated with acetylcholinesterase insensitivity in *Ae. aegypti* in West Sumatra to temephos.[33] *ace-1* gene was shown to be responsible for AChE insensitivity in resistant strains of two mosquito species, *Anopheles gambiae* and *Culex pipiens*.[31] Mutations in the AChE gene at the molecular level may interfere with the synthesis of the AChE enzyme causes the decreases in AChE levels.

This current study found differences in mutations between dead larvae and survived larvae. More transitions and transversions were found at positions 554-580 in dead larvae, while more insertion types were found at positions 262–581 of the sequence in survived larvae, and transitions were more commonly found in positions 500–600. A different study found that deletion and insertion occurred considerably in *T. castaneum* after being treated with lethal concentration 99 (LC99) of *Bolanthus turcicus* extract.[34]

Mutation in *ace-1* gene of *Ae. aegypti* were G119SA, F290V and F455W.[35] However, another study did not find F290V and F455W mutations in temephose resistant *Ae. aegypti* in West Sumatra, Indonesia, instead, a new mutation of T506T was found.[33] Interestingly, G119S was also found in a carbamates resistant *Anopheles gambiae* (sensu lato) populations from Mali.[36] Those kinds of mutation did not find in this current study. The point mutations found in this study apparently are typical for extract-post-treated mutations.

The methanolic extract of *C. hystrix* leaves contains alkaloids, flavonoids, terpenoids, and phenols which have antioxidant activity. The extract also contains anti-fungal compounds, namely flavonoids and tannins.[37] Phytochemical screening of extracts of *C. hystrix* herbs for IPA, aqueous, acetone and benzene from India had revealed the presence of flavonoids, tannins, terpenoids, alkaloids, cardiac glycosides, proteins, carbohydrates and quinones.[38] Secondary metabolites are toxic and cause death in insects, including mosquitoes. The n-hexane extract of *C. hystrix* leaves at a dose of 4,000 ppm was reported to cause the death of *Cx. quinquefasciatus* larvae *in vitro* at 93.33% after 4 hours of observation.[39] Nano emulsion of essential oil of *C. hystrix* showed larvicidal and pupicidal activity on larva and pupa of *Ae. aegypti* and it is more environmentally friendly than temephos. β-pinene and limonene are the main compounds with the highest content in the essential oil of *C. hystrix*.[40] Different origins of *C. hystrix* showed different compound content. A total of 26 compounds were detected in the leaves of *C. hystrix* from East Sumba whereas, 21 compounds were identified in the leaves of *C. hystrix* from Central Java. Two oxygenated monoterpene groups, Linalool and Citronellal, were detected as the main compounds with a high percentage. Linalool is known to have biological activities such as antimicrobial, anti-inflammatory, anticancer, antioxidant properties, a lead compound in the synthesis of vitamins A and E. Citronellal's biological activities were antinociceptive and anti-inflammatory effects.[41] The volatile organic compounds in *C. hystrix* leaf methanolic extracts and fractions have a variety of biological activities, including anticancer (cytotoxicity, apoptosis inducing activity, anti-proliferative), antimicrobial (antibacterial, antifungal, antiviral), antioxidant, anti-inflammatory, lipid lowering effect, anxiolytic-like effect, anti-neoceptive, and analgesic-like effect.[42]

The compound which inhibits AChE enzyme in *Anopheles stephensi* mosquito has been reported previously was Plumbagin from the rhizome of *Plumbago zeylanica*.[43] Other compounds that have same inhibition effect were Coumaran (2,3-dihydrobenzofuran) from *Lantana camara* L. (Verbenaceae) and methanolic extract from *Cassia fistula* L. (Fabaceae) roots, and also increasing the concentration levels of acetylcholine in the synapse cleft, causing excessive neuroexcitation due to the neurotransmitter's prolonged biding to its post-synaptic receptor.[11] Phenol groups and essential oil can bind to the steric center of AChE, inhibit its activity and cause an increase in acetylcholine in nerve endings, resulting in continuous stimulation of the central nervous system and the insect's paralysis and death.[28] AChE activity has also been shown to be inhibited by essential oils containing monoterpenes as major compounds. α-pinene, α-terpinene, (−)-linalool, geraniol, (−)-carvone, thymol, carvacrol, E-anet- hole, estragole, (+)-camphor, 1,8-cineole, cuminaldehyde, L-fenchone, (−)-limonene, (−)-menthol, and myrcene have also been shown to cause AChE inhibition.[44] Two novel chromone flavonoid compounds from a methanolic extract of date palm pits are considered inhibitors for the AChE enzyme in *Culex pipiens* according to experimental and in silico molecular docking technique analysis. The inhibition of AChE in larvae treated with both chromones 1 and 2 shows that these chromones may prevent any message from being transmitted to the receptor, causing the insect to lose neurological orientation.[45] Four flavonoids (caranjin, karanjachromene, pongamol, and pongarotene), oleic acid, and palmitic acid from *Millettia pinnata* seeds were tested against the AChE activity of *Ae. albopictus* larvae. Karanjachromene, pongarotene, pongamol, and oleic acid were the most potent AChE inhibitors. The AChE inhibitory activity of palmitic acid was the lowest of any of the compounds examined.[46]

Recently, global attention for vector control has shifted from chemical insecticides to botanicals.[26] Several phytochemicals from several plant families are identified with larvicidal activities against different mosquito species, such as from plant's barks, leaves, roots, flowers, fruits, seeds, cloves, twigs, woods, herbs, rhizomes, and stems. Myrtaceae, Lauraceae, Rutaceae, Lamiaceae, Asteraceae, Apiaceae, Cupressaceae, Poaceae, Zingiberaceae, Piperaceae, Liliaceae, Apocynaceae, Solanaceae, Caesalpinaceae, Sapotaceae are plant families that have been reported to contain bioactive compounds with activity against important insect.[26,43,47] When mosquitos come into contact with these plants' secondary metabolites, a relatively unambiguous response is elicited that has a non-specific influence on a wide range of molecular targets such as proteins, nucleic acids, and bio-membranes. As a result, the physiology is disrupted at numerous receptor sites, eventually leading to a nervous system abnormality. Plant metabolites GABA-gated influence several vital physiological functions, including the inhibition of AChE and GABA-gated chloride channels, the disruption of Na-K ion exchange, and the restriction of cellular respiration. As a result of the altered enzyme levels, several anomalies occur, including the obstruction of nerve cell membranes and octopamine receptors, as well as calcium channel blockage, resulting in hormonal imbalance, mitotic poisoning, and modifications to the molecular basis of morphogenesis.[43]

Citrus species from Rutaceae family is a great source of essential oil because of numerous oil glands in various parts of the body. Kaffir lime *(C. hystrix)* leaves contained alkaloids, steroids, reducing sugar, and carbohydrates in all methanolic extracts. Flavonoid only found in methanol extract.[48] Essential oils extracted from the leaf and peel of *Citrus aurantifolia*, and one of its main constituents was citral.[26] Citral showed the highest affect as ovicide, larvicidal and adulticidal activities, and can be used in mosquito control programs against the *Ae. aegypti* mosquito.[26] Alkaloids, saponins, flavonoids, triterpenoids and tannins which are known to have insecticidal and pesticidal properties against *Anopheles gambiae, Ae. aegypti,* and *Culex quinquefasciatus*.[49,50] Study on the effects of *Baccharis dracunculifolia* leaf essential oil on *Cx. quinquefasciatus* larvae based on morphology and biochemistry, such as total glucose, triacylglyceride (TAG), protein, and AChE levels, indicated that the essential oil can significantly decreased AChE levels after exposure for 24 h.[28] Alkaloid can degrade the cell membrane of the digestive tract, and interfere with the nervous system of larvae by inhibiting AChE activities.[39]

The mutations in the *ace-1* gene in survived larvae may play a role in the

detoxification of the poison content in the extract leading to the survival of larvae. The ace-1 mutation in the *Anopheles gambiae* mosquito population from Cameroon is associated with most mosquitoes being alive after carbamate exposure.[51] The G119S mutation has been associated with the survival of *An. gambiae* populations. The *ace-1* gene was associated with *An. arabiensis* resistance to bendiocarb in Dangassa, *Anopheles coluzzii* in Koula and Dangassa, and *An. gambiae* in all surveyed localities.[36] The sequencing analysis of the *ace-1* gene revealed the absence of the F290V and F455W mutations in survived larvae of *Ae. aegypti* treated with temephos, but a point mutation was detected at codon 506. This mutation shifts the ACA codon to ACT but still codes for the same amino acid, threonine.[33] Three VGSC mutation alleles, S989P, V1016G, and F1534C, were identified from specimens of *Ae. aegypti*. The presence of a silent mutation (TTG to TTA) at position (Leu, L) was found. No I1011M or F1552C mutations were identified.[52]

On the other hand, the mutations found in dead larvae may cause damage or disruption of AChE synthesis that cause AChE function becomes abnormal and many acetylcholine accumulated in the synapse.[53] Mutations in the form of transitions, transversions, deletions, and insertions at different nitrogenous bases and amino acids were also found in the sequence of the CYP345A1 region of *cyp* gene in the insect of *Tribolium castaneum* treated with *Bolanthus turcicus* extract compared with that in control where deletion and insertion occurred in a significant amount.[34] The more mutations in mosquitoes relate to the resistance to insecticides has also been reported.[54] Three mutations (S989P, V1016G and F1534C) and novel mutation (A1007G) were associated with pyrethroid resistance within *Ae. aegypti* population in Penang, Selangor, and Kelantan (Malaysia).[55] As a conclusion, the secondary metabolite components in the methanolic extract of *C. hystrix* leaves might cause the variety of point mutations in the *Ae. aegypti* larvae treated with this extract.

The limitation of the study is that the analysis was only based on the DNA sequences to find out the effect of the extract. There was no histological observation on the extract post-treatment. Nevertheless, the findings will serve as the primary data for future research and further analysis of the *ace-1* gene.

CONCLUSION

This first report indicated that many mutations in the form of deletions and insertions in nitrogenous bases and different amino acid variations of the *ace-1* gene of either dead or survived third instar larvae of *Ae. aegypti* after 24 h treated with methanolic extract of *C. hystrix* leaves than those in control group. These findings indicated the typical extract-post-treated mutations, and prove this bio larvicide can cause the damage of DNA in a short time exposure.

SUMMARY

This study reports the first discovery of point mutations of the *ace-1* gene of *Aedes aegypti* larva treated with methanolic extract of *C. hystrix* leaves.

CONFLICTS OF INTEREST

All authors declare no conflicts of interest.

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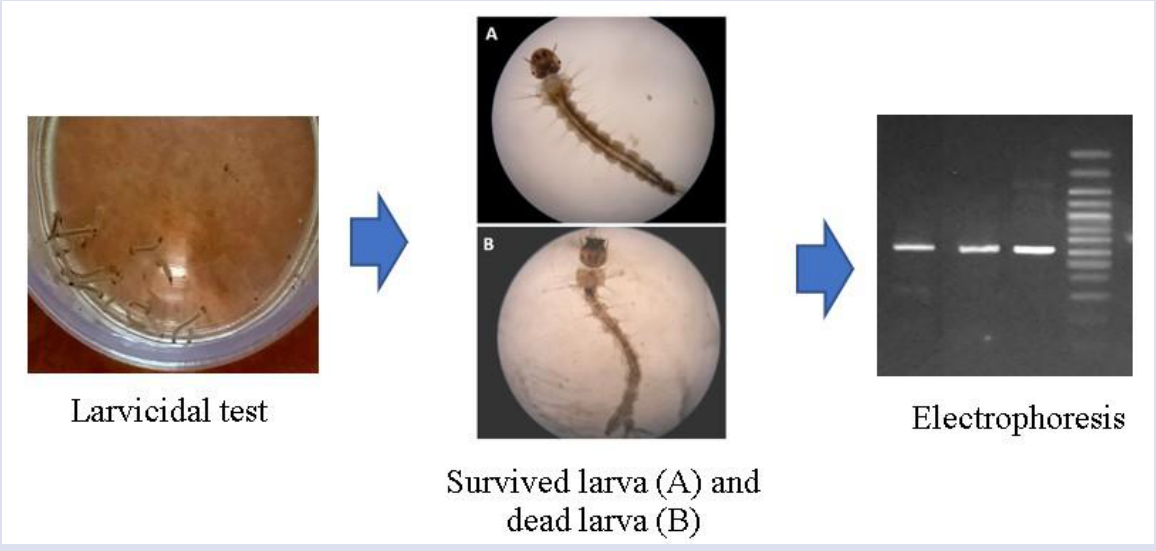
GRAPHICAL ABSTRACT



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