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DNA Barcoding and Sensitivity of Dengue Vector *Aedes aegypti* (Linnaeus, 1762) (Diptera: Culicidae) Aquatic Stages to Different Insecticides with Reference to Non-Target Organisms, *Danio rerio* and *Daphnia magna*

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ABSTRACT

Aedes aegypti is a major vector of Dengue fever, whose slight reappearance was recorded in the Red Sea Governorate in 2017. The present study was carried out to determine the DNA barcoding of *Ae. aegypti* and investigate the sensitivity of its aquatic immature stages to different insecticides, with reference to non-target organisms, zebrafish, *Danio rerio*, and *Daphnia magna*. Genetic identification of *Ae. aegypti* was confirmed through PCR amplification and subsequent sequencing. The obtained DNA sequences were deposited in the National Center for Biotechnology Information (NCBI) sequence database with the accession number OR76138. Methomyl, α-cypermethrin, and chlorpyrifos were tested against immature stages of *Ae. aegypti*. Additionally, the effect of tested insecticides on acetylcholinesterase (AChE) and glutathione S-transferase (GST) activities were assessed. Results showed that the activity of the tested insecticides varied according to the concentration of the insecticides, exposure time and the immature stage tested. Complete larval mortality (100%) of the first instar was attained at 0.007, 0.0009, and 0.005ppm of methomyl, α- cypermethrin, and chlorpyrifos after 48h of exposure, respectively. Generally, α- cypermethrin was the most effective insecticide, followed by chlorpyrifos and methomyl. In addition, methomyl caused the least inhibition of AChE activity (5.53 and 5.90U/ L) in larvae I after 24 and 48h of exposure, compared to 5.74 and 6.13U/ L for the control group. In addition, the GST activity was significantly increased ($P< 0.05$) by all tested insecticides. The highest GST values (1.73 and 1.59U/ g tissue) were recorded for pupae after 24 and 48h of exposure to α-cypermethrin. On the other hand, methomyl recorded LC_{50} of 0.862, 0.011, and 0.575, 0.009 ppm against zebrafish and *Daphnia* after 24 and 48h, respectively. Generally, methomyl, α-cypermethrin, and chlorpyrifos are effective agents against *Ae. aegypti*, however, their environmental footprint cannot be overlooked.

INTRODUCTION

Today, vector-borne diseases are considered one of the most critical issues worldwide; it is responsible for about 700 thousand deaths every year **(Zulfa *et al.*, 2022)**. In tropical and subtropical countries, the prevalence of different mosquito species enhances the

opportunities of contracting serious diseases such as Malaria, Dengue, Rift Valley Fever (RVF) and chikungunya **(Ferguson, 2018; Mordecai *et al.* 2019; Shehata *et al.* 2021; WHO, 2023)**. *Aedes aegypti* L. (Diptera: Culicidae) is distributed through Africa, Asia in addition to North & South America **(Shehata *et al.*, 2022)**. Remarkably, *Ae. aegypti* is the main vector of Dengue fever, which has become endemic in more than 125 countries worldwide, with more than 390 million new infections per year **(Stanaway *et al.*, 2016; Wilder-Smith *et al.*, 2019; Zulfa *et al.*, 2022)**.

For decades, Egypt witnessed an absence of *Ae. aegypti* **(Holstein, 1967)**. The re-appearance of *Ae. aegypti* in Egypt with a slight Dengue outbreak in the Red Sea Governorate in 2017 highlighted the urgent need for effective control strategies, especially due to the lack of antiviral treatments **(Abozeid *et al.*, 2018; El-Ansary *et al.*, 2022)**. Although a licensed vaccine for Dengue fever treatment is available today, the protection is still incomplete **(Satoto *et al.* 2019)**. Thus, the prevention of Dengue fever outbreak still depends on *Ae. aegypti* control **(El-Ansary *et al.*, 2022)**. Hence, targeting *Ae. aegypti* with chemical insecticides such as Organophosphates and Pyrethroids remained a key intervention for Dengue fever prevention **(Putra *et al.*, 2016).**

Methomyl, an oxime carbamate, was created in 1968 to manage numerous insect classes, including Hemiptera, Lepidoptera, Diptera, Homoptera, and Coleoptera. Like several carbamates, methomyl can inhibit acetylcholine (AChE) function **(Van Scoy *et al.*, 2013)**. Moreover, although pyrethrum is extracted from chrysanthemum flowers, synthetically modified plant-based insecticides like pyrethroids have different effects. Alpha-cypermethrin is among the most widely used pyrethroids, and it is harmless and less hazardous to animals because of their fast metabolism and high surface area **(Kakati, 2023)**. Pests have also been managed using chlorpyrifos, a broad-spectrum organophosphate that is only mildly harmful **(Sparks & Nauen, 2015)**. It is used to control several mosquito species, such as *Culex pipiens* and *Cx. quinquefasciatus* **(Aney *et al.*, 2018; Mohamed *et al.*, 2022)**.

On the other hand, the potential impact on non-target organisms remains a significant concern. Zebrafish and *Danio rerio* have become popular models for studying the toxicological effects of various substances; their responses to toxins often mirror those seen in other vertebrates, making them a valuable tool for preliminary screenings **(Martins *et al.*, 2007)**. Besides, *Daphnia magna* is a small planktonic crustacean commonly found in freshwater ecosystems. Their sensitivity to environmental changes and toxins makes them an essential bioindicator species. Interestingly, studies have shown that zebrafish and daphnia exhibit distinct sensitivities and responses to different pesticides **(Hussain *et al.*, 2020)**. Methomyl, a carbamate insecticide, has been studied for its effects on fish species. For instance, in male tilapia, exposure to low doses of methomyl resulted in irreversible endocrine disruption, indicating a potential threat to fish populations **(Meng *et al.*, 2016)**.

The present research was conducted to assess the DNA barcoding of *Ae. aegypti*, as well as investigating the sensitivity of its aquatic immature stages to methomyl, α-cypermethrin, and chlorpyrifos. *Dan. rerio* and *Daphnia magna* were used to investigate the toxicity of pesticides on non-target species.

MATERIALS AND METHODS

1. Tested insect

Larvae of *Ae. aegypti* were collected from the Red Sea Governorate, Egypt (27°5'36.413N, 33°49'56.965E). The collected larvae were morphologically identified according to **Rueda (2004)** and then subjected for molecular identification. The larvae were reared for five generations under controlled optimum temperature conditions (25±2ºC), RH (70±10%), and 12 light-dark *Aedes aegypti* adults were kept in a standard wooden cage (30×30×30 cm) and daily provided with 10.0% sucrose solution via cotton pads. The rearing procedure mentioned by **Shehata *et al.* (2020)** was followed to provide *Ae. aegypti* different immature stages.

2. Genetic identification of *Aedes aegypti* larvae

2.1. Extraction of DNA from mosquito larvae

Small pieces of the *Ae. aegypti* specimens were inserted in 1.5µL Eppendorf tubes for study. The PureLink® Genomic DNA Kits were used for DNA extraction (Invitrogen, Waltham, Massachusetts, USA). In summary, 180-250µL of tissue lysis buffer was added to each sample, and 10µL of proteinase K was added to each 180µL of tissue lysis solution. Then, for 4 hours, the combination was kept at 56 degrees Celsius. The supernatant was transferred to a fresh tube as directed by the manufacturer (Invitrogen; Waltham, Massachusetts; USA). Following the addition of 200µL of ethanol and 200µL of Lysis/Binding Buffer, the lysate was vertexin. The solution was then placed in a spin column and centrifuged at 10,000xg for one minute. DNA was eluted in 50µL of elution solution after two washes with wash buffers and then kept at -20°C.

2.2. Polymerase chain reaction (PCR)

As detailed by **Folmer *et al*. (1994)**, LCO1490 (5'-GGTCAACAAATCA TAAAGATATTGG-3') and LCO1490-R (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') were used as the forward and reverse primers, respectively. A total of 50µl was used for the PCR amplification process, with 25µl used for the 2X master mix solution (i-Taq, iNtRON, Seongnam, Korea), 2µl of each of the 0.2 M primers, 4µl of template DNA, 2µl of BSA at 0.2mg/ mL, and 14.5µl of nuclease-free water. First, tick DNA was denatured at 95 degrees Celsius for 10 minutes, then it was subjected to 40 cycles of denaturation at 95 degrees Celsius for 1 minute, annealing at 46 degrees Celsius for 1 minute, and extension at 72 degrees Celsius for 1 minute. The last 10-minute extension was carried out at 72 degrees. The amplified DNA was seen on a 1% agarose gel stained with ethidium bromide and examined under a transilluminator to

determine the PCR product's quality and quantity (U.V. transilluminator, Spectro line, Westbury, USA).

2.3. Sequence analysis

Using a Macro-gen reagent, the PCR products were isolated and purified (Seoul, Korea). Nucleotide sequences of *Ae. aegypti* COI were aligned following single-strand DNA sequencing.

2.4. Bioinformatics

Chromas Pro 1.5 beta was used to assemble the resulting sequences (Technelysium Pty., Tewantin, QLD, Australia). Basic Local Alignment Search Tool (BLAST), available at http://blast.ncbi.nlm.nih.gov/Blast.cgi, was used to compare the newly obtained COI sequences of *Ae .aegypti* (accession number: OR726138) to sequences in GenBank. The MEGA 11.0 software's muscle alignment was used to align the sequences. The Tamura three-parameter model was used to determine the degree of sequence dissimilarity **(Tamura, 1992)**. A neighbor-joining tree was constructed using the Tamura three-parameter approach to illustrate patterns of species divergence **(Tamura, 1992)**. Bootstrapping is done with 1000 replications, as **Kumar *et al.* (2004)** outlined. ITOl data visualization software were then used to further refine the data **(Letunic & Bork, 2021)**. A minimal spanning network was built in PopArt v.3.0 to further evaluate haplotype divergence.

3. Tested insecticides

The three insecticides used for the study, methomyl ($C_5H_{10}N_2O_2S$), α- cypermethrin ($C_{22}H_{19}C_{l2}NO_3$), and chlorpyrifos ($C_9H_{11}C_{l3}NO_3PS$), (Fig. 1) were purchased from KZ-Kafr El Zayat Company for Pesticides and Chemicals.

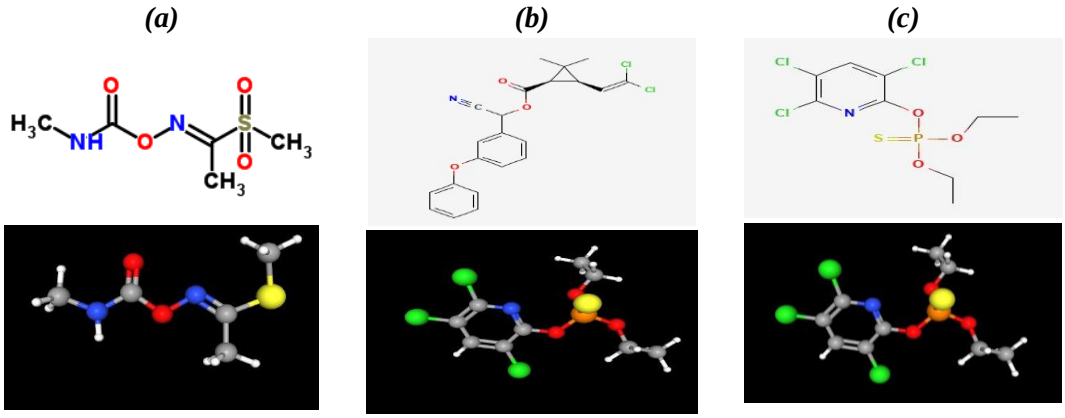

Fig. 1. Tested insecticides: *(a)* Methomyl, *(b)* α-cypermethrin, and *(c)* Chlorpyrifos

4. Toxicity on *Aedes aegypti* different immature stages

A standard procedure described by **Hassanain *et al.* (2019)** was adopted to test the sensitivity of *Ae. aegypti* immature aquatic stages to tested insecticides. Different

concentrations of each tested insecticide were prepared in 250ml of distilled water. Twenty-five *Ae. aegypti* larvae (I-IV) and pupae were separately placed into beakers containing different insecticide concentrations. Mortality of larvae and pupae was recorded at 24 and 48h post-treatment and indicated by failure to respond to mechanical stimulation. Each test was repeated thrice, and mean values were considered.

5. Determination of acetylcholinesterase (AChE) and glutathione S-transferase (GST) activities

The half-lethal concentrations (LC_{50}) were used to study the effect of tested insecticides on acetylcholinesterase (AChE) and glutathione S-transferase (GST) activities in *Ae. aegypti* different immature stages. About 10ml solutions of 0.1 M-phosphate buffer, pH 7.5 (KH_2PO_4 -NaOH), containing 1% Triton X-100, 1% Triton X-100, 1% ethanol, and 1% Triton X-100, respectively, were used to homogenize 3 batches of larvae (obtained from each tested LC_{50}). Hereaeus Labofuge 400R, Kendro Laboratory Products GmbH, Germany, was used to centrifuge the homogenates for 60 minutes at 4ºC and 15.000 x g. Ten microliter aliquots of supernatant were subjected without further purification for *in vitro* inhibition assay of AChE (U/L) **(Ellman *et al.*, 1961)**. In addition, biodiagnostic kits were used to spectrophotometrically assess GST activity (U/g tissue). following the technique of **Habig *et al.* (1974)**. Measuring the conjugation of S-2, 4-dinitrophenyl glutathione (CDNB) with reduced glutathione is the method's underlying premise. A net increase in absorbance at 340nm relative to the blank was used to track the adduct formation of CDNB.

6. Toxicity on non-target organisms

6.1. Zebrafish, Danio rerio model

The zebrafish (*Danio rerio*) specimens were captured from the aquariums at Al-Azhar University's Animal House and Zoology Department in the university's Faculty of Science campus in Cairo. The animals used in the experiments were humanely treated, according to Al-Azhar University's Animal Research Ethics Committee's regulations (Egypt). They were acclimated in 1000mL circular aquaria. 10 fish sample were distributed in each tank. Tanks were artificially aerated for 24 hours daily. The fish were given fish food pellets of the appropriate size. Experiments were performed in triplicate following **Norberg-King *et al.* (1991)**. To shed light on the effect of tested materials on our non-target model, thirty adults of healthy zebrafish were exposed to 0, 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2ppm of methomyl and α- cypermethrin. The concentrations of 0, 0.01, 0.02, 0.03, 0.04, 0.05, and 0.06 ppm chlorpyrifos were used. Mortality rates were adjusted at 24- and 48 hours post-treatment for both the treatment group and the control group. The percentage of toxicity was calculated using the formula of **Deo *et al.* (1988)**, where the toxicity (%) was calculated as LC_{50} of target vector species /LC_{50} of non- target organisms × 100.

6.2. Daphnia magna model

From the Laboratory of Invertebrate Rearing, we collected several *Daphnia magna*. Raised in 10µL water tanks and fed a yeast powder solution, both nymphs and adults were eventually released into the wild. The physical and chemical parameters were as follows: a pH range of 7.1 to 7.5; a constant temperature of 25°±1°C; a conductivity of around 160S cm^{-1}; a dissolved oxygen concentration of about 4mg/ l, and a total hardness of 3.5% to 5% $CaCO_3$ **(Pavela, 2014)**.

Organization for Economic Co-operation and Development (OECD) criteria (Test no. 202, *Daphnia* sp., Acute Immobilization Test) **(OECD, 2004)** were followed for the acute toxicity testing, with the required adjustments explained below. Each treatment group included 60 *Daphnia* overall, with 20 neonatal *Daphnia* exposed in the three test vessels. The *Dap. magna* individuals were placed in containers with 250mL of pure water and each tested materials was used with the following concentrations for 24 and 48h. *Daphnia magna* were exposed to 0, 0.003, 0.006, 0.009, 0.012, 0.015, and 0.018 ppm of methomyl; 0, 0.0003, 0.0006, 0.0009, 0.0012, 0.0015, and 0.0018 ppm of α-cypermethrin;0, 0.0006, 0.0012, 0.0018, 0.0024, 0.0030, and 0.0036 ppm of chlorpyrifos. The number of dead neonates in the three replicates was recorded after the acute toxicity experiments were completed, and the organisms were viewed using a stereomicroscope. In this experiment, death was determined by the presence or absence of a pulse when observed via a stereomicroscope **(Day *et al.*, 1993)**.

7. Statistics

The data were statistically analyzed using the technique of **Lentner *et al.* (1982)**. The half-lethal concentrations (LC_{50}) were calculated using multiple linear regression **(Finney, 1971)**. SPSS V.22 was used for data encoding and entry. Parametric test assumptions were examined using the chi-square and F- test, and the Shapiro-Wilk and Kolmogorov-Smirnov tests for normality were used for continuous data. Arcsine square root was used to ensure normality in the probability and percentile statistics. Mean and standard deviation were used to display data. Analysis of variance (ANOVA) was performed on the mortality and enzyme activity observed across experimental groups, with at least three replicates per group. Post- hoc analysis was performed using Tukey pairwise comparison, and a P-value of 0.05 was deemed significant. With the release of Minitab V 14, Chi 2 was included. When feasible, data were displayed in R studio V 2022.02.4.

RESULTS

1. DNA barcoding of *Aedes aegypti*

The evolutionary history was inferred using the neighbor-joining method **(Saitou & Nei, 1987)**. The optimal tree, illustrating the relationships among taxa, is presented in Fig. (2*i*). Each node in the tree represents the proportion of bootstrap samples in which

the linked taxa grouped (1000 replicates) **(Felsenstein, 1985)**. Tamura's 3-parameter method was utilized to calculate evolutionary distances, and those numbers were then used to create a scaled tree **(Tamura, 1992)**, defined by the average number of base changes per position. All unclear locations in the 12 nucleotide sequences included in the investigation were eliminated by paired deletion. The whole dataset included 428 unique items.

Furthermore, a minimum haplotype-spanning network was constructed to visualize the haplotype distribution and geographical location of the evaluated nucleotide sequences. This network provides insight into the diversity and changes in haplotypes across different geographical regions. Nucleotide diversity was calculated, resulting in a *P*-value of 5.71057e+06. The dataset contained 53 segregating sites, with only one being parsimony informative. Tajima's D statistic was computed to be 7.9283e+07, with a *P*-value (P) of 0 for the condition D >= 7.9283e+07 (Fig. 2*ii*). All evolutionary analyses were performed using MEGA11 **(Tamura *et al.*, 2021)**.

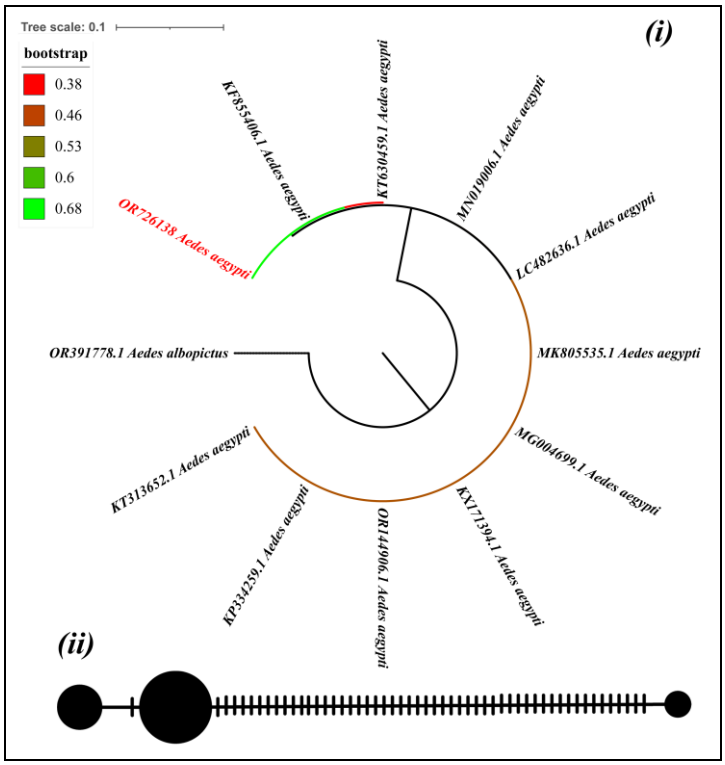

Fig. 2. ***(i)***Neighbor-Joining phylogenetic evolutionary tree; ***(ii)*** Minimum haplotype spanning network.

2. Sensitivity of *Aedes aegypti* different immature stages to tested insecticides

2.1. Toxicity of tested insecticides on Aedes aegypti

The toxicity of tested insecticides against *Ae. aegypti* immature aquatic stages after 24 and 48h was recorded in Figs. (3- 5). The mortality rates increased with increasing insecticide concentration and exposure time ($P< 0.05$). Among the tested insecticides,

chlorpyrifos recorded the highest mortality (98.66%) in *Ae. aegypti* first larval instar at 0.005ppm at 24h post-treatment, while 100% complete larval mortality of first instar took place at 0.007, 0.0009, and 0.005ppm of methomyl, α- cypermethrin, and chlorpyrifos after 48h of exposure, respectively.

Additionally, methomyl, α- cypermethrin, and chlorpyrifos caused 89.33, 97.33, and 94.66% mortality in larvae III at 0.08, 0.03, and 0.04 ppm after 24h of exposure. In comparison, after 48h of exposure, 100.0% mortality in larvae III was recorded at the same concentrations. In addition, complete pupal mortality (100.0%) was recorded by 0.12 and 0.16 ppm of α-cypermethrin, and chlorpyrifos after 48h post-treatment; meanwhile, 0.2ppm of methomyl recorded 98.66% pupal mortality at 48h post-treatment, respectively, compared to no mortality in the untreated congers at the same time of exposure (Figs. 3- 5).

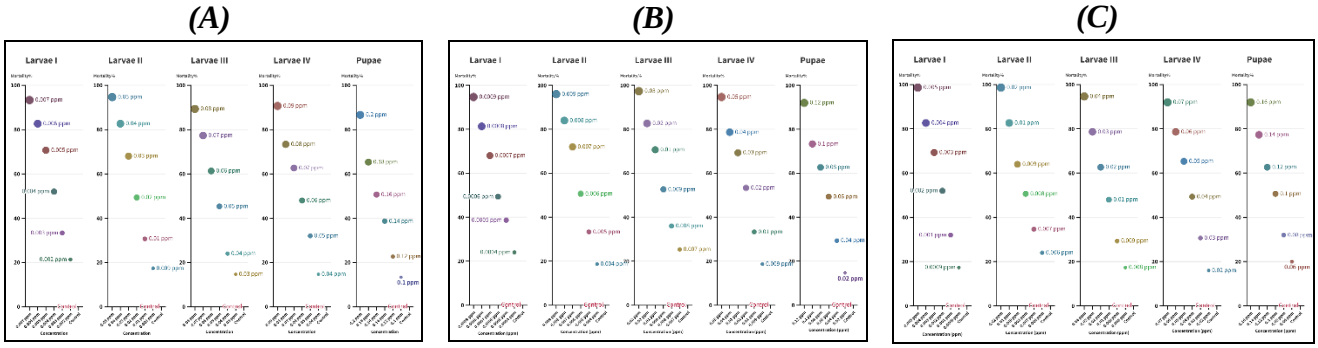

Fig. 3. Bubble chart representing the effect of tested insecticides on *Aedes aegypti* in different immature aquatic stages after 24h of exposure to *(A):* methomyl, *(B):* α- cypermethrin, and *(C):* chlorpyrifos.

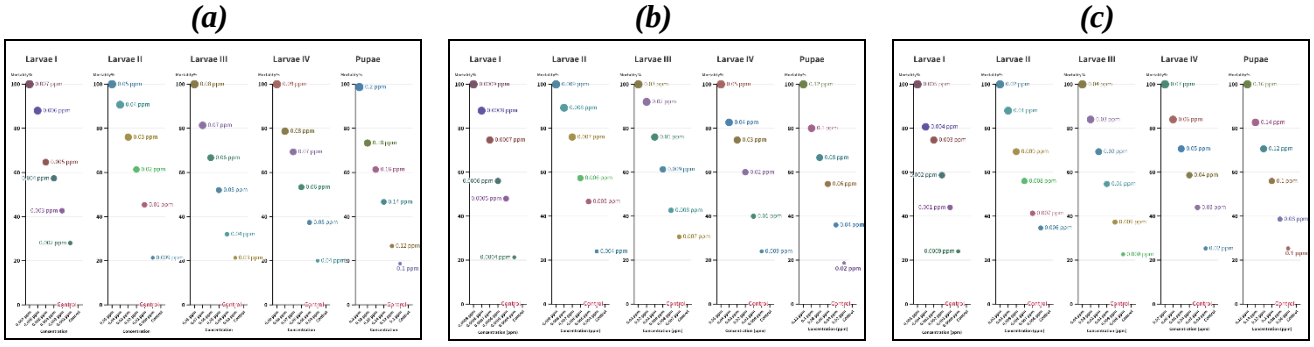

Fig. 4. Bubble chart represents the effect of tested insecticides on *Aedes aegypti* different immature aquatic stages after 48 h of exposure to *(a):* methomyl, *(b)* α- cypermethrin, and *(c)* chlorpyrifos.

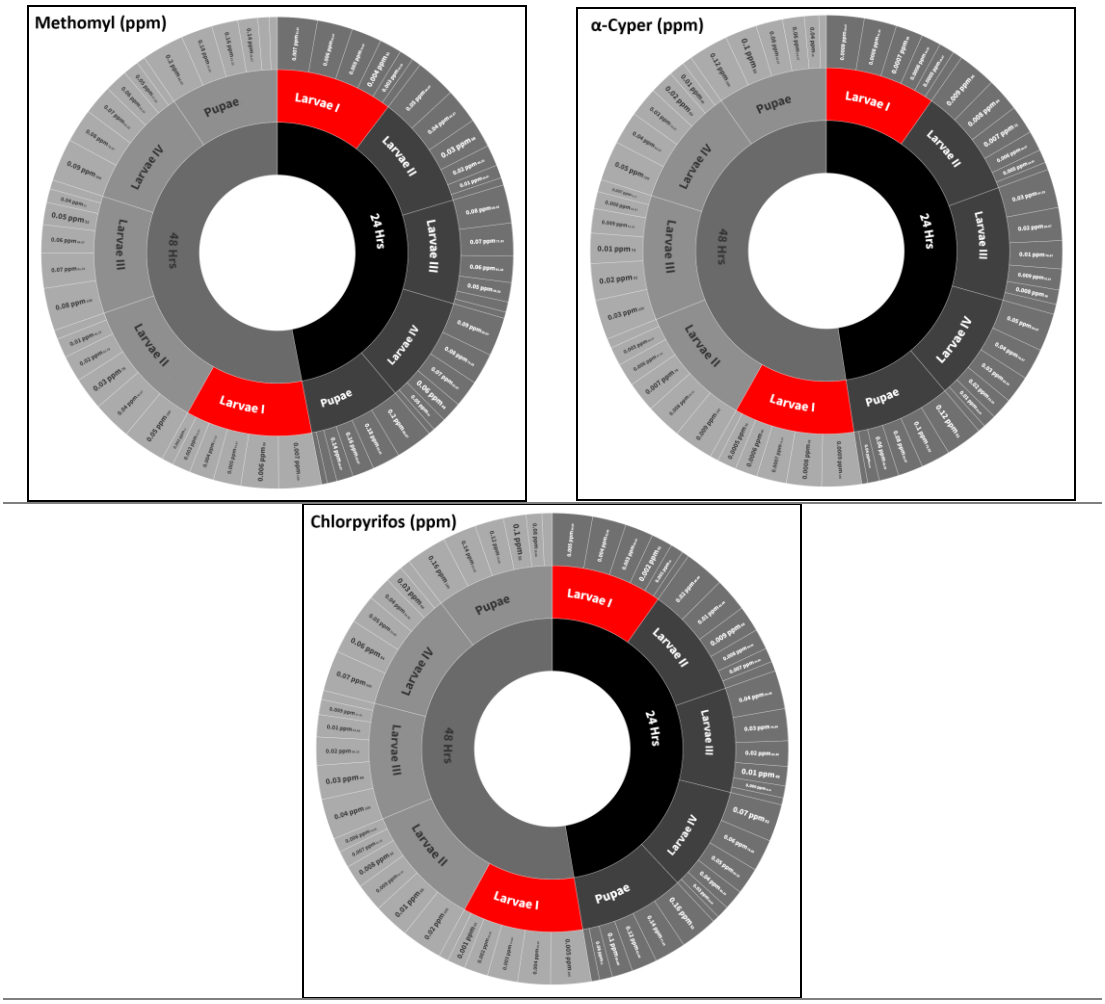

Fig. 5. Sunburst chart representing toxicity of tested insecticides against *Aedes aegypti* in different immature aquatic stages

On the other hand, Table (1) & Fig. (6) show the LC_{50} values (ppm) of tested insecticides against *Ae. aegypti* different immature aquatic stages after 24 and 48h of exposure. The χ^2 values were calculated to determine if there were any significant differences in the LC_{50} values between the different target stages. The LC_{50} values for methomyl ranged from 0.0039 to 0.155ppm after 24h of exposure. The lowest LC_{50} value (0.0039ppm) was observed against larvae I after 24h. Whereas, the highest LC_{50} value (0.155 ppm) was observed against pupae after 48h of exposure. For α-cypermethrin, LC_{50} values against different stages ranged from 0.00058 to 0.065ppm. The LC_{50} values recorded fluctuated between 0.0098 and 0.0073ppm against *Ae. aegypti* 3rd larval instar at 24 and 48h post-treatment, respectively. Finally, LC_{50} values of chlorpyrifos against different stages ranged from 0.0022 to 0.102 ppm after 24h of treatment. For all experimental insecticidal groups, there were no significant differences in the LC_{50} values between the different target stages.

Based on calculated LC_{50} values, all tested insecticides were effective against *Ae. aegypti* immature aquatic stages; however, α-cypermethrin was the most effective, followed by chlorpyrifos and methomyl (Table 1 & Fig. 6).

Table 1. The LC_{50} values (ppm) of tested insecticides against *Aedes aegypti* in different immature aquatic stages

Insecticide	Target	LC_{50}(LCL-UCL) (ppm)		χ^2	
		24 Hours	48 Hours	24 Hours	48 Hours
Methomyl	Larvae I	0.0039 (0.0037-0.0042)	0.0036 (0.0034-0.0038)	1.143^{NS}	1.320^{NS}
	Larvae II	0.023 (0.020-0.026)	0.017 (0.016-0.019)	1.107^{NS}	0.610^{NS}
	Larvae III	0.054 (0.052-0.055)	0.050 (0.048-0.051)	0.647^{NS}	0.893^{NS}
	Larvae IV	0.063 (0.061-0.064)	0.059 (0.057-0.060)	1.677^{NS}	0.860^{NS}
	Pupae	0.155 (0.151-0.159)	0.145 (0.141-0.149)	0.967^{NS}	0.860^{NS}
α- Cypermethrin	Larvae I	0.00058 (0.00055- 0.00062)	0.00055 (0.00054-0.00057)	1.037^{NS}	0.064^{NS}
	Larvae II	0.0059 (0.0057-0.0061)	0.0055 (0.0053-0.0057)	0.893^{NS}	1.213^{NS}
	Larvae III	0.0098 (0.0091-0.0106)	0.0073 (0.0062-0.0084)	0.467^{NS}	1.357^{NS}
	Larvae IV	0.022 (0.020-0.023)	0.018 (0.015-0.021)	0.610^{NS}	1.107^{NS}
	Pupae	0.065 (0.061-0.069)	0.058 (0.055-0.062)	1.357^{NS}	0.503^{NS}
Chlorpyrifos	Larvae I	0.0022 (0.0017-0.0027)	0.0018 (0.0016-0.0020)	0.753^{NS}	1.177^{NS}
	Larvae II	0.0081 (0.0079-0.0082)	0.0065 (0.0056-0.0074)	1.357^{NS}	0.50^{NS}
	Larvae III	0.017 (0.016-0.019)	0.014 (0.012-0.016)	0.540^{NS}	0.930^{NS}
	Larvae IV	0.041 (0.038-0.045)	0.036 (0.034-0.037)	0.750^{NS}	0.50^{NS}
	Pupae	0.102 (0.100-0.105)	0.094 (0.088-0.099)	1.143^{NS}	1.213^{NS}

LC_{50} Half Lethal Concentrations; ppm part per million; LCL Lower Confidence Limit; UCL Upper Confidence Limit; χ^2Chi-square value; NS non-significant. The effect of tested compounds was non-significant at the level of LC_{50} irrespective of time of exposure.

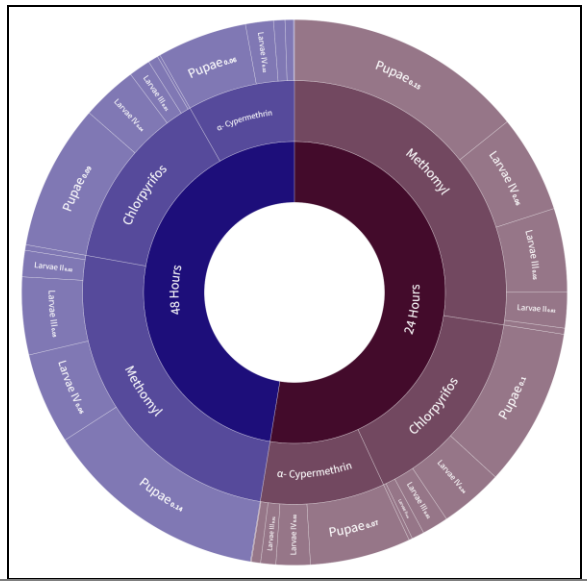

Fig. 6. Sun-brust chart representing LC_{50} values (ppm) of tested insecticides against *Ae. aegypti* different immature aquatic stages.

2.2. Effect of tested insecticides on Aedes aegypti acetylcholinesterase (AChE) and glutathione S-transferase (GST) activities

The effect of tested insecticides on acetylcholinesterase (AChE) and glutathione-S-transferase (GST) activities of *Ae. aegypti* different immature aquatic stages is recorded in Table (2) and illustrated in Fig. (7). All tested insecticides inhibited the AChE activity significantly. Methomyl caused the least inhibition of AChE activity (5.53 and 5.90U/ L) in larvae I after 24 and 48h of exposure, respectively. The lowest AChE value (5.55 U/l) was recorded in larvae I after 48h of exposure to α-cypermethrin.

On the other hand, the GST activity was significantly increased ($P< 0.05$) by all tested insecticides. The highest GST values (1.73 and 1.59 U/g tissue) were recorded for pupae after 24 and 48h of exposure to α-cypermethrin (Table 2 & Fig. 7).

Table 2. Effect of tested insecticides on acetylcholinesterase (AChE) and glutathione-S-transferase (GST) activity of *Aedes aegypti* different immature aquatic stages

Insecticidal	Larval stage	AChE (U/l)		GST (U/g tissue.)	
		24 Hours	48 Hours	24 Hours	48 Hours
Methomyl	**Larvae I**	5.53±0.03	5.9±0.15	0.69±0.02	0.59±0.04
	Larvae II	5.91±0.06	6.28±0.07	0.8±0.04	0.61±0.02
	Larvae III	6.23±0.09	6.47±0.11	1.08±0.05	1.07±0.05
	Larvae IV	6.8±0.1	7.15±0.18	1.17±0.04	1.09±0.08
	Pupae	7.45±0.05	7.79±0.08	1.32±0.03	1.18±0.06
α- Cypermethrin	**Larvae I**	5.15±0.04	5.55±0.1	0.98±0.05	0.85±0.06
	Larvae II	5.26±0.06	5.71±0.09	1.09±0.05	0.96±0.03
	Larvae III	5.02±0.11	5.26±0.12	1.5±0.03	1.27±0.04
	Larvae IV	5.74±0.1	6.11±0.08	1.66±0.04	1.5±0.05
	Pupae	6.67±0.17	6.95±0.09	1.73±0.04	1.59±0.01
Chlorpyrifos	**Larvae I**	5.35±0.07	5.45±0.06	0.82±0.03	0.67±0.04
	Larvae II	5.66±0.07	5.87±0.04	0.96±0.03	0.86±0.07
	Larvae III	5.67±0.1	5.86±0.12	1.26±0.03	1.11±0.07
	Larvae IV	6.24±0.05	6.55±0.05	1.37±0.05	1.22±0.03
	Pupae	6.83±0.19	7.36±0.08	1.5±0.07	1.36±0.07
Control	**Larvae I**	5.74±0.04	6.13±0.14	0.56±0.04	0.48±0.02
	Larvae II	6.2±0.14	6.56±0.11	0.68±0.04	0.61±0.05
	Larvae III	6.55±0.07	6.97±0.19	0.93±0.02	0.7±0.02
	Larvae IV	7.08±0.07	7.43±0.13	1.03±0.04	0.87±0.05
	Pupae	7.7±0.07	7.96±0.04	1.17±0.06	1.02±0.02

3. Effect of tested insecticides on non-target organisms

The lethal concentration values (LC_{50}) of three insecticides (methomyl, α-cypermethrin, and chlorpyrifos) on two non-target organisms (Zebrafish and *Daphnia*) at two different exposure durations (24and 48 h) are displayed in Table (3).

Methomyl recorded LC_{50} of 0.862, 0.011 and 0.575, 0.009ppm against zebrafish and *Daphnia* after 24 and 48h, respectively. Moreover, α- cypermethrin recorded LC_{50} of 0.941, 0.0011 and 0.673, 0.0007ppm against zebrafish and *Daphnia* after 24 and 48h, respectively. In addition, LC_{50} values of chlorpyrifos against zebrafish and *Daphnia* were 0.041, 0.0031 and 0.026, 0.0013ppm after 24 and 48h, respectively, as shown in Table (3).

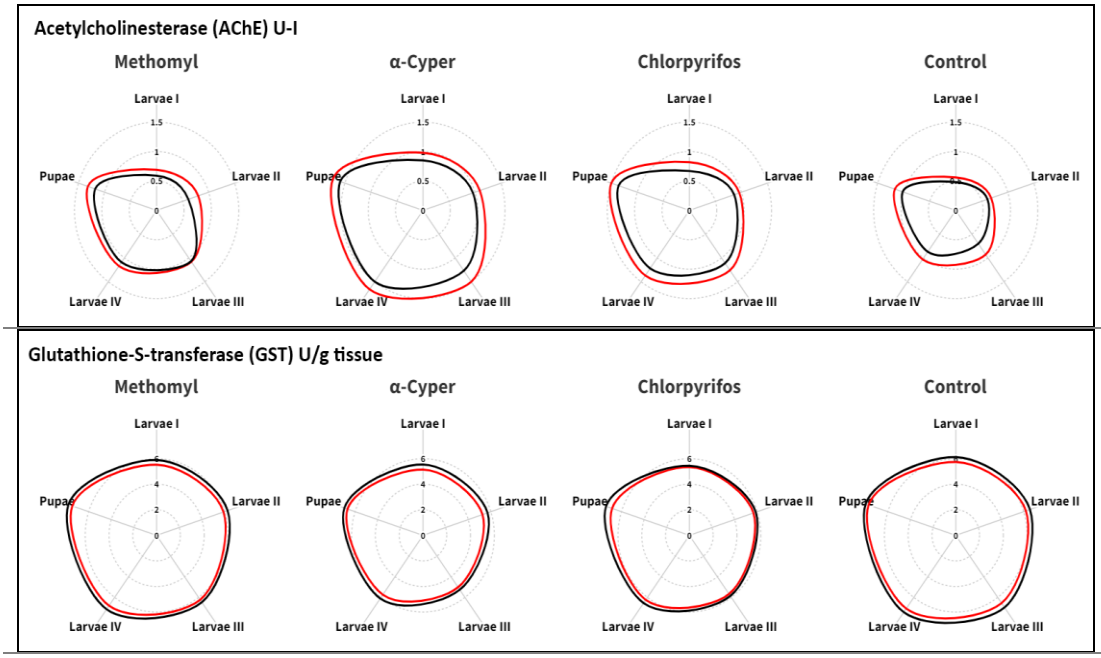

Fig. 7. Radar bar chart displaying the effect of tested insecticides on acetylcholinesterase (AChE) and glutathione- S- transferase (GST) activity of *Aedes aegypti* in different immature aquatic stages

Table 3. The LC_{50} values (ppm) of tested insecticides against two non-target organisms, zebrafish, *Danio rerio,* and *Daphnia magna*

Non-target organisms	Tested insecticides	LC_{50}(LCL-UCL) (ppm)		χ^2	
		24 Hours	48 Hours	24 Hours	48 Hours
Zebrafish, *Danio rerio*	Methomyl	0.862 (0.763-0.985)	0.575 (0.490-0.658)	3.62^{NS}	7.79^{NS}
	α- cypermethrin	0.941 (0.837- 1.081)	0.673 (0.582-0.767)	5.31^{NS}	8.12^{NS}
	Chlorpyrifos	0.041 (0.036-0.047)	0.026 (0.0022-0.030)	5.00^{NS}	9.23^{NS}
Daphnia magna	Methomyl	0.011 (0.010-0.012)	0.009 (0.008- 0.010)	8.25^{NS}	6.88^{NS}
	α- cypermethrin	0.0011 (0.0010-0.0012)	0.0007 (0.0006-0.0008)	4.99^{NS}	10.59^{NS}
	Chlorpyrifos	0.0031 (0.0027-0.0038)	0.0013 (0.0010-0.0015)	22.92^{NS}	15.11^{NS}

DISCUSSION

The findings of the present study revealed that methomyl, α- cypermethrin, and chlorpyrifos were effective against *Aedes aegypti* immature aquatic stages. The effectiveness of the tested insecticides varied according to the concentration of the insecticides and the immature stage tested. Overall, a decrease was recorded in LC_{50} values for samples exposed to insecticides for 48 hours, compared to 24h, indicating increased toxicity with prolonged exposure. The $\chi 2$ values vary, reflecting the goodness of fit of the data. Generally, α- cypermethrin was the most effective insecticide, followed by chlorpyrifos and methomyl. These results confirm the previous findings of **Ali *et al.* (1995)**, who studied the larvicidal effect of five organophosphates and three pyrethroids

against *Ae. albopictus*, and they found that cypermethrin (LC_{50} = 0.0026ppm) was more effective than chlorpyrifos (LC_{50} = 0.0033ppm) in addition to **Zidan *et al.* (2012)** who examined the field evaluation of different pesticides against cotton bollworms and sucking insects. They concluded that the tested synthetic pyrethroids (α- cypermethrin) were more effective than organophosphorus and carbamate compounds (chlorpyrifos and methomyl).

In addition, all tested insecticides have the potential to disrupt the nervous and detoxification systems of *Ae. aegypti* immature aquatic stages. This could lead to reduced survival and developmental rates, as well as increased susceptibility to other stressors. Insecticidal efficacy was evaluated using assays for enzymes, including acetylcholinesterase (AChE) and glutathione S- transferase (GST), and α-cypermethrin was shown to be the most effective. Smaller size, lower body temperature, and increased expression of sensitive sodium channels may all contribute to α-cypermethrin's effectiveness **(Costa, 2015)**. On the other hand, the spatial contact is also involved in the insecticidal effects of α-cypermethrin, which primarily result in paralysis by blocking repolarization of the voltage-gated Na^+ channel in the axonal membrane **(Kakati, 2023)**. There is an increase in acetylcholine at the synapse between neurons when chlorpyrifos is present, and the drug also impacts other neurotransmitters, enzymes, and cell signaling pathways at concentrations below those needed to block AChE **(Sparks & Nauen, 2015)**. However, the magnitude and underlying mechanisms of these impacts remain unclear. Chlorpyrifos has a similar impact on *Ae. aegypti* aquatic juvenile stages as it does on other contact- and ingestion-killed agricultural pests such as *Cydiapomonella, Philosamiaricini,* and *Spodoptera littoralis* **(Dewer *et al.*, 2016; Kalita *et al.*, 2016; Parra Morales *et al.*, 2017).** In addition, methomyl (like several carbamates) inhibited AChE function, which results in nerve and/or tissue insufficiency, perhaps leading to death. Methomyl is thought to be highly metabolically destroyed via mixed-function oxidase due to its considerable toxicity to insects at different life stages, such as larva and adult **(Van Scoy *et al.*, 2013).** Despite its own acute toxicity, methomyl is occasionally used in unintended ways, probably as a result of the substance's poor stability, low bio accumulative activity, and more rapid dispersal from virtually all environmental matrices **(Mortensen & Serex, 2014)**.

On the other hand, data presented the impact of tested insecticides on non-target organisms, specifically zebrafish, *Danio rerio* and *Daphnia magna.* The marked difference in LC_{50} values between *Ae. aegypti* and these aquatic organisms underscore the differential susceptibility of species to the same chemicals. For instance, while methomyl exhibited relatively low LC_{50} values against *Ae. aegypti* larvae, its impact on zebrafish was considerably less pronounced, with LC_{50} values of 0.862ppm at 24h, respectively. Such variations can be attributed to differences in physiology, uptake, and detoxification mechanisms among different species. However, though the data provides valuable insights, it also raises concerns. The pronounced effects of these insecticides on non-target organisms like zebrafish and *Dap. magna* emphasize the potential ecological implications of insecticide use. Daphnia, for instance, are keystone species in freshwater

ecosystems, and any perturbation in their populations can have cascading effects on the food web. It is worth noting that, chlorpyrifos has been shown to cause developmental abnormalities in zebrafish embryos, including malformations and altered heart rates **(Chang *et al.*, 2012)**. Similarly, α-cypermethrin, a synthetic pyrethroid insecticide, has been linked to neurotoxic effects in zebrafish, leading to behavioral changes and altered neurotransmitter levels **(Chang *et al.*, 2012)**. These findings highlight the potential risks these chemicals pose to aquatic ecosystems, even at low concentrations.

CONCLUSION

In conclusion, the efficacy of methomyl, α-cypermethrin, and chlorpyrifos in controlling the Dengue vector, *Aedes aegypti*, is undeniable; however, their environmental footprint cannot be overlooked. Striking a balance between effective *Ae. aegypti* control and environmental conservation necessitates the development of targeted insecticides with minimal off-target effects.

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