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Impact of NaCl on the morphology and biochemical aspects of Asian tiger mosquito, *Aedes albopictus* Skuse (Diptera: Culicidae)

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Abstract

The main objective of this investigation is to analyse NaCl-induced morphological and biochemical changes in *Aedes albopictus*. *Ae. albopictus* larvae were treated with distinct concentrations of NaCl to establish morphological as well as biochemical responses. Larval mortality was recorded after 24-hour exposure period to determine the LC_{50} value by using Probit Analysis, IBM's SPSS 29 Programme and Student's t-test. The NaCl solution caused a notable mortality after 24h experimental period. The LC_{50} value of NaCl in *Ae. albopictus* was found to be 1.7% and hence the sub-lethal dose, 1.3% NaCl, was used for further analyses. Morphological studies of eggs and fourth instar larvae of *Ae. albopictus* displayed severe morphological alterations in the treated samples as contrasted with those of the untreated groups. In the fourth instar larvae of *Ae. albopictus*,the total protein, total free amino acid and major antioxidant enzymes such as GST and GPx were found to be increased, and the activities of SOD, CAT and AChE were substantially declined as compared with those of the control group. NaCl adversely affects the growth and development of *Ae. albopictus* and leads to mortality due to metabolic as well as biochemical imbalances.

Keywords: *Aedes albopictus,* NaCl, LC_{50}, Morphology, Biochemical assay.

Introduction

Mosquitoes are highly decisive vector species in terms of public health, and they have a great economic and social impact, especially in tropical and subtropical countries [1]. *Aedes albopictus* is the most important vector for the transmission of several vector-borne diseases [2]. Worldwide, synthetic pesticides are the primary method used to control mosquitoes.

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However, the indiscriminate use of chemicals imposes several negative impacts on humans as well as on the environment [3,4,5]. Salts are a safer and more eco-friendly alternative to chemical pesticides [5]. There is an urgent need to develop new strategies to eliminate mosquito breeding habitats [6].

Habitat transformation from aquatic to terrestrial life encounters several environmental stressors that adversely affect their successful adaptations, such as physical, chemical and biological origins [7,8]. The major natural abiotic stress in aquatic habitats is water salinity and ionic composition, which is one of the main obstacles to the development of the basic ecological niche of aquatic organisms [7]. Salinity is typically linked with salty or alkaline water or soil. An accumulation of salt in water or soil can retard the development and water absorption of living organisms residing in it, resulting in osmotic or water-deficit stress in *vivo* [9]. Saline stress from NaCl induces the high accumulation of reactive oxygen species (ROS), resulting in oxidative damage to the larval body [10,11], which ultimately leads to the organism's death.

The purpose of this research is to assess the larvicidal potency of NaCl on early fourth instar larvae of *Aedes albopictus* and to investigate salt-induced changes in development and metabolism, with the goal of developing alternative, comparatively safer strategies for mosquito larval management.

2. Materials and Methods

1.1 Collection and rearing of *Ae. albopictus*

Eggs, larvae collected by placing a tray with 2L of water containing dry leaves in the garden areas where *Ae. albopictus* lay their eggs. The collected eggs and larvae were then transferred to water-filled containers and given crushed dog biscuits as food [12].

1.2 Experimental media

Standard taxonomic keys were used for the identification of the collected eggs and larvae [13,14]. Different concentrations of NaCl (Sodium Chloride) were tested, and the mortality rate was recorded. The Lethal Concentration (LC_{50}) value of NaCl was determined through the probit analysis with IBM's SPSS 29 program software from IBM, USA [15]. Hence, a sub-lethal dose of NaCl was selected as the exposure dosage. Twenty eggs and larvae were placed in separate cups with 100 mL of a sub-lethal dose of NaCl solution for further studies. As control groups, eggs and larvae were maintained in dechlorinated tap water. Six replicates have been prepared to evaluate the effect of a NaCl solution on egg and larval morphology, as well as developmental and biochemical studies of *Ae. albopictus.*

1.3. Morphological study of eggs and larvae

After 24-hour treatment, the morphology of eggs and fourth-instar larvae of *Ae. albopictus* were analysed under a Stereomicroscope (AxioCam208color, ZEISS), changes were recorded, and photographs were taken.

1.4. Biochemical analyses

After 24 hours of exposure to NaCl, the larvae were blotted using blotting paper and weighed. Larvae weighing 100 mg were chosen from both the experimental and untreated groups. A homogenizer filled with an appropriate amount of buffer solution was used to homogenize the weighed samples. In a chilled centrifuge, homogenization was done for 10 minutes at 40°C and 10,000 rpm. Following standard laboratory procedures, the recovered supernatant was used for further biochemical and enzyme studies.

1.4.1. Estimation of whole-body protein

The amount of protein was estimated [16]. 100mg of the test sample was homogenized in a mortar and pestle with 0.1M Phosphate buffer and made up to 1.5ml. Centrifuged at 5000rpm for 15minutes. From this extract, 200 µl was taken and made up to 1.5 ml for estimation. Pipetted aliquots of standard solutions of protein 20,40,60,80, and 100 µl and transferred to test tubes. Diluted to 1.5 ml with phosphate buffer. Added 1.5ml Bradford reagent to all the tubes. After five minutes at room temperature and thorough mixing, the absorbance at 595 nm was measured.

1.4.2. Total free amino acid estimation

After homogenizing 100 mg of the complete larval body in 3000 µl of perchloric acid, the sample was centrifuged for 10 minutes at 3000 rpm. After neutralizing one milliliter of the subsequent supernatant with one milliliter of 4N KOH, and was diluted with distilled water to a final volume of 2.5 milliliters. After extracting 0.1 ml from this solution, 0.9 ml of Ninhydrin solution was applied. After 20 minutes of incubation at 37°C in a water bath, the mixture was allowed to cool to room temperature before 2 mL of diluent solution was added. Absorbance was measured at 570nm, and a standard graph with leucine was generated alongside the experiments. The total free amino acid present in the test samples was explored from the standard calibration curve [17].

- Enzyme Assays:

2.5. Antioxidant Enzyme Assays:

2.5.1 Superoxide Dismutase (SOD)

The reaction mixture comprised 1.2 millilitres of buffer solution containing Sodium pyrophosphate (0.052 M, pH 8.3), 0.1 millilitres of Phenazine methosulphate (186 mM), 300 µl of Nitro blue tetrazolium (300 mM), 200 µl of Nicotinamide adenine dinucleotide (NADH, 780 mM), appropriately diluted enzyme preparation, and 300 ml of water. 1 ml of Acetic acid (glacial) was added to stop the reaction after 90 seconds of incubation at 30°C. The concoction used for the reaction was then strenuously mixed and agitated with 4 ml n-butanol, permitted to stand for within ten minutes, and subsequently centrifuged at 2500 rpm for about 10 minutes. The layer containing butanol is removed by pipetting. Using a UV-Visible Spectrophotometer, the chromogen concentration in the butanol layer was determined at 560 nm and compared with a blank containing butanol. The amount of SOD needed to reduce the optical density of chromogen production at 560 nm by about 50% in a minute under the given test conditions was regarded as one unit of enzyme expression. The total SOD action was reported as IU/mg of protein [18].

2.5.2. Catalase (CAT)

The product of the reaction is composed of 3ml Phosphate buffer (0.01M, pH 7.00), 0.2ml Hydrogen peroxide (2mM) and 1000 µl of substantially diluted extract obtained by mixing the larval whole tissue in 10 mM buffer. The blank sample did not contain any extract. The precise activity was given in terms of mg of protein/minute/n moles of H_2O_2 decomposition [19].

2.5.3. Glutathione Peroxidase (GPx)

The 0.1g sample was blended in 1ml Sucrose and centrifuged at 10,000rpm for 10 minutes. The resulting unsinkable samples were utilized for further procedures. With the activity being quantified by 2ml Phosphate buffer (0.01M, pH 7.4) containing 300µl Sodium azide (1mM), 300µl EDTA, 100µl of reduced glutathione (1mM), 100µl H_2O_2, 100µl NADPH (0.1M) and 200µl tissue extract. The solution was rapidly mixed. Except for the tissue extract, Blank included all the reagents. For one minute, the change in absorbance at 340 nm was monitored at 15-second intervals. Enzyme activity was expressed as IU/mg protein [20].

2.5.4 Glutathione-S-transferase(GST)

Combination of reactions contained 100µl sample, 1ml Phosphate buffer, 100µl CDNB (1-chloro, -2, 4-dinitrobenzene, 30mM) and 0.1ml reduced glutathione (30mM). At 340 nm, the enzyme's increase in optical density

was contrasted with that of the blank. For sample tissue, enzyme activity was reported as n moles of CDNB-GSH conjugate formed/minute/mg of protein [21].

2.6. Non-Antioxidant Enzyme Assay:

2.6.1. Acetylcholinesterase (AChE)

3600 µl assay cocktail included 2400µl of 100Mm Phosphate buffer (pH 7.4), 100µl supernatant, 100µl of 0.075 M Acetylthiocholine-iodide and 0.01M 5, 5'- dithio-bis (2-nitrobenzoic acid). After 15 minutes of incubation at around 27°C, the resulting combination was incubated with 0.001M eserine. The optical density was measured at 412nm. The µmole/min/mg of protein was used to express the quantification of AChE activity in insects [22].

2.7. Statistical Analyses

Statistical evaluations were conducted by employing IBM SPSS version 29.0 software. Mean ± Standard Error was employed for presenting the results. Average of six observational replicates. For comparison between the groups, the Student's t-test was performed.

3. RESULTS

3.1. Determining the LC_{50}

By conducting a probit analysis, the 50% lethal concentration of NaCl for *Ae. albopictus* was established at 1.7%. Hence, a sub-lethal dose of NaCl (1.3%) was selected as the dosage for exposure. Eggs and larvae (20 numbers each) were exposed to a sub-lethal concentration of NaCl solution for 24h. Although there was no mortality at lower NaCl concentrations, all experimental larvae were found to be in a moribund state. LC_{50} results are shown in Figure. 1.1.

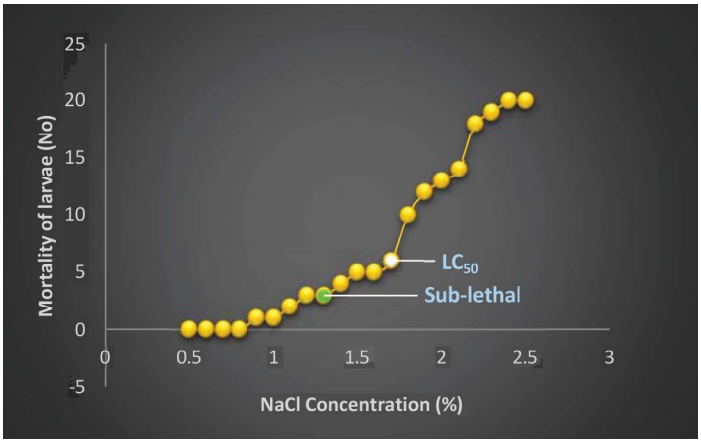

Figure. 1.1: LC_{50} - 24h determination of 4th instar larvae of *Aedes albopictus.*

3.1. Analysis of egg morphology after 24h exposure

The stereomicroscopic observations of the morphological features of the control eggs of *Ae. albopictus* have normal morphological characters, such as shiny-jet black cigar-shaped eggs with narrow dorso-ventral distortion and presence of outer intact chorionic cells (Plate.1 A). However, 1.3% NaCl-treated eggs appeared as deformed and boat-shaped with a lack of outer chorionic cells and destruction of egg shell membranes (Plate.1 B, C and D). The length and width of all treated eggs increased as compared to the control, and they shrank inward and were found to be broken (Figure 2.1 and Figure 2.2).

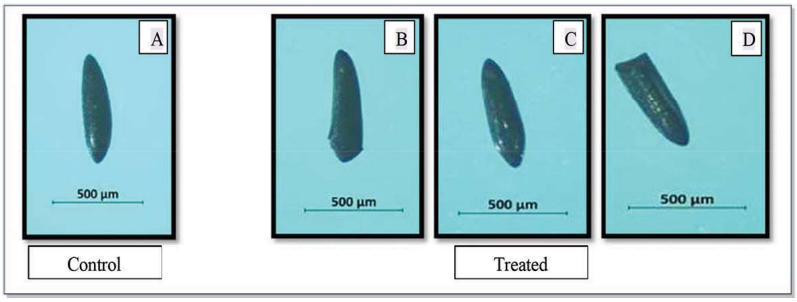

Plate.1: Effect of sub-lethal concentration of NaCl on *Aedes albopictus* eggs. A-Control egg; B, C, D- Treated eggs

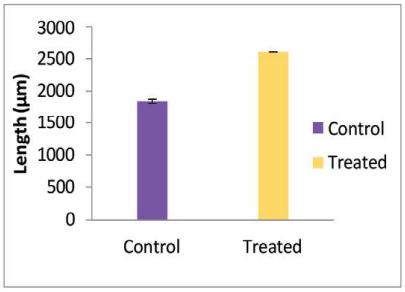

Figure.2.1: Effect of NaCl on egg length

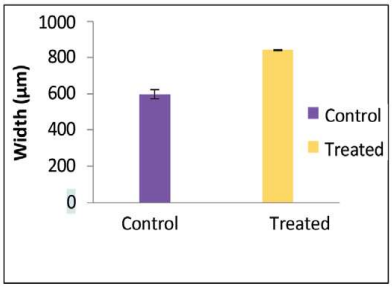

Figure.2.2: Effect of NaCl on egg width

3.3. Evaluation of larval morphology

Control larvae of *Ae. albopictus* have normal morphological body parts such as head, thorax, abdomen, respiratory siphon and no apparent abnormalities were noted in their body segments, cuticle, respiratory tract, midgut and siphon (Plate.2 A). Change in body colour was observed from the beginning of the head and extending to the siphon, loss of body pigmentation and blackening of the midgut, lack of clarity was mainly observed in the segmentation of thorax and abdomen compared to the control, shrunken body with an intensely damaged cuticle, damaged and disordered midgut and elongated siphon (Plate.2 B). However, the larvae treated with 1.3% NaCl showed Abnormalities such as the whole-body length and length of head, thorax, abdomen and siphon were increased with respect to the control group (Figure 3.1).

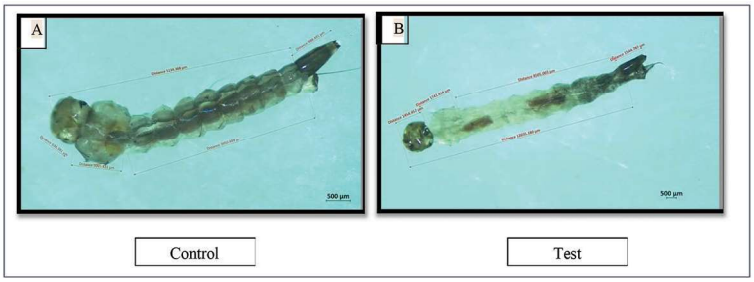

Plate.2: Effect of NaCl on fourth instar larvae of *Aedes albopictus*. Control (A) and treated (B).

Figure 3.1: Effect of NaCl on larval morphology.

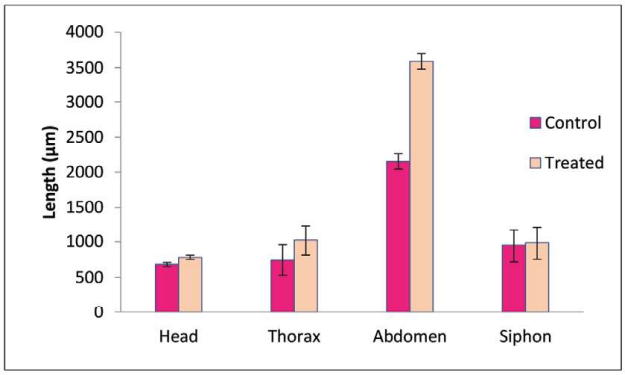

Figure 3.1: Effect of NaCl on larval morphology.

3.4. Estimation of the whole-body protein and amino acid

Ae. albopictus larvae fed with NaCl after 24 hours showed a significant increase [P<0.001] in whole-body protein compared to the control (Figure 4.1). Furthermore, compared to the control group, the treated larvae had considerably increased levels of total free amino acids [P<0.001] (Figure 4.1).

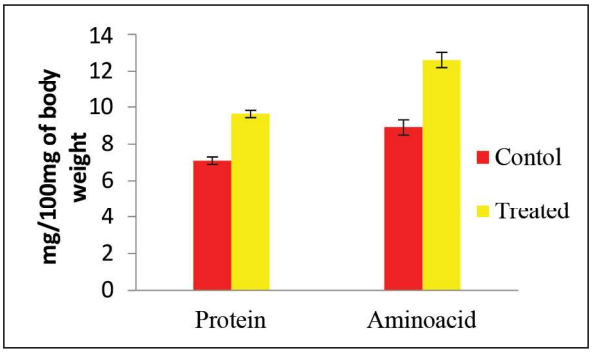

Figure 4.1: The effect of NaCl on protein and amino acids in *Aedes albopictus*.

3.5. Enzyme Assays:

Action of SOD in *Ae. albopictus* larvae exposed to NaCl for 24h showed a significant decrease [P<0.001] when compared to the control (Figure 5.1).

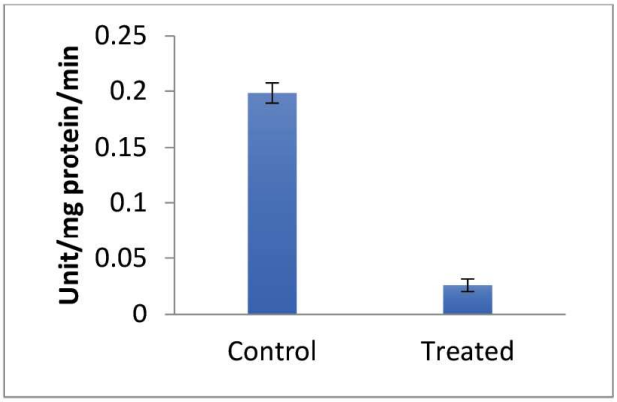

Figure 5.1: The impact of NaCl on Superoxide Dismutase activity.

As contrasted with the untreated sample group, the activity of CAT in the treated larvae of *Ae. albopictus* decreased significantly [P<0.001] (Figure 6.1).

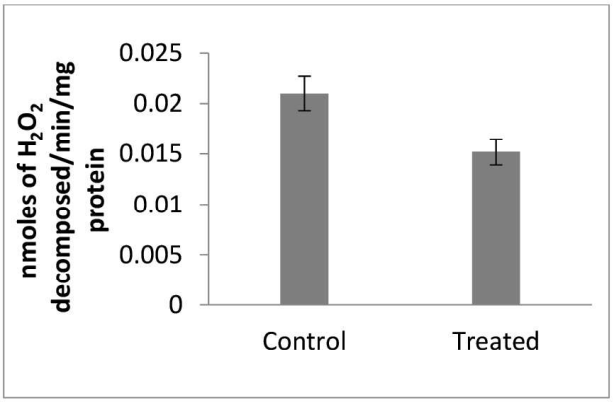

Figure 6.1: The impact of NaCl on catalase activity.

Additionally, compared to the control, the results showed a significant increase in GPx activity [P<0.001] in the larvae of *Ae. albopictus* that were exposed to 1.3% NaCl for 24 hours (Figure 7.1).

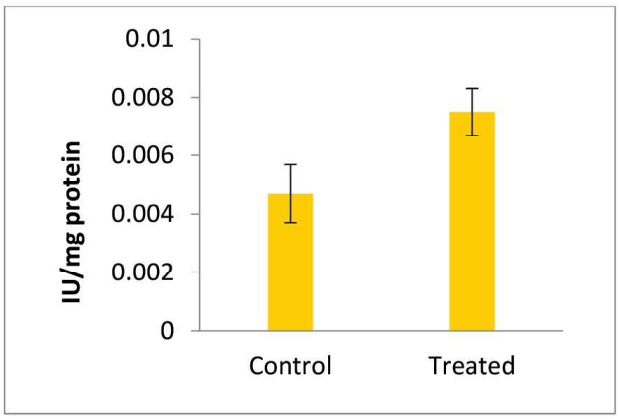

Figure 7.1: The effect of NaCl on the activity of Glutathione Peroxidase.

CDNB is used as the substrate to measure the activity of GST. Fourth instar larvae of *Ae. albopictus* exposed to 1.3% NaCl for 24h showed a considerable increase [P<0.001] after treatment (Figure 8.1).

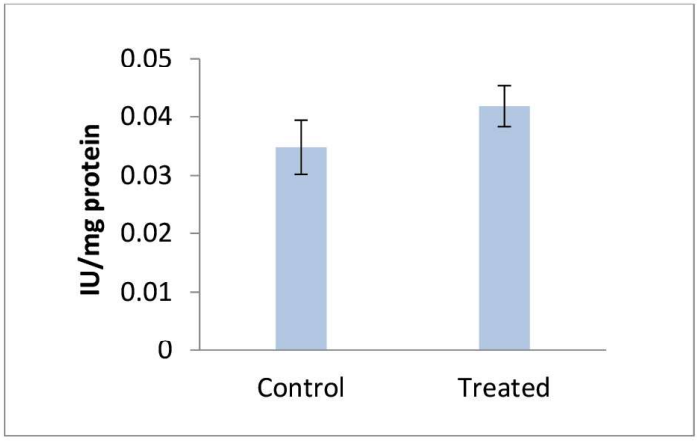

Figure 8.1: Glutathione-S-transferase's activity in response to NaCl.

After 24 hours of treatment, the AChE activity in the final instar larvae of *Ae.albopictus* exposed to NaCl was found to be decreased [P<0.001] in comparison to that of the control (Figure 9.1).

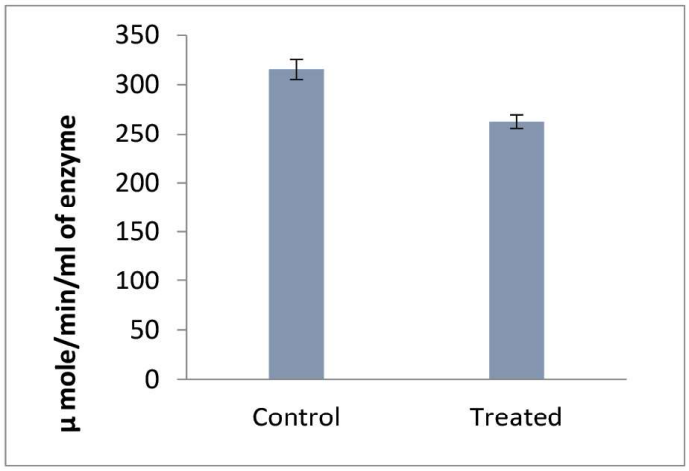

Figure 9.1: Acetylcholinesterase activity in relation to NaCl.

4. Discussion

This study focuses on how *Ae. albopictus* larvae in their final instar adapt to and are affected by NaCl stress through various morphological, developmental, and biochemical alterations. The biochemical analyses conducted after 24 h of NaCl treatment showed notable variations in the concentrations of biochemicals, including protein, amino acids, enzymes, SOD, CAT, GPx, GST, and AChE, compared to the control.

The LC_{50} value of NaCl on experimental larvae was found to be 1.7%. The length and width of treated eggs exhibited some structural alterations, and after 24 hours of exposure to NaCl, larvae showed modifications in their head, thorax, abdomen, and respiratory siphon compared to the control groups.

The distinction of *Aedes aegypti* and *Ae. albopictus* eggs morphology using SEM, observed some morphological changes in their eggs [23]. Similar results were also reported for another important vector species, *Ae. aegypti* [24]. The investigation into the insecticidal characteristics of the ethanol-based solution made from *Acmella oleracea* on *Ae. aegypti* and *Culex quinquefasciatus* demonstrated notable morphological abnormalities in the larvae treated for 24 hours, in comparison to the control group [25]. The findings from this research also showed specific alterations in the larval body, including whole-body discolouration and abnormal body segmentation. These results indicate that the chemical substance in the test media intensely interacts with their exoskeleton and midgut of the larvae [25].

Morphological studies on fourth instar larvae of *Ae. aegypti* showed abnormalities induced by the *Achyranthes aspera* mediated silver nanocomposites [26]. Morphological abnormalities such as, increase in egg length, width and an increase in the length of head, thorax, abdomen and siphon were visibly observed in our 24 h-treated larvae.

The current data shows that the impact of NaCl on larval physiology and metabolism is mainly through altering the concentration of major biochemicals such as protein, amino acids, SOD, CAT, GPx, GST and AChE. Larvae treated with NaCl showed a substantial increase in total body protein and amino acid content, which suggests that insects develop the primary level of resistance against salt stress.

It has been demonstrated that when botanical extracts and silver nanoparticles are exposed to *Ae. albopictus* and *Cx. pipiens*, the concentration of total protein decreases noticeably [27]. Another significant study examined the influence of low-temperature exposure on the metabolism of major biomolecules in *Philosamia ricini,* reporting an increase in both protein and amino acids following the treatment period [28].

Insects have a well-developed and sophisticated enzymatic antioxidant and esterase system that shields them from the abnormal production of reactive oxygen free radicals during certain unfavorable stressed conditions [29]. According to NaCl-induced stress responses, 1.3% NaCl exposure dramatically raised GPx and GST activity while considerably lowering SOD, CAT, and AChE activity. SOD is a crucial antioxidant enzyme in insects that transforms superoxide into oxygen

and hydrogen peroxide [29]. The action of SOD was found to be reduced in treated larvae than in controls. A notable surge in SOD was recorded in the midgut of *Helicoverpa armigera* larvae when exposed to metal ions such as Mn, Fe, Zn and Cu [29]. The decreased activity of SOD may be due to the excessive accumulation of superoxide radical, mainly in H2O2, which highly inhibits the action of SOD in the treated larvae [30,31].

Another important antioxidant enzyme in the insect body is CAT. CAT activity was found to be elevated in the mosquito vector *Anopheles* exposed to $CuSO_4$ [32]. In the current investigation, *Ae. albopictus* larvae in their fourth instar, exposed to the sub-lethal dose of NaCl, showed a reduction in the activity of CAT, which might be the reason for the abnormal production of H_2O_2 [31].

GPx activity exhibited a notable increase in NaCl-treated larvae, with this enhancement being dependent on the duration of exposure time. GPx is a selenium-dependent antioxidant enzyme present in hematophagous insects [33], and it primarily inactivates ROS and H_2O_2. The increase in GPx activity might be attributed to the increased production of H_2O_2, hydroxyl radical and O_2 under salt stress [34,35]. The same GPx elevation was also observed in the different algal extracts in the filariasis vector, *Cx. pipiens* [35].

GST activity increased significantly in NaCl-exposed *Ae. albopictus* larvae. GST represents one of the most important phase-II detoxification antioxidant enzymes that holds an important responsibility for the existence of insects that encounter chemical substances of both internal and external origin [36]. The increased expression of GST thus indicates a resistance mechanism in the treated larvae to overcome oxidative damage due to salt stress and thereby reducing the high risk of larval death [37]. The populations of *Ae. aegypti* and *An. arabiensis* showed comparable results [38,39].

In insects, the non-antioxidant AChE plays an important role in ending nerve signals at the synaptic cleft by breaking down the neurotransmitter acetylcholine through hydrolysis [40]. AChE activity level remarkably lowered after 24h of NaCl treatment. Similarly, *Ae.albopictus* larvae in their fourth instar showed a decline in AChE activity after exposure to silver nanoparticles derived from the water-based extract of *Cassia fistula* [27]. Such a reduced level of AChE is possibly due to the high accumulation of NaCl in the larval body.

The study's findings clearly demonstrate that the NaCl solution exerts an adverse effect on *Ae. albopictus* morphology, development, and biochemical characteristics. The drastic metabolic imbalances might be the reason for morphological as well as developmental abnormalities, which finally resulted in the mortality of both eggs and larvae. It can be concluded that the ovicidal as well as larvicidal effect of NaCl solution can be effectively

exploited for the management of *Ae. albopictus*, the potent vector of many communicable diseases. This can target larvae in their breeding habitat itself before adult emergence and find application in mosquito larval source management

Conclusions

This study clearly demonstrates that NaCl has the potential to be used as a secure larvicide in mosquito control methods due to its easy availability and lower toxicity, thereby enabling the development of comparatively safer strategies for mosquito larval management.

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Author Contribution

The research plan and article were authored by Neethu Karan, while Ajitha V.S. coordinated the data compilation and discussions. The final article has been reviewed and authorised by all authors.

Conflict Of Interest

There are no conflicts of concern in the content of the paper or with the authors.

Abbreviations

NaCl, Sodium chloride; SOD, Superoxide Dismutase; CAT, Catalase; GST, Glutathione-S-tranasferase; GPx, Glutathione Peroxidase; AChE, Acetylcholine esterase; KOH, Potassium hydroxide; ROS, Reactive Oxygen Species; NADH, Nicotinamide adenine dinucleotide;H_2O_2, Hydrogen Peroxide; Ethylenediaminetetraacetic acid; NADPH, Nicotinamide adenine dinucleotide phosphate; CDNB, 1-chloro-2,4-dinitrobenzene; GSH, Reduced glutathione; $CuSO_4$, Copper sulfate;

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