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Synergistic larvicidal potential of neem leaf aqueous extract and common salt against *Aedes aegypti* Linnaeus

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ABSTRACT

The transmission of dengue virus by the Aedes aegypti mosquito is a serious global concern. Neem leaf aqueous extract (NLAE) was prepared and tested for its larvicidal potential against Ae. aegypti 3rd/4th instars @ 1, 2, 3, 4, and 5 % and 4% NLAE was found to be the effective larvicidal concentration. Nine different combinations of NLAE and common salt (NaCl) obtained with the help of response surface methodology (RSM) were tested under laboratory conditions and two combinations having NLAE + common salt @ 2+0.5% and 1+0.25% were found to be the effective ones which were further tested under simulated conditions. The best effective combination obtained after a simulated trial having NLAE + common salt @ 2+0.5% showed larvicidal retention efficacy up to 7 days determined for two consecutive seasons. The effective concentration of NLAE either alone or in combination with common salt showed morphological changes like darkening of anal segments, loss of body hairs, and extensive swelling of the thorax and the alimentary canal leading to larval mortality. Thus, such synergistic formulations may prove to have a good potential for their usage as eco-friendly bio-larvicides against Ae. aegypti.

Keywords-*Aedes aegypti, common salt, larvicidal potential, mosquito control, plant-based extracts*

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INTRODUCTION

Mosquitoes play a predominant role in the transmission of malaria, dengue fever, yellow fever, filariasis, and several diseases which are today among the greatest health problems in the world [1 and 2]. *Aedes* mosquitoes are vectors for the transmission of the world's most life-threatening and debilitating diseases, of which dengue and chikungunya are the major global health concern. Dengue, in particular, is the most rapidly spreading mosquito-borne disease being transmitted by *Aedes aegypti*. As per World Health Organization (WHO) about half of the world's population is now at risk of dengue with an estimated 100–400 million infections occurring each year[3].According to the recent report of the National Vector Borne Disease Control Programme, in India, 31,464 dengue and 1,794 chikungunya cases have been reported in the country till 31st July 2023[4].

Ae. aegypti commonly known as the 'urban mosquito' is active during daylight hours and generally prefers to lay eggs in man-made clean containers having freshwater lying in peridomestic areas found in and around households, construction sites, and factories[5and6].This mosquito has been targeted over the years for control of dengue and the most common way being used is by applying chemical insecticides. The most often used insecticides includepyrethroids (reserermethrin, sumithrin, pyrethrin, and permethrin), carbamates (carbaryl), organochlorines (aldrin), and organophosphates (malathion, parathion)[7].However, the repeated use of such insecticides has made the control of mosquitoes more difficult because of the development of insecticide resistance [8], along with imposing negative impacts on humans and the environment [9].Larval source management is recognized as a successful solution to disrupt the completion of immature mosquito development and in turn controlling adult mosquitoes.

Nowadays, there is a prompt interest in the use of plant-based products as bio-insecticides being a rich source of natural insect repellents and a safe replacement for synthetic pesticides[10and11]. Due to their excellent insecticidal capabilities, plant extracts have drawn a lot of attention as effective bioactive chemicals against mosquito larvae also [12]. Botanicals are user-friendly because of their lower toxicity to non-target organisms and their innate biodegradation ability, thus acting as promising alternatives for mosquitoes [13].Plant-derived products, in contrast to chemical larvicides, contain several active ingredients that work in integration, thus making these more effective.The Indian neem tree (*Azadirachta indica A. Juss*), one of the important limonoid-producing plants from the Meliaceae family, has long been recognized as a source of environment-friendly biopesticide[14]. Most work has been focused on azadirachtin and other related compounds mainly limonoids, azadirachtin, and nimbinexhibiting a variety of actions against insects including highly toxic, antimitotic, antifeedant, growth controlling, fecundity suppressing, sterilization effects and attract and kill strategies [15]. Azadirachtin affects the insect's reproductive organs, body development, and other endocrine events [16]. Integration of such plant-based extracts especially the aqueous one with some non-lethal chemicals like common salt may result in the formulation of products having enhanced larvicidal potential along with better solubility and sustainability for the control of mosquitoes in their breeding habitats. The novelty of the present study is using aqueous extracts from leaves of a tree i.e. neem which is very easily available in Punjab (India) and the other component is common

salt (with very low concentration) which is also very economical. Thus, keeping all this in mind, the present study was planned to study the effect of neem leaf aqueous extract in combination with common salt against *Ae. aegypti* larvae.

MATERIALS AND METHODS

Collection of Ae. aegypti larvae

Water samples were collected from various small freshwater collections such as desert coolers, earthen pots, plastic containers, rubber tyres, flower pots, and roadside ditches lying in peri-domestic areas of the urban region of Ludhiana district of Punjab state (India) from May to November 2022.Water samples were brought to the laboratory in plastic bottles and transferred to plastic trays. These samples were allowed to settle for approximately 30 minutes. *Ae. aegypti* larvae were identified and separated out from other species of mosquito larvae (if present) based on their morphological characteristics by following the standard keys[17].

Preparation of neem leaf aqueous extract (NLAE)

Fresh leaves were collected from neem trees present in and around Punjab Agricultural University in Ludhiana, Punjab (India) from May to July 2022.Large fabric bags were used to bring the collected leaves to the laboratory. The infectedleaves were rejected and the fresh green leaves were cleaned properly to remove the dust. The cleaned neem leaves were shade dried by spreading these over the filter paper for three to five days atroom temperature. The filter paper was changed daily to avoid any bacterial or fungalinfection. Dried leaves were delicately mashed with hands and then fine powder wasprepared by using electric grinder mixture. Powder was autoclaved for 30 minutes to avoidany kind of contamination. For the preparation of aqueous extract, 20 g of neem leaf powderwas thoroughly mixed in 100 ml of distilled water taken in a conical flask. The flask waswrapped with aluminium foil and kept overnight. Next morning the mixture was filtered usingmuslin cloth and filtrate was collected in clean polycarbonate vials. These vials were tightly losed and wrapped in aluminium foil. To avoid any kind of contamination these vials werestored in a deep freezer at -18°C for further use.

Dose response bioassay for larvicidal potential of neem leaf aqueous extract (NLAE) against Ae. aegypti

The standard protocol proposed by[18]was slightly modified for the conduct of mosquito larvicidal assay under laboratory conditions. Preliminary testing for the larvicidal potential of NLAE was carried out against *Ae. aegypti* $3^{rd}/4^{th}$ instars by random selection of higher and lower concentrations of NLAE. Based on preliminarydose screening, twenty *Ae. aegypti* larvae ($3^{rd}/4^{th}$ instars) wereexposed to five concentrations of NLAE prepared @ 1, 2, 3, 4 and 5%. A control set having250 ml of de-chlorinated water with twenty *Ae. aegypti* larvae ($3^{rd}/4^{th}$ instars) was also runsimultaneously. The treated and control experimental sets were performed in triplicate in plastic beakers with 250 ml capacity and these beakers were properly covered with net using arubber band. A mixture of dog biscuits and crushed yeast @ 3:1 (2mg/100gm) was also addedas feed to all larvae of experimental sets.Mortality of *Ae. aegypti* larvae exposed to different concentrations of prepared NLAE and in control set was calculated by counting the number of dead larvae after 3, 6, 9,12, 24, 36 and 48 hours. Larvae that showed no response after being disturbed with a brush were considered dead.The minimal concentration of NLAE which resulted in maximum mortality within less duration (hours) was considered as the effective concentration for its usage in further experiments. For calculating $LC_{50}$and LC_{90} values after 24 hours of post-exposure, the log concentration-mortality regression was worked out by the log probit technique [19] employing the computer programme POLO[20].

Larvicidal potential of neem leaf aqueous extract (NLAE) in combination with common salt against Ae. aegypti

(a) Under laboratory conditions

The range of NLAE concentration (below its effective larvicidal concentration determined during dose-response larvicidal bioassay) and range of common salt concentration (below its effective larvicidal concentration i.e. 0.9% NaCl as already determined by[21]) were selected for making different combinations byusing Response Surface Methodology (RSM) software using central composite design (CCD)for testing their synergistic larvicidal potential and to acquire best possible combinations against. *Ae. aegypti* by "Design-Expert-9.0.3" (Stat Ease, Inc., Minneapolis, USA). A 13 factorial centralcomposite experimental design, with two factors and five replicates at the center point,leading to a set of 13 experiments, was obtained and out of these nine combinations wereconsidered (as there was the repetition of one combination i.e. combination-V for five times as shown in table 1) forfurther larvicidal bioassay.Obtained nine different combinations of NLAE + common salt were prepared inconcentration (per cent) @ 2+0.15, 1+0.25, 3+0.25, 0.6+0.5, 2+0.5, 3.4+0.5, 1+0.75, 3+0.75 and2+0.85 (as calculated by RSM software and shown in table 1) in plastic beakers having de-chlorinated water tomake total volume of 250 ml and twenty *Ae. aegypti* larvae ($3^{rd}/4^{th}$ instars) were exposed tothese combinations. A control set having 250 ml de-chlorinated water with twenty $3^{rd}/4^{th}$instars larvae in each beaker was also run along with the respective treatment trial. The treatmentand control experiments were performed in triplicate sets in plastic beakers with 250 mlcapacity and these beakers were properly covered with net using a rubber band. Thelarval mortality was recorded after 3, 6, 9, 12, 24, 36and 48 hours of exposure in all thetreatment and control sets. NLAE with the common salt combination(s) showing the highestmortality rate in the shortest period among the tested combinations was considered aseffective combination(s) and taken for further experimental study.

(b) Under simulated conditions

To conduct simulated trials, water was collected from small freshwater collections (i.e. breeding sites of *Ae. aegypti*) and filtered before use. Simulated experiments were performed in small containers like plastic cups with 250 ml capacity and large containers like plastic buckets with 10 L capacity. Fifty $3^{rd}/4^{th}$ instars of *Ae. aegypti* were exposed to two effective combinations of NLAE with common salt @ 1+0.25% and 2+0.5% (obtained from laboratory trials) and were tested under simulated conditions. The total volume of different combinations of NLAE and common salt in plastic cups and plastic buckets having water was made to 250 ml and 1000 ml respectively. Control sets having 250 ml and 1000 ml de-chlorinated water in plastic cups and plastic buckets with fifty $3^{rd}/4^{th}$ instars larvae were also run along with their respective treatment sets. The treated combinations and control sets were performed in triplicate sets. The number of larvae killed after 24 and 48 hours of exposure were recorded in all the treatment and control sets.

Larvicidal retention efficacy of best effective larvicidal combination of neem leaf aqueous extract (NLAE) and common salt against Ae. aegypti

Twenty *Ae. aegypti* larvae ($3^{rd}/4^{th}$ instars)were exposed to the best effective combination havingNLAE and common salt @2+0.5%. To this 1.25 ml of sodium azide (0.1%) was added, so as to avoid any fungal contamination. Total volume of an effective combination of NLAE with common salt along with 0.1% sodium azide in plastic beakers was made to 250 ml with de-chlorinated water. After 24 hours of exposure, number of dead larvae were counted and the remaining live larvae were removed and replenished with a new batch of 20 larvae. Two control sets one having de-chlorinated water and other containing 0.1% sodium azide in de-chlorinated water have same number of *Ae. aegypti* larvae as that of treatment sets were also run simultaneously. The experiment was performed in triplicate for each combination. The data for mortality of larvae and their replenishment with new larvae was repeated after every 24 hours until the percent larval mortality was found to reduce below 25% [22].

Effect of neem leaf aqueous extract (NLAE) alone and in combination with common salt on morphological characters of Ae. aegypti larvae

Ae. aegypti larvae ($3^{rd}/4^{th}$ instars) after their exposure to the effective concentration of NLAE (4%) alone and effective combination having NLAEandcommon salt @ 2+0.5% were observed minutely for any kind of morphological changes. For this, the moribund (about to die) and deceased *Ae. aegypti* larvae were separated and examined under light microscope (Olympus CX21i) and photographed with a SONY colour video camera (SSC-G818) to study any kind of morphological changes/abnormalities in the treated larvae and compare with that of control larvae.

Statistical analysis

Data was statistically analyzed by comparing larval mortality observed in treatment and control sets by using ANOVA (Tukey's method test) at 5% level of significance.

RESULTS

General characteristics of neem leaf aqueous extract (NLAE)

NLAE prepared in the laboratory was dark green with a pungent garlic-like smell and was soluble in water. Approximately 125-130 ml of NLAE was obtained from 50 g of neem leaf powder after its extraction with 250 ml of water by keeping overnight.

Larvicidal potential of neem leaf aqueous extract against Ae. aegypti

Exposure of *Ae. aegypti* larvae to 1% NLAE resulted from no larval mortality till 12 hrs and this mortality rate increased up to 50.00±17.32, 63.33±5.77 and 73.33±5.77 percent after 24, 36, and 48 hrs, respectively. However, no mortality was observed after 48 hrs onwards, and the larvae that survived got transformed to the next developmental stage i.e. pupae. NLAE @ 2 and 3% showed no larval mortality till 6 hrs and per cent, larval mortality was found to increase with the increase in exposure time and reached up to 100%after 48 hrs. However, exposure of larvae to 4 and 5% resulted in 100% larval mortality with 12 hours of treatment.Overall, the percent larval mortality was found to increase with increase in the concentration of NLAE as well as with the increase in exposure periodconcerning each concentration. Comparative analysis of larval mortality among the tested concentrations revealed that NLAE @ 4% was found to be a statistically efficient larvicidal concentration, as it resulted in maximum mortality(100%) in 12 hrs (Table 2). No larval mortality was observed in the control sets performed along with the treatment sets. LC_{50} and LC_{90} toxicity values computed for *Ae. aegypti* larvae based on record of mortality till 24 hrs of exposure to NLAE were worked out to be1.18 and 3.24 mg/L respectively (Table 3).

Larvicidal potential of neem leaf aqueous extract (NLAE) in combination with common salt against Ae. aegypti

(a) Under laboratory conditions

Out of the tested nine combinations of NLAE + common salt obtained with RSM design, combination-I and IV showed 100 and 90% larval mortality respectively within 48 hrs. Three combinations i.e. II, V, and VII showed 100% larval killing within 36 hrs of exposure. However, combinations- III, VIII, and IX showed 100% mortality within 12 hours of treatment. While combination-VI showed killing of all larvae within 9 hrs of exposure. Out of these nine combinations, two combinations i.e. combination-II having NLAE + common salt @1+0.25% and combination-V having NLAE + common salt @ 2+0.5% were found to be the best larvicidal effective combinations which resulted in 100% larval mortality within 36 hrs of treatment with minimum concentration of NLAE and common salt (Table 4).

(b) Under simulated conditions

Two effective combinations of NLAE + common salt i.e. combination-II and V @1+0.25% and 2+0.5% were taken into consideration for their validation under simulated conditions and their testing in plastic cups and plastic buckets which showed percent larval mortality @ 63.33±5.03 and 77.33±3.05 in plastic cups and 58.00±4.00 and 76.00±5.29 in plastic buckets respectively after 24 hrs of the treatment whereas 100% larval killing was found in both the types of containers after 48 hrs of each type of treatment. However, no larval mortality was observed in the control sets. Combination-V having NLAE + common salt @ 2+0.5% was found to be a better combination in comparison to combination-II as it showed statistically higher larvicidal potential within 24 hrs of treatment (Table 5).

Larvicidal retention efficacy of neem leaf aqueous extract (NLAE)integrated with common salt against Ae. aegypti

The best effective combination having NLAE and common salt @ 2+0.5% obtained from simulated trials (Table 5) was tested for its retention efficacy and 76.66±5.77 percent larval mortality for first day during season-1 (September 2022) and 70.00±10.00 per centduring season-2 (July, 2023) was observed.Then, a gradual decline in larvicidal activity was observed during both seasons as shown in table 6. Larval mortality was found to decrease by25% by 8^{th} day in both seasons as it got reduced to 23.33 and 16.66% during season I and II respectively (Table 6). The larvicidal retention period of this combination was found to be effective till 7 days as per the efficacy determination method given by [22]according to which larval mortality below 25% is not considered as the effective one. No larval mortality was observed in two control sets i.e.C1 having de-chlorinated water and C2 having de-chlorinated water and 0.1% sodium azide which were run along with the respective treatments every season.

Effect of neem leaf aqueous extract (NLAE) alone and in combination with common salt on morphological characters of Aedes aegypti larvae

Exposure of *Ae. aegypti* larvae to effective larvicidal concentration of NLAE i.e. 4.0% resulted in visible swollen alimentary canal (Fig. 1a) and darkening of anal segments (Fig. 1b), while in case of treatment having NLAE + common salt

@2+0.5%,swelling in thorax region was also observed (Fig. 1c) along with complete loss of anal gills (Fig. 1d).While untreated/controllarvae were found to be intact and normal (Fig. 1e and f).

DISCUSSION

Neem based extracts/oils are known to have larvicidal properties in addition to repellency action because of the presence of various active compounds such as limonoids, azadirachtin, salannin, deacetyl geduum and deacetylnimbin[23]. Literature reveals that concentration of neem leaf aqueous extracts have a variable range of larval mortality (45 to 98%) depending upon the type of larval instar stage and exposure period[24]. In the present study also, it was observed that with an increase in the concentration of neem leaf aqueous extract (NLAE), the extent of larval mortality goes higher and even with the increase in time of treatment (Table 2) and 4% NLAE was found to be the effective larvicidal concentration against *Ae. aegypti*. Significant larval killing observed after treatment with neem leaf oil revealed that this oil has survivability-reducing properties against *Ae. aegypti* larvae[25].A previous research work done in our laboratory has shown that 500 ppm of neem oil extracted from seed kernels acted as the effective larvicidal concentration against *Ae. aegypti*[26]. In another study, researchers observed LC_{50} and LC_{90} values as 7852 and 10,092 ppm respectively after exposure of *Ae. aegypti* larvae to neem oil[27]. In the present study, toxicity values of LC_{50} and LC_{90}after exposure of NLAE observed in terms of mg/L were 1.18 and 3.24 against*Ae aegypti* larvae (Table 3).

Ae. aegypti is a freshwater mosquito, its larvae can only withstand a narrow range of salinity. The survival of larvae is hampered by any rise in salinity which causes tremendous stress [28]. Even our previous study has demonstrated 100% mortality of 1st instar larvae of *Ae. aegypti* after their exposure to 9ppt of common salt i.e. NaCl [21]. Those *Aedes* species that cannot tolerate higher concentrations of common salt because of the lack of an additional rectum segment, it becomes impossible for their larvae to eliminate excess of ions, resulting in their mortality [29]. So the idea was to integrate neem leaf aqueous extract with common salt to enhance*Ae. aegypti* larval killing and the present study got successful results,as their integration (with a concentration lesser than their effective one if used alone) evaluated the larvicidal potential of such combinations firstly under laboratory conditions (Table 4) and then its confirmation under simulated trials (Table 5). The best combination obtained at the end having neem leaf aqueous extract (NLAE) and common salt @ 2+0.5% was found to have larvicidal efficacy till 7 days recorded for two consecutive seasons (Table 6). If NLAE was taken alone, it was effective @ 4% (Table 2) but mixing with common salt @ 0.5% reduced the concentration of NLAE to half i.e.2% indicating the synergistic larvicidal effect of NLAE and common salt. This integrated approach resulted in morphological larval damages like swelling of the alimentary canal and darkening of anal gills after exposure to NLAE and observation of swollen thorax along with complete loss of anal gills when larvae were kept in treatment trial having NLAE and common salt (Fig. 1) because of their synergistic effect. Similar degenerative changes and necrosis have also been observed in *An. stephensi*larvae after exposure to crude eucalyptus oil [30]. The darkening of the midgut and hindgut, as well as its contents, can occur for a variety of reasons and is influenced by some mechanisms, including melanization caused by phenoloxidase cascade activation [31]. The primary absorption regions of the mosquito larval gut include the midgut epithelium, which has well-developed microvilli with a brush border in the cell apex. As a result, any modification or disruption in these structures resulted in poor or no absorption [32]. The effects of *Melia azedarach* extract on the midgut of *Culex quinquefasciatus* larvae included vacuolization, microvilli damage, cell lysis and death [33].

CONCLUSION

The present study revealed the synergistic larvicidal potential of neem leaf aqueous extract (NLAE) and common salt against *Ae. aegypti*when used in combination @ 2+0.5% and its efficacy up to seven days. Such type of neem extract blended with common salt can be used as efficient bio-larvicidal agents for the control of dengue spreading *Ae. aegypti,*because of its aqueous nature it will disperse well in the breeding habitats of mosquitoes and will be environmentally safe too being plant-based in nature.

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Table 1: Optimization of different combinations of common salt with neem leaf aqueous extract (NLAE) against Aedes aegypti larvae (3rd/4th instars) obtained by RSM design

Run	Common salt (%)	NLAE (%)	Combinations taken for testing in per cent (Common salt + NLAE)	
1	0.1464	2	0.15+2	Combination-I
2	0.25	1	0.25+1	Combination-II
3	0.25	3	0.25+3	Combination-III
4	0.5	0.5858	0.5+0.6	Combination-IV
5	0.5	2	0.5+2	Combination-V
6	0.5	3.4142	0.5+3.4	Combination-VI
7	0.5	2	0.5+2	Repeat
8	0.75	1	0.75+1	Combination-VII
9	0.5	2	0.5+2	Repeat
10	0.5	2	0.5+2	Repeat
11	0.75	3	0.75+3	Combination-VIII
12	0.5	2	0.5+2	Repeat
13	0.8536	2	0.85+2	Combination-IX

Table 2: Effect of different concentrations of neem leaf aqueous extract (NLAE) on mortality of Aedes aegypti larvae (3rd/4th instars)

Concentration (%)	Per cent larval mortality upto (Mean±S.D) (n=20)							Range of mortality (Within hours)
	3hr	6hr	9hr	12hr	24hr	36hr	48hr	
1	0.00±0.00^{a} (0)	0.00±0.00^{a} (0)	0.00±0.00^{a} (0)	0.00±0.00^{a} (0)	50.00±17.32^{b} (6-12)	63.33±5.77^{b} (12-14)	73.33±5.77^{b} (14-16)	24-48
2	0.00±0.00^{a} (0)	0.00±0.00^{a} (0)	13.33±5.77^{a} (2-4)	23.33±5.77^{b} (4-6)	66.66±5.77^{bc} (12-14)	86.66±5.77^{c} (16-18)	100.00±0.00^{c} (20)	9-48
3	0.00±0.00^{a} (0)	0.00±0.00^{a} (0)	13.33±5.77^{a} (2-4)	36.66±5.77^{c} (6-8)	80.00±10.00^{c} (12-18)	96.66±5.77^{c} (18-20)	100.00±0.00^{c} (20)	9-48
4	**0.00±0.00^{a} (0)**	**26.66±5.77^{b} (4-6)**	**46.66±5.77^{b} (8-10)**	**100.00±0.00^{d} (20)**	**-**	**-**	**-**	**6-12**
5	23.33±5.77^{b} (4-6)	50.00±0.00^{c} (10)	70.00±0.00^{c} (12-16)	100.00±0.00^{d} (20)	-	-	-	3-12
0 (Control)	0.00±0.00^{a} (0)	0.00±0.00^{a} (0)	0.00±0.00^{a} (0)	0.00±0.00^{a} (0)	0.00±0.00^{a} (0)	0.00±0.00^{a} (0)	0.00±0.00^{a} (0)	-

- n represents number of larvae taken
- Figures in parenthesis indicate the range in number of dead larvae from the start of experiment till that period
- Figures followed with different superscripts indicate significant difference ($p<0.05$) with respect to control and treatment sets by using Tukey's test

Table 3: Toxicity values of neem leaf aqueous extract (NLAE) against Ae. aegypti larvae(3rd/4th instars) after 24 hours of exposure

Toxicity Value (mg/L)	Fiducial limits		χ^2
	Lower limit (mg/L)	Upper limit (mg/L)	
LC_{50}=1.18	0.28	1.76	15.68
LC_{90}=3.24	2.19	12.22	

Table 4: Synergistic larvicidal effect of RSM designed combinations having neem leaf aqueous extract (NLAE)and common saltagainstAedes aegypti(3rd/4th instars) tested under laboratory conditions

NLAE+ Common salt (%)	Per cent larval mortality upto (Mean±S.D) (n=20)							Range of mortality (Within hours)
	3hr	6hr	9hr	12hr	24hr	36hr	48hr	
Combination-I (2+0.15)	0.00±0.00^{a} (0)	0.00±0.00^{a} (0)	10.00±10.00^{ab} (2-4)	26.66±5.77^{b} (4-6)	53.33±5.77^{b} (10-12)	66.66±5.77^{b} (12-14)	90.00±10.00^{b} (16-18)	9-48
Combination-II (1+0.25)	**0.00±0.00^{a} (0)**	**0.00±0.00^{a} (0)**	**23.33±5.77^{abc} (2-6)**	**53.33±5.77^{c} (10-12)**	**70.00±0.00^{c} (14)**	**100.00±0.00^{d} (20)**	**-**	**9-36**
Combination-III (3+0.25)	0.00±0.00^{a} (0)	0.00±0.00^{a} (0)	40.00±10.00^{cde} (6-10)	80.00±10.00^{d} (14-18)	96.66±5.77^{d} (18-20)	100.00±0.00^{d} (20)	-	9-36
Combination-IV (0.6+0.5)	0.00±0.00^{a} (0)	0.00±0.00^{a} (0)	23.33±5.77^{abc} (4-6)	56.66±5.77^{c} (10-12)	73.33±5.77^{c} (14-16)	86.66±5.77^{c} (16-18)	100.00±0.00^{b} (20)	9-48
Combination-V (2+0.5)	**0.00±0.00^{a} (0)**	**6.66±5.77^{a} (2)**	**30.00±10.00^{bcd} (4-8)**	**60.00±0.00^{c} (12)**	**80.00±0.00^{c} (14-18)**	**100.00±0.00^{d} (20)**	**-**	**6-36**
Combination-VI (3.4+0.5)	0.00±0.00^{a} (0)	33.33±5.77^{b} (6-8)	66.66±11.54^{e} (12-16)	100.00±0.00^{e} (20)	-	-	-	6-36
Combination-VII (1+0.75)	0.00±0.00^{a} (0)	26.66±5.77^{b} (4-6)	53.33±15.27^{de} (8-14)	100.00±10.00^{e} (20)	-	-	-	6-12
Combination-VIII (3+0.75)	0.00±0.00^{a} (0)	30.00±10.00^{b} (4-8)	63.33±15.27^{e} (10-16)	100.00±0.00^{e} (20)	-	-	-	6-12
Combination-IX (2+0.85)	0.00±0.00^{a} (0)	40.00±10.00^{b} (6-10)	100.00±0.00^{f} (20)	-	-	-	-	6-9
0 (Control)	0.00±0.00^{a} (0)	0.00±0.00^{a} (0)	0.00±0.00^{a} (0)	0.00±0.00^{a} (0)	0.00±0.00^{a} (0)	0.00±0.00^{a} (0)	0.00±0.00^{a} (0)	-

- n represents number of larvae taken
- Figures in parenthesis indicate the range in number of dead larvae from the start of experiment till that period
- Figures followed with different superscripts indicate significant difference ($p<0.05$) with respect to control and treatment sets by using Tukey's test

Table 5: Larvicidal potential of RSM designed effective combinations having neem leaf aqueous extract (NLAE) and common salt against Aedes aegypti ($3^{rd}/4^{th}$ instars) under simulated conditions

NLAE + Common salt (%)	Per cent larval mortality upto (Mean±S.D) (n=50)			
	24hr		48hr	
	Plastic Cups	Plastic Buckets	Plastic Cups	Plastic Buckets
Combination-II **(1+0.25)**	65.33±5.03^{b} (30-35)	58.00±4.00^{b} (27-31)	100.00±0.00^{b} (50)	100.00±0.00^{b} (50)
Combination-V **(2+0.5)**	77.33±3.05^{c} (37-40)	76.00±5.29^{c} (35-40)	100.00±0.00^{b} (50)	100.00±0.00^{b} (50)
0 (Control)	0.00±0.00^{a} (0)	0.00±0.00^{a} (0)	0.00±0.00^{a} (0)	0.00±0.00^{a} (0)

- n represents number of larvae taken
- Figures in parenthesis indicate the range in number of dead larvae from the start of experiment till that period
- Figures followed with different superscripts indicate significant difference ($p<0.05$) with respect to control and treatment sets by using Tukey's test

Table 6: Larvicidal retention efficacy of RSM designed effective combination of neem leaf aqueous extract (NLAE) and common salt against Aedes aegypti ($3^{rd}/4^{th}$ instars) for two consecutive seasons

Duration (days)	Treatment sets having effective combination of NLAE and common salt (2+0.5%)		Retention efficacy
	Per cent larval mortality (Mean±S.D) (n=20)		
	Season-1 (September 2022)	Season-2 (July 2023)	
Day 1	76.66±5.77	70.00±10.00	Yes
Day 2	73.33±5.77	66.66±11.54	Yes
Day 3	63.33±5.77	53.33±5.77	Yes
Day 4	60.00±0.00	46.66±5.77	Yes
Day 5	56.66±5.77	36.66±5.77	Yes
Day 6	40.00±10.00	36.66±5.77	Yes
Day 7	36.66±11.54	26.66±5.77	Yes
Day 8	23.33±5.77	16.66±5.77	No

- n represents number of larvae taken
- no larval mortality was observed in control sets (C1-control having de-chlorinated water and C2-control having 0.1% sodium azide in de-chlorinated water)

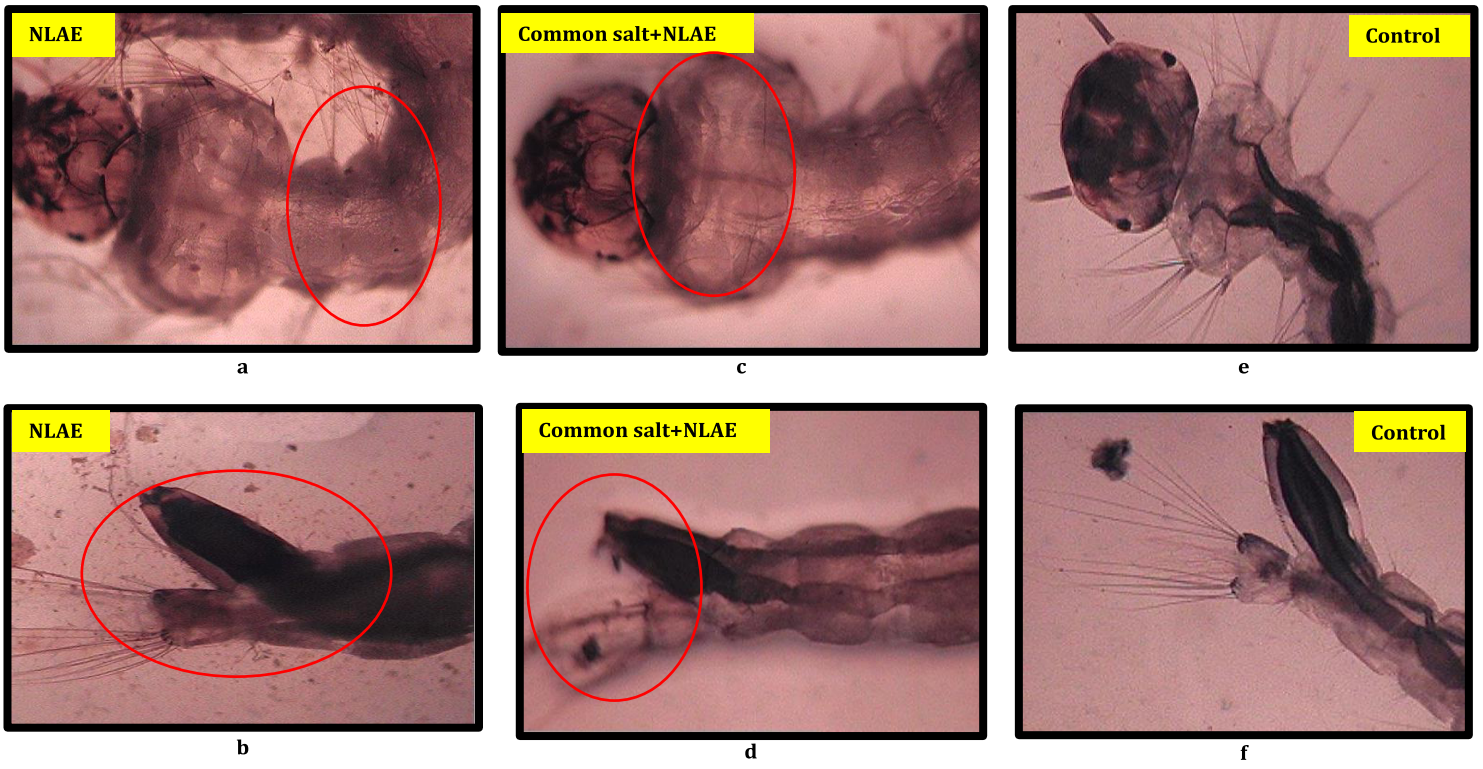

Fig. 1. Morphological changes observed in Aedes aegypti larvae after exposure to 4% neem leaf aqueous extract (NLAE) and 2% NLAE + 0.5% common salt (10X x4X)

(a) Swollen alimentary canal (c) Swollen thorax (e) and (f) Control larvae
(b) Darkening of anal segments (d) Loss of anal gills

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