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Research Article

Synthesis and Testing of Zinc Sulfide Based Hybrid Nanoemulsion of *Eucalyptus* Oil as a Safe Larvicide against *Aedes aegypti*

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ABSTRACT

The growing threat of vector-borne diseases and environmental pollution has prompted nanotechnology based investigations. The present study aimed to use one of the nanotechnological applications with larvicidal potential against *Aedes aegypti* by preparing aqueous hybrid nanoemulsion of zinc sulfide nanoparticles and *Eucalyptus globulus* oil. The mean droplet size of prepared and most stable hybrid nanoemulsion (9.5 ppm) was found to be 60 ± 10.00 nm. The hybrid nanoemulsion exhibited LC_{50} and LC_{90} values of 7.63 and 9.22 ppm. The findings obtained from larvicidal assay were corroborated with SEM, histological and biochemical profiles of *Aedes* larvae after treatment. Optimum larvicidal potency was observed under simulated conditions after 48 hrs of exposure. Larvicidal concentration of nanohybrid was found to be non-toxic to *Scapholebris kingi*. Thus, the following research explains larvicidal efficacy of zinc sulfide based hybrid nanoemulsion formulated during the present study is a step towards safe and efficient approach against *Aedes aegypti*.

Keywords: *Aedes aegypti*; Hybrid nanoemulsion; Larvicidal; Biosafety; Simulated conditions; Zinc sulfide

INTRODUCTION

The *Aedes aegypti* (Linnaeus, 1762) mosquito is responsible for transmitting diseases like dengue, chikungunya and zika in tropical and sub tropical countries. This mosquito breeds on stagnant water existing in artificial containers. In more than one hundred countries, dengue is endemic [1] and major cause of hospitalization [2]. Mosquito itself transmits diseases to more than 700 million people every year [3].

Therefore, the control of vector mosquito is an important public health concern [4] and until now, there has been no vaccine against such diseases. Vector control measures include chemical and biological methods for their immature and adult stages [5]. In this context, insecticides are the most widely used products. But, their usage in bulk is non selective, toxic to non-target species, harmful for environment and also results in mosquito resistance [6].

Plethora of scientific studies reported the larvicidal properties of phytoconstituents and plant based extracts with varied potential [7]. The main drawback of using essential oils as larvicidal agent in the natural habitat of larvae, which are water bodies, is immiscibility of water and oil. This problem can be overcome by downsizing the oils using nanotechnology and by making oil nanoemulsions.

Furthermore, researchers have checked the efficacy of different metal nanoparticles against insect vectors and revealed the potency of nanoparticles in vector control, out of these silver, gold, palladium, copper, zinc, silica and carbon nanoparticles have been best exemplified for mosquito control [8].

In the recent times, scientists have synthesized nanoformulations of heteroleptic metal complexes and developed them as larvicides against *Ae. aegypti*. Metal complexes of Cu (II), Co (II) and Fe (III) with heteroleptic ligands *viz*. 1,2,4-triazole-dithiocarbamate, triphenyl phosphine and isothiocyanate in different ratios prepared and converted into water dispersed nano-formulations were found to act as better larvicidal agents due to lower toxicity to non target organisms and higher water dispersibility [9].

Oil in water nanoemulsion of carbendazim (a fungicide) showed effective anti larval activity against *Culex* mosquito, as compared to organophosphorus and less persistant than organochlorine

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pesticides [10]. Different reports on antimicrobial, antitumor, and antilarval activities of transition metal complexes derived from amino acid Schiff bases have also been well documented and as compared to other bioactive metals, copper complexes with best potential against *Ae. Aegypti* [11].

Recently, *Azadirachta indica* leaf extract mediated bimetallic copper-zinc nanoparticles have been found to show larvicidal potential against *Culex quinquefasciatus* [8]. Green synthesis of copper oxide nanoparticles (CuO NPs) has also shown the larvicidal potential against *Ae. Aegypti* [12] and these CuO NPs were found to result in malformations of the larvae and pupae of cotton leafworm, *Spodoptera littorals* [13].

When *Scadoxus multiflorus* leaf powder aqueous extract was used as a capping and stabilizing agent during the synthesis of pure zinc oxide nanoparticles (ZnO NPs), it acted as larvicidal and ovicidal agent against *Ae. Aegypti* [14]. Antilarval activity of ZnO NPs using *Momordica charantia* leaf extract against *Anopheles stephensi*, *Culex quinquefasciatus* was reported [15].

Thus, eminent bioefficacy and safe nature of metal sulfide nanoparticles [16] are drawing the attention of scientists for their use against variety of environmental menaces and vectors responsible of public health concerns. Therefore, the present study was planned to synthesize and test the larvicidal efficacy of *Eucalyptus globulus* oil based nanohybrid emulsions having zinc sulfide nanoparticles against *Ae. aegypti* along with testing its safety against other non target organism which in the present study was *Scapholebris kingi*.

MATERIALS AND METHODS

Preparation of hybrid nanoemulsions (nanohybrids): Synthesis of ZnS nanoparticles

Zinc acetate $Zn(CH_3COO)_2$ (0.044 g, 0.2 m mol) was dissolved in 200 ml ethylene glycol in 250 ml conical flask to get 0.001 M solution. In another 250 ml flask, sodium sulfide Na_2S (0.016 g, 0.2 m mol) was dissolved in 200 ml distilled water to get 0.001 M solution. Twenty ml aqueous solution of sodium sulfide (0.001 M) was mixed dropwise into equal quantity of zinc acetate solution (0.001 M) under sonication which was continued for additional 30 minutes to obtain zinc sulfide nanoparticles (0.0005 M, 48.74 ppm).

Preparation of non polar *Eucalyptus* oil nanoemulsion

The oil was fractionated into polar and non polar fractions. The non polar portion of the oil was formulated in different ratios and sonicated along with water and surfactant [17].

Hybrid nanoemulsion preparation

The most stable oil nanoemulsion and zinc sulfide nanoparticles (ZnS NPs) were mixed in five different ratios i.e. 1:1, 1:2, 1:3, 1:4 and 1:5. All the different formulations were sonicated along with drop of Tween 20 so that metal sulfide nanoparticles get decorated on the non polar oil nanoemulsion to make hybrid nanoemulsions.

Screening and morphological studies of hybrid nanoemulsions

Stable hybrid nanoemulsions were screened by visually observing the transparency followed by various stress tests [18]. The morphometric properties of zinc sulfide based hybrid nanoemulsions (drop) on a copper grid was examined *via* Transmission Electron Microscopy (TEM-Hi 7650) operating at an accelerated voltage of 80 kV in the Electron Microscopy and Nanotechnology (EMN) Laboratory, PAU, Ludhiana.

Larvicidal bioassay of *Ae.aegypti* larvae

Mosquito larvae were collected from various peri-domestic water collections in urban areas of Ludhiana, Punjab by using plastic dippers and *Aedes aegypti* larvae identified using standard keys [19,20]. Zinc sulfide based hybrid nanoemulsions of *Eucalyptus* oil were tested @ 10.00, 9.50, 9.00, 8.50 and 8.00 ppm concentrations. Twenty early fourth instar *Ae. agypti* larvae per concentration were used for all the experiments in triplicate.

Control and vehicle control sets were also run simultaneously with twenty larvae in each set. During experimental duration, no food was provided. Dead larvae were identified from their discoloration and by probing needle in their siphon region. Mortality of the larvae in different concentrations was recorded after 3, 6, 12, 24 and 48 hours of treatment.

Morphological studies of Larvae

Morphological changes were studied by usage of Scanning Electron Microscope at Electron Microscopy and Nanotechnology Laboratory, PAU, Ludhiana. Moribund larvae from treated and untreated sets were primarily fixed for two hours in 2.5% glutaraldehyde solution followed by one hour fixationin 1% osmium tetroxide at 4°C.

Then, larvae were properly washed with 0.1 M sodium cacodylate buffer and in graded ethyl alcohol series for 15 minutes. Larvae were dried in vacuum desiccators for 1 hour and mounted on aluminium stub having a double sticky carbon tape. In iron sputter coater (E-1010), larvae were covered with a thin layer of approximately 20 nm to 30 nm of gold. Samples were examined and imaged under SEM-S3400N operated at an accelerating voltage of 15 kV.

Histological studies

For histological studies, the treated and untreated larvae were collected and washed properly in 0.9% saline solution followed by their embedding in 10% Neutral Buffered Formalin solution. Larvae were further processed, sectioned and stained following the standard histological procedure [21].

Biochemical assays

The treated and control larvae were collected, washed in double distilled water, homogenized and centrifuged, the supernatant was collected and used for estimation of total proteins [22] and activity of digestive enzymes i.e., α-amylase [23], protease [24] and lipase [25].

Larvicidal bioassay under simulated conditions

Experiments were performed in plastic tubs (with 25 litre capacity) having dried leaves, soil and algae. Control, vehicle-control and treated sets were performed in triplicate by introducing hundred fourth instar *Ae. aegypti* larvae. Mortality was recorded after 3, 6, 12, 24 and 48 hours in all experimental sets.

Bioefficacy of zinc sulfide based hybrid nanoemulsions against non-target organism, *Scapholebris kingi*

Samples of crustaceans (natural enemies of *Aedes* larvae) were collected from pond water using zooplankton net and *Scapholebris kingi* were specifically identified on basis of morphological characters [26,27]. In small plastic containers, twenty *S. kingi* were introduced in control, vehicle-control and treated sets in triplicate. All sets were observed for mortality and toxicity after 12, 24 and 48 hours initially and then at weekly intervals upto 21 days.

Statistical analysis

Mortality data was statistically analyzed by comparing treated and control groups by using ANOVA (Duncan multiple range test) on SPSS software version 16. Log probit method [28] using POLO [29] software was used for calculating LC_{50} and LC_{90}.

RESULTS

Screening and morphological analysis of hybrid nanoemulsions

The different non polar fractions were screened on the basis of certain parameters given in Table 1. Prepared hybrid nanoemulsions in ratio 1:5 (ZnS NPs: Oil) was found to be most stable after performing thermodynamic stability tests. TEM micrographs showed average size of droplets i.e. 60 ± 10.00 nm with slightly irregular in shape (Figure 1). The other ratios 1:1, 1:2, 1:3 and 1:4 did not show any coating of zinc sulfide nanoparticles (ZnS NPs) and hence were not considered for larvicidal studies.

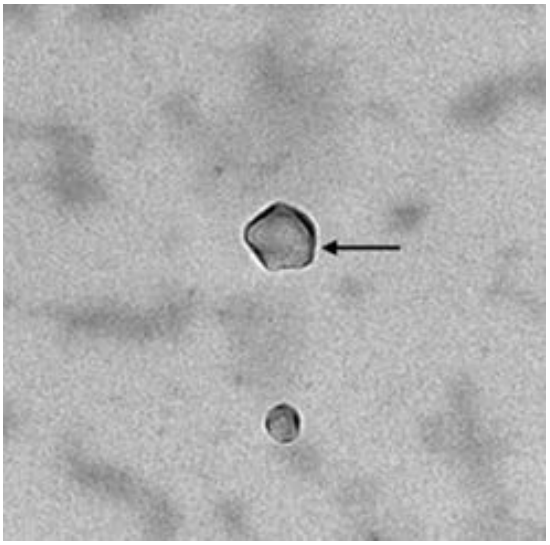

Figure 1: Transmission electron microscope image of stable ZnS based hybrid nanoemulsion of *Eucalyptus* oil (1:5).

Table 1: Characteristic features of zinc sulphide based hybrid nanoemulsions of *Eucalyptus* oil.

ZnS NPs: Oil	Thermodynamic Stability	Optical transparency	Appearance	Phase separation
1:1	-	Moderate	Clear	No
1:2	-	Moderate	Clear	No
1:3	-	Moderate	Clear	No
1:4	-	Moderate	Clear	No
1:5	Most stable	Highest	Clear	No

Effect of zinc sulfide based hybrid nanoemulsions on *Aedes aegypti* larvae

Larvicidal effect and toxicity: Exposure of *Ae. Aegypti* larvae to 8 ppm of stable zinc sulfide (1:5) based hybrid nanoemulsion showed 5.00 ± 0.00, 20.00 ± 5.00, 45.00 ± 5.00, 73.33 ± 15.28 and 95.00 ± 5.00 per cent mortality after 3, 6, 12, 24 and 48 hrs, respectively. With the increase in concentration to 8.5 ppm, the per cent mortality was observed as 11.67 ± 2.89, 26.67 ± 2.89, 51.67 ± 2.89, 75.00 ± 5.00 and 95.00 ± 5.00 after 3, 6, 12, 24 and 48 hrs respectively.

The per cent larval killing was found to increase statistically with the increase in concentration of zinc sulfide based hybrid nanoemulsion. On exposure to 9.5 ppm of concentration of zinc sulfide based hybrid nanoemulsion, 100% mortality of *Ae. aegypti* larvae was observed within 24 hrs.

Thus, 9.5 ppm concentration was found to be statistically the most effective concentration in comparison to other concentrations, as all larvae were killed at this concentration only within 24 hrs before conversion to pupae in comparison to 10 ppm concentration where 100% mortality was observed

within 12 hrs (Table 2). LC_{50} and LC_{90} of zinc based hybrid nanoemulsion were calculated to be 7.63 and 9.22 ppm respectively (Table 3).

Table 2: Effect of different concentrations of stable zinc sulfide (1:5) based hybrid nanoemulsion of eucalyptus oil on mortality of 4th instar *Aedes aegypti* larvae.

ZnS based nanohybrid concentration (ppm)	Per cent mortality (Mean ± SD) (n=20)					Range of mortality (Within hours)
	3 hr	6 hr	12 hr	24 hr	48 hr	
8	5.00 ± 0.00^{e}(1-1)	20.00 ± 5.00^{c}(3-5)	45.00 ± 5.00^{e}(8-10)	73.33 ± 15.28^{b}(12-18)	95.00 ± 5.00^{b} (18-20)	3-48
8.5	11.67 ± 2.89^{d}(2-3)	26.67 ± 2.89^{c}(5-6)	51.67 ± 2.89^{d}(10-11)	75.00 ± 5.00^{b}(14-16)	95.00 ± 5.00^{b}(18-20)	3-48
9	25.00 ± 5.00^{b}(4-6)	45.00 ± 5.00^{b}(8-10)	60.00 ± 5.00^{c}(11-13)	83.33 ± 7.64^{b}(15-18)	100.00 ± 0.00^{a}(20)	3-48
9.5	20.00 ± 5.00^{c}(3-5)	45.00 ± 5.00^{b}(8-10)	83.33 ± 7.64^{b}(15-18)	100.00 ± 0.00^{a}(20)	-	3-24
10	50.00 ± 0.00^{a}(10-10)	76.67 ± 7.64^{a}(14-17)	100.00 ± 0.00^{a}(20)	-	-	3-12
0 (Control)	0.00 ± 0.00^{f}(0)	0.00 ± 0.00^{d}(0)	0.00 ± 0.00^{f}(0)	0.00 ± 0.00^{c}(0)	0.00 ± 0.00^{c}(0)	-
0 (Vehicle-control)	0.00 ± 0.00^{f}(0)	0.00 ± 0.00^{d}(0)	0.00 ± 0.00^{f}(0)	0.00 ± 0.00^{c}(0)	0.00 ± 0.00^{c}(0)	-

- n represents number of larvae taken.
- Figures in parenthesis indicate the range of number of dead larvae from the start of experiment till that period.
- Figures with various superscripts predict significant difference ($p<0.05$) with respect to Control and Vehicle-control sets by using Duncan multiple range test (DMRT).

Table 3: Toxicity values of zinc sulfide based hybrid nanoemulsion against fourth instar larvae of *Aedes aegypti*.

Toxicity Value (ppm)	Fiducial limits		Slope	x^2
	Lower limit (ppm)	Upper limit (ppm)		
LC_{50}=7.63	6.56	8.06	15.586 ± 2.953	4.1528
LC_{90}=9.22	8.87	10.04	15.586 ± 2.953	4.1528

Morphological changes: Control larvae were found to have smooth head surface, clearly visible eye and antennae (Figure 2a) while distortion was observed in mouth region, mouth brushes and constriction on head capsule in treated larvae (Figure 2b and 2c).

Abdominal surface of control larvae was smooth as well as intact with no signs of disruption and cracking along with a clearly visible median spine (Figure 3a). Treatment led to reduction in thickness of exoskeleton, degradation of segmental cuticle, cracking and surface destruction of this region (Figure 3b and 3c).

Control larvae had smooth siphon with distinct spiracular valves (Figure 4a) while treatment resulted in shrinkage of siphon as well as anal papillae (Figure 4b).

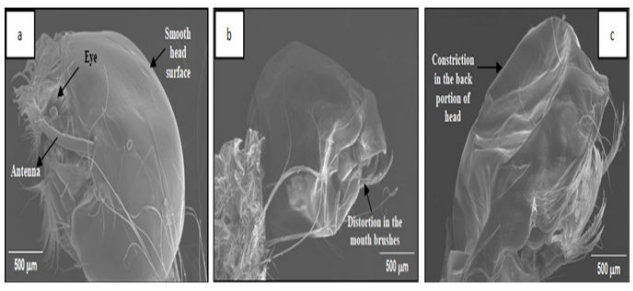

Figure 2: Scanning electron micrographs showing morphology of head region of *Aedes aegypti* larvae; (a) Control; (b,c) Treated with ZnS based hybrid nanoemulsions.

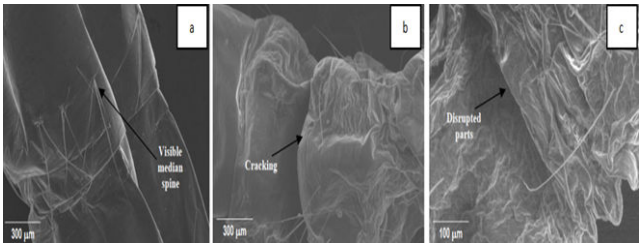

Figure 3: Scanning electron micrographs showing morphology of abdominal segments of *Aedes aegypti* larvae; (a) Control; (b,c) Treated with ZnS based hybrid nanoemulsions.

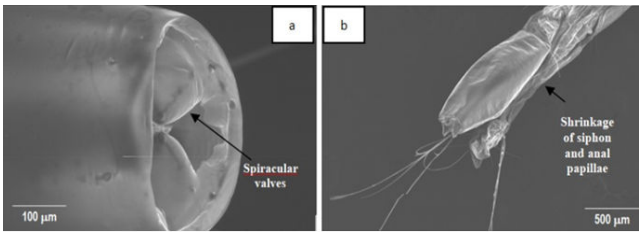

Figure 4: Scanning electron micrographs showing morphology of terminal region of *Aedes aegypti* larvae; (a) Control; (b) Treated with ZnS based hybrid nanoemulsions.

Histological changes: Control larvae were having imaginary eyes (E), an imaginary pair of antennae bud (IBA), a pair of brush inner retractor muscle (IRB) and brush outer retractor muscle (ORB) and brain with a pair of optic lobes (OL), all these parts in the head region were found to have no damage (Figure 5a).

Hybrid nanoemulsions based on ZnS induced alterations in the head region structure in the form of complete absence of optic lobes, and total disintegration of the brushes of inner and outer retractor muscles (Figure 5b).

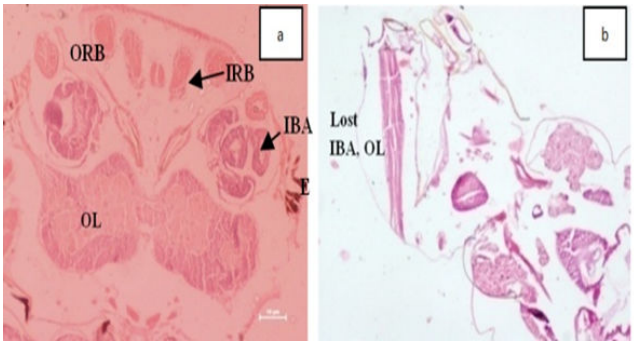

Figure 5: Longitudinal sections of head region of 4th instar *Ae. aegypti* larvae; (a) Control larva showing imaginal eyes (E), imaginal bud of antenna (IBA), inner retractor muscle of brush (IRB), outer retractor muscle of brush (ORB) and optic lobes (OL) (100X); (b) ZnS based nanohybrid treated larva showing absence of OL and IBA (100X).

IBA were found to be intact, complete and normal in the control larva (Figure 6a) while treatment induced stretching and elongation (Figure 6b).

Cylindrical epithelial cells (EC), vesicles (V), nucleus (N), peritrophic membrane (PM), basement membrane (BM) and muscle fibers (MF) were clearly visible along with well-developed microvilli (MV) present in the control gastric caeca located in thorax region (Figure 7a) while rifts were observed in peritrophic membrane in treated larvae (Figure 7b).

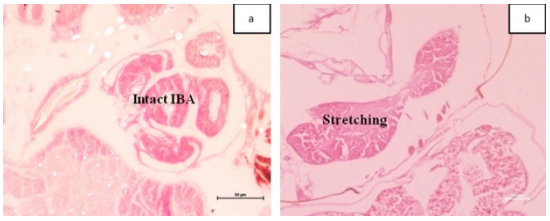

Figure 6: Longitudinal sections of head highlighting the region of imaginal bud of antennae (IBA) of 4th instar *Ae. aegypti* larvae (400X); (a) Control larva showing intact IBA; (b) ZnS based nanohybrid treated larva showing stretching and elongation in IBA.

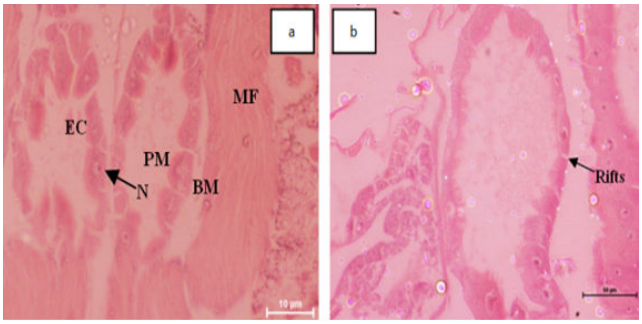

Figure 7: Longitudinal section of thorax highlighting the gastric caeca of 4th instar *Ae. aegypti* larvae (400X); (a) Control larva having epithelial cells (EC), nucleus (N), peritrophic membrane (PM), basement-membrane (BM), muscle fibres (MF); (b) ZnS based nanohybrid treated larva showing rifts at peritrophic membrane (PM).

The control larva showed complete abdominal region with a clearly visible food channel showing lumen (L), muscle fibers (MF), and gut lumen was filled with columnar cells of epithelium (Figure 8a) whereas treated larvae has disrupted alimentary canal with lesions (Figure 8b).

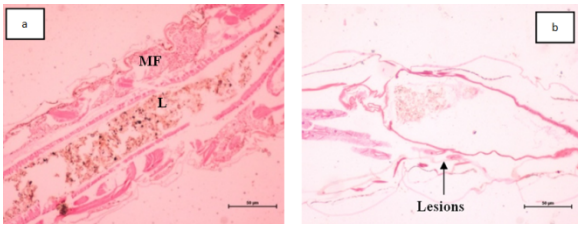

Figure 8: Longitudinal sections of abdomen of 4th instar *Ae. aegypti* larvae (100X); (a) Control larva showing lumen (L) and muscle fibres (MF); (b) ZnS based nanohybrid treated larva showing perturbation and lesions in the alimentary canal.

The lumen content of anterior midgut showed well-developed fat body (FB) tissues in control (Figure 9a) and very less distortion of FB was observed in treated larvae (Figure 9b). Intact epithelium layer in hindgut region of the control larva with presence of food in lumen was reported (Figure 10a) and disorganization was seen in epithelial layer of treated larvae (Figure 10b).

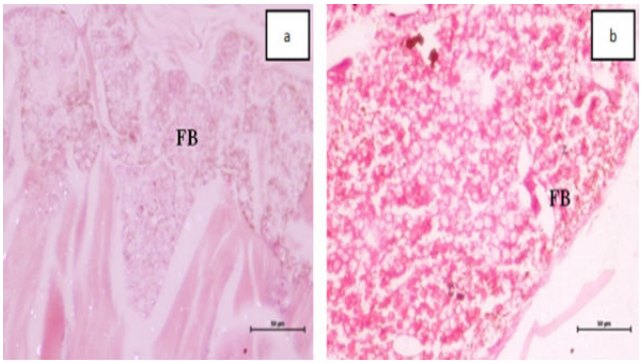

Figure 9: Longitudinal sections of midgut region highlighting fat bodies of 4th instar *Ae. aegypti* larvae (400X); (a) Control larva showing deposition of fat bodies (FB); (b) ZnS based nanohybrid treated larva showing very less disruption of fat bodies (FB).

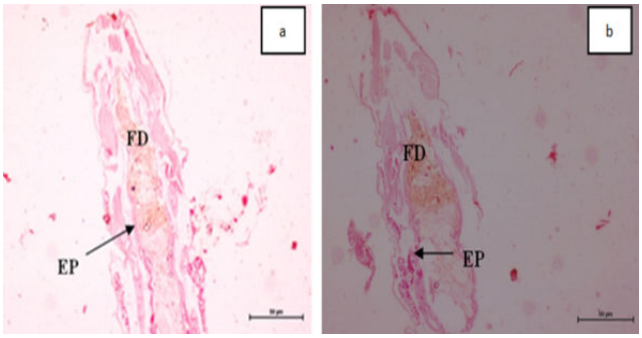

Figure 10: Longitudinal sections of terminal region highlighting hindgut of 4th instar *Ae. aegypti* larvae (400X); (a) Control larva showing intact epithelial layer and food in gut lumen (FD); (b) ZnS based nanohybrid treated larva showing degeneration of hindgut epithelium (EP).

Changes in biochemical parameters: Significant reduction in protein content, specific activity of α -amylase and protease was observed in treated larvae. On the other hand, lipase activity was neither decreased nor increased in treated as compared to control larvae (Table 4).

Table 4: Changes in biochemical parameters of 4th instar larvae of *Aedes aegypti* treated with effective concentration of ZnS based hybrid nanoemulsion (9.5 ppm).

Biochemical Parameters	Control	ZnS based hybrid nanoemulsion treated
Protein content (mg/g)	24.38 ± 1.24[a]	10.56 ± 2.48[b]
α -Amylase activity (nmol/min/mg protein)	0.041 ± 0.08[a]	0.003 ± 0.01[b]
Lipase activity (µmol/min/mg protein)	0.0052 ± 0.002[a]	0.0053 ± 0.00[a]
Protease activity (µmol/min/mg protein)	0.0032 ± 0.15[a]	0.0003 ± 0.07[b]

- Values are Mean ± SD
- Mean values followed with different superscripts are significantly different ($p<0.05$) using Duncan Multiple Range Test.

Testing the larvicidal potential of effective concentration of zinc sulfide based hybrid nanoemulsion under simulated conditions

Under simulated conditions, treatment of effective concentration of ZnS based hybrid nanoemulsion (9.5 ppm) resulted in more than 90% mortality after 48 hrs (Table 5). The mortality rate was found to be 19.33 ± 4.04, 32.00 ± 1.00, 51.67 ± 1.53, 79.00 ± 3.61 and 92.67 ± 2.52 per cent after 3, 6, 12, 24 and 48 hrs respectively. No larval mortality was observed in control and vehicle-control sets (Table 5).

Table 5: Larvicidal potential of effective concentration of zinc sulfide based hybrid nanoemulsion (9.5 ppm) against *Aedes aegypti* under simulated conditions.

ZnS based hybrid nanoemulsion concentration (ppm)	Percent mortality (Mean ± SD) (n=100)					Range of mortality (Within hours)
	3 hr	6 hr	12 hr	24 hr	48 hr	
ZnS based nanohybrid (9.5)	19.33 ± 4.04[a](15-23)	32.00 ± 1.00[a](31-33)	51.67 ± 1.53[a](50-53)	79.00 ± 3.61[a](75-82)	92.67 ± 2.52[a](90-93)	3-48
0 (Control)	0.00 ± 0.00[b](0)	0.00 ± 0.00[b](0)	0.00 ± 0.00[b](0)	0.00 ± 0.00[b] (0)	0.00 ± 0.00[b](0)	-

0 (Vehicle-control)	0.00 ± 0.00^{b}(0)	0.00 ± 0.00^{b}(0)	0.00 ± 0.00^{b}(0)	0.00 ± 0.00^{b}(0)	0.00 ± 0.00^{b}(0)	-

- n represents number of larvae taken.
- Figures in parenthesis indicate the range of number of dead larvae from the start of experiment till that period.
- Figures with various superscripts show significant difference ($p<0.05$) with respect to Control and Vehicle-control sets by using Duncan Multiple Range Test (DMRT).

Non toxicity tests of ZnS based hybrid nanoemulsions against *Scapholebris kingi*

During the present study, the exposure of selected non target organism i.e., *S. kingi* to 9.5 ppm of ZnS based hybrid nanoemulsion resulted in its 0.00 ± 0.00 per cent mortality (Table 6). Microscopic examination of these *S. kingi* (after exposure) showed no morphological changes as their eyes, mouth and antennae were normal and clearly visible (Figure 11). Therefore, this nanohybrid was found to have no toxic effect on non target organism studied during the present research.

Table 6: Toxicity test of zinc sulfide based hybrid nanoemulsion (9.5 ppm) on the non target organism *Scapholebris kingi*.

ZnS based hybrid nanoemulsions concentration (ppm)	Per cent mortality (Mean ± SD) (n=20)					Range of mortality (Within hours)
	3 hr	6 hr	7th day	14th day	21st day	
ZnS based nanohybrid (9.5)	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 (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 (Vehicle-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)	-

- n represents number of *Scapholebris kingi* taken.
- Figures in parenthesis indicate the range of number of dead organisms from the start of experiment till that period.
- Figures followed with same superscripts indicate non significant difference ($p<0.05$) with respect to Control and Vehicle-control sets by using DMRT test.

Figure 11: Microscopic photograph showing no morphological changes in *Scapholebris kingi* after treatment with 9.5 ppm of zinc sulfide based hybrid nanoemulsion (40X).

DISCUSSION

Among various prevailing approaches to mosquito control, larvicidal approach has been shown to be more effective, target-specific, and safer [30]. Herbal larvicidal agents are safer, simple to use and more environment friendly and can be used as alternative to chemical formulations [31]. Essential oil from *Lantana camara* leaves against VK7 and Kisumu strains of *Anopheles gambiae* and showed higher sensitivity of Kisumu strain to tested oil compared with VK7 strain [32].

Allium sativum, *Ferula asafetida* essential oils were evaluated for larvicidal activity against *Culex pipiens* and *Culex restuans* and calculated EC_{50} as 2.7 and 7.5 µg/mL, respectively, indicating high larvicidal potential of A. sativum oil [33]. Larvicidal activity of essential oils from seven plants (*Cuminum cyminum*, *Citrus aurantifolia*, *Cinnamomum verum*, *Syzygium aromaticum*, *Laurus nobilis*, *Lippiaber landieri* and *Pimpinella anisum*) against *Culexquinquefasciatus* larvae was also investigated [34]. Brazilian researchers developed *Rosmarinus officinalis* nanoemulsion against *Ae. aegypti* larvae and mortality recorded after treatment with 250 ppm was 80 per cent after 24 hrs [35].

Non-emulsified *R. officinalis* EO had LD_{95} of 408 ppm after 24 hours of exposure which, in contrast, suggests greater larvicidal efficacy of the nanoemulsion [36]. Iranian researchers published a work on inhibiting *Anopheles stephensi* larvae through action of

Artemisia dracunculus derived nanoemulsion with LC50 of 11.36 ppm [37]. Major nanoparticles synthesized by plant extracts are gold, silver, copper, copper oxide, palladium, platinium, titanium Dioxide, zinc oxide, indium oxide, iron oxide, lead and selenium nanoparticles [38].

Silver nanoparticles synthesis has been achieved using Kiwi fruit juice, *Rumex hymenosepalus* extract, *Annonas quamosa* leaf extract, *Podophyllum hexandrum* leaf extract, extracts of *Acalyphaindica Linn.*, *Hibisicus cannabinus* leaf extract, *Macrotyloma uniflorum* seed extract [39]. ZnS nanoparticles synthesised by sonochemical route employing zinc chloride and sodium sulphide, displayed significant anti-fungal property against pathogenic yeast *Candida albicans* at a minimum fungicidal concentration of 300 µg/ml [40]. Hybrid nanomaterial based on chlorine-e6 and surface-active maghemite nanoparticles with water self-assembly was synthesized and has a photocidal potential against *Ae. Aegypti* [4].

Morphological alterations by SEM in treated and control larvae of *Ae. aegypti* after exposure of ethanolic extracts of Piper longum was studied [41] and observed that larvae become shrunken and anal papillae got damaged led to death of larvae, similar observations have also been recorded in our present study. Cashew nut shell liquid nanoemulsion showed effect on morphological characters by SEM in *Anopheles culicifacies* larvae which revealed adherence of nano droplets to larval body resulting in complete larval deformation [42].

Our observations coincided with other authors who considered the impact of plant extracts, their nanomaterials and other such agents on mosquitoes and even on gut section. For example, larvicidal potential and histopathological changes induced after treatment of ethanolic extracts of Piper nigrum [43]. The most characteristic disordered signs visualized in the mid gut region of treated larvae were elongation and vacuolization of epithelial cells, rupturing of the peritrophic membrane, detachment of basement membrane, disorganization of microvilli, and furthermore, degeneration of muscle layer.

Histological changes in midgut of *Bacillus thuringiensis var. israelensis* (Bti) infected *Ae. albopictus* larvae, where disorganization in intestinal cells, dilation and disintegration in rough endoplasmic reticulum with intense cytoplasmic vacuolization was observed [44]. *Cx. tritaeniorhynchus* larvae treated with nanopermethrin colloidal dispersion and histological studies revealed normal midgut content (MC), EC and PM in control larvae, but these structures got damaged in treated larvae [45]. Partial damage in epithelial cells (EC) and peritrophic membrane (PM) in cashew nut shell liquid nanoemulsion treated *Anopheles culicifacies* larvae but in control larvae undamaged EC and unbroken PM were found [42].

Permethrinnanoformulation against *Cx. tritaeniorhynchus* larvae and studied its effect on glutathione S-transferase, acetylcholine esterase and acid and alkaline phosphatase assays were performed and decreased enzyme activity in treated as compared to control larvae was reported [45]. Their results were substantiated with our results as activity of digestive enzymes got decreased which led to death of the larvae. Neem-laced urea nanoemulsion exposed to *Ae. aegypti* and *Cx. tritaeniorhynchus* and total protein, lipid, carbohydrate and biomarker enzymes like GST, acid and alkaline phosphatase activity got also decreased [46].

Larvicidal efficacy of *Cryptomeria japonica* oil against *An. gambiae* was studied and observed 31.75 to 100% mortality of larvae under laboratory and 17.75 to 99.5% in semi field conditions [47]. The LC_{50} value of *J. procera* against larvae of *An. arabiensis* were 14.42 mg/L under laboratory conditions and 24.51 mg/L under semi-field conditions [48].

Lvandulyl acetate cytotoxicity on three non target mosquito predators *Anisops bouvieri*, *Diplonychus indicus* and *Gambusia affinis* was demonstrated and found to be 206, 336 and 534 µg/mL [49]. Effect of *Syzygium lanceolatum* essential oil on *A. bouvieri*, *D.indicus*, *G. affinis* and *Poecilia* reticulate with LC_{50} ranging from 4148 to 15762 µg/ml was reported [50].

CONCLUSION

Nanohybrid material based on chlorine-e6 and surface-active maghemite nanoparticles showed no adverse effects on Daphnia magna. Hence this nanohybrid reduced environmental concerns and may be used as a safer alternative. Therefore, biosafety assessment of formulated ZnS based hybrid nanoemulsion against non target species depicted minimal/no toxicity proves non toxic behavior of applied nanohybrid which portrays its environmental benevolence as an efficient and biosafe larvicidal agent.

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