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RESEARCH ARTICLE

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Formulation of eucalyptus oil-zinc sulphide hybrid nanoemulsion and evaluation of its larvicidal potential against *Aedes aegypti*

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The growing threat of vector-borne diseases and their associated health risks has prompted the nanotechnology-based investigations. The present study aimed to use a nanotechnological application with larvicidal potential against *Aedes aegypti* by preparing aqueous hybrid nanoemulsion of zinc sulphide 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 with rectangular shape. The hybrid nanoemulsion exhibited LC_{50} and LC_{90} values of 7.63 and 9.22 ppm, respectively, against larval stages of *Aedes aegypti*. The findings obtained from the larvicidal assay were corroborated with SEM, histological and biochemical profiles of *Aedes* larvae after treating with hybrid nanoemulsion. Under simulated conditions, nanohybrid treatment demonstrated optimum larvicidal potency after 48 hours of exposure. Further, biosafety studies of hybrid nanoemulsion were carried out against *Scapholebris kingi* and this larvicidal concentration of nanohybrid was found to be non-toxic to this species. Thus, the following research explains the larvicidal efficacy of zinc sulphide-based hybrid nanoemulsion of eucalyptus oil formulated during the present study is a step towards safe and efficient approach against the dengue-spreading vector mosquito, *Aedes aegypti*.

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INTRODUCTION

Aedes aegypti (Linnaeus, 1762) 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 (Vezzani 2007). In more than 100 countries, dengue is endemic (Pessoa et al. 2018) and major cause of hospitalisation (Santosh Kumar et al. 2018). The mosquito transmits diseases to more than 700 million people every year (Taubes 2000). Therefore, the control of this vector mosquito is an important public health concern (Magro et al. 2019) and until now, only few vaccines exist against arboviruses and especially for humans are still under clinical trials. Vector control measures include mainly the chemical methods and to some extent use of biocontrol agents for their immature and adult stages (George et al. *2015).* 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 the environment and also results in mosquito resistance (Margulis-Goshen et al. 2013).

Various scientific studies reported the larvicidal properties of phytoconstituents and plant-based extracts with varied potential (Piplani et al. 2019). The main drawback of using essential oils as a 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 examined the efficacy of different metal nanoparticles against insect vectors and revealed the potency of nanoparticles (NPs) in vector control. Out of these silver, gold, palladium, copper, zinc, silica and carbon nanoparticles have been best exemplified for mosquito control (Minal & Prakash 2019).

Recently, nanoformulations of heteroleptic metal complexes have been developed as potential 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 (Gumber et al. 2017). Oil in water nanoemulsion of carbendazim (a fungicide) showed effective anti-larval activity against *Culex* mosquito, as compared to organophosphates like temephos (Gumber et al. 2017). Different reports on antimicrobial and anti-larval 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* (Santha Lakshmi & Geetha 2016). Recently, *Azadirachta indica* leaf extract mediated bimetallic copper-zinc NPs have been found to show larvicidal potential against *Culex quinquefasciatus* (Minal & Prakash 2019).

Green synthesis of copper oxide nanoparticles (CuO NPs) has also shown the larvicidal potential against *Ae. aegypti* (Selvan et al. 2018) and these CuO NPs were found to result in malformations of the larvae and pupae of cotton leafworm, *Spodoptera littorals* (Shaker et al. 2016). When *Scadoxus multiflorus* leaf powder aqueous extract was used as a capping and stabilising agent during the synthesis of pure zinc oxide nanoparticles (ZnO NPs), it acted as larvicidal and ovicidal agent against *Ae. aegypti* (Dhabi & Arasu 2018). Antilarval activity of ZnO NPs using *Momordica charantia* leaf extract against *Anopheles stephensi*, *Culex quinquefasciatus* was reported by Gandhi et al. (2017).

The bioefficacy and safety of metal sulphide nanoparticles are of interest for public health use. Therefore, the present study was planned to synthesise and test the larvicidal efficacy of *Eucalyptus globulus* oil based nanohybrid emulsions having ZnO NPs against *Ae. aegypti* along with testing its safety against other non-target organisms which in the present study was *Scapholebris kingi*.

MATERIALS and METHODS

Preparation of hybrid nanoemulsions (nanohybrids)

Synthesis of ZnS nanoparticles

A 20 ml aqueous solution of sodium sulphide (0.001 M) was mixed dropwise into equal quantity of zinc acetate solution (0.001 M) under ultrasonic irradiation at temperature of 40.25 °C having pulse of 05 on and 01 off and amplitude of 60%, which was continued for additional 30 minutes to obtain zinc sulphide 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 (Kaur et al. 2019).

Preparation of nanoemulsion

The most stable oil nanoemulsion and zinc sulphide nanoparticles (ZnS NPs) were mixed in five different ratios viz. 1:1, 1:2, 1:3, 1:4 and 1:5. All the different formulations were sonicated along with a drop of Tween 20® so that metal sulphide nanoparticles were 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 described by Shafiq et al. (2007). The morphometric properties of zinc sulphide-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*

Aedes aegypti larvae were collected from various peri-domestic water collections in different zones of Punjab Agricultural University, Ludhiana, Punjab (30.9010 °N 75.8573 °E) by using plastic dippers from June to August 2019. *Aedes aegypti* larvae identified using the standard keys given by Becker et al. (2010) and Bar & Andrew (2013). Zinc sulphide based hybrid nanoemulsions of eucalyptus oil were tested at 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. The experimental sets were kept in a Bio-Oxygen Demand incubator at 26 ± 2 °C. Control (having 100 ml de-chlorinated water only) and vehicle control (having Tween 20 in de-chlorinated water in same ratio as treatment set) sets were also run simultaneously with 20 larvae in each set. During the 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 in triplicate.

Morphological studies of larvae

Morphological changes were studied by using a Scanning Electron Microscope (SEM-S3400N operated at 15 kV) at Electron Microscopy and Nanotechnology Laboratory, PAU, Ludhiana. Larvae from treated (larvae about to die) and untreated sets were primarily fixed for two hours in 2.5% glutaraldehyde solution followed by one hour fixation in 1% osmium tetroxide at 4 °C. Then, larvae were 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. Larvae were covered with a thin layer of approximately 20 nm to 30 nm of gold in the iron sputter coater (E-1010).

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 given by Luna (1968).

Biochemical assays

The treated and control larvae were collected, washed in double distilled water, homogenised and centrifuged, the supernatant was collected and used for estimation of total proteins (Lowry et al. 1951) and activity of digestive enzymes, i.e. α-amylase, protease and lipase by the methods given by Bernfeld (1955), Elpidina et al. (2001) and Tsujita et al. (1989), respectively.

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

For the conduct of experiments under simulated conditions, experiments were performed in plastic tubs (with dimensions of 45 × 34 × 26 cm) having dried leaves, soil and algae. Control, vehicle-control and treated sets were performed in triplicate by introducing 100 fourth instar *Ae. aegypti* larvae. Mortality was recorded after 3, 6, 12, 24 and 48 hours in all experimental sets in triplicate.

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

Samples of various zooplanktons like copepods, polychaetes and cladocerans were collected from pond water using a zooplankton net and *Scapholebris kingi* were specifically identified on the basis of morphological characters given by Battish (1992) and Jamwal (2015). In small plastic containers, 20 *S. kingi* were introduced in control, vehicle-control and treated sets in triplicate. All the sets were observed for mortality and toxicity after 12, 24 and 48 hours initially and then at weekly intervals up to 21 days.

Statistical analysis

Mortality data was statistically analysed by comparing treated and control groups by using ANOVA (Duncan multiple range test; DMRT) on SPSS statistical analysis software version 16 of log probit method (Finney 1971) using the POLO (Robertson et al. 1980) 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 (ZnSNPs: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 sulphide nanoparticles (ZnS NPs) and hence were not considered for larvicidal studies.

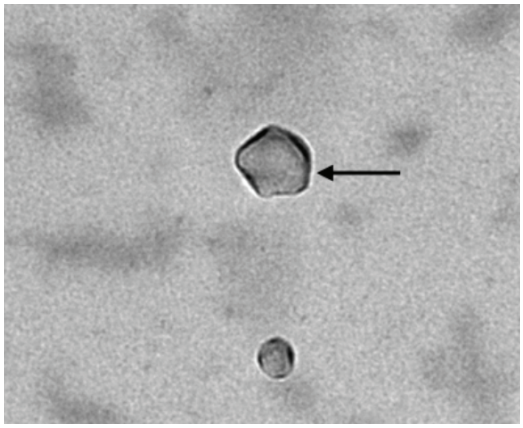


Figure 1. Transmission electron microscope image of stable ZnS-based hybrid nanoemulsion of eucalyptus oil (1:5)

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 sulphide-based hybrid nanoemulsions on Aedes aegypti *larvae*

Larvicidal effect and toxicity

Exposure of *Ae. aegypti* larvae to 8 ppm of stable zinc sulphide (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 hours, 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 hours respectively. The per cent larval mortality was found to increase statistically with the increase in concentration of zinc sulphide-based hybrid nanoemulsion. On exposure to 9.5 ppm of concentration of zinc sulphide based hybrid nanoemulsion, 100% mortality of *Ae. aegypti* larvae was observed within 24 hours. 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 hours before conversion to pupae in comparison to 10 ppm concentration where 100% mortality was observed within 12 hours (Table 2).

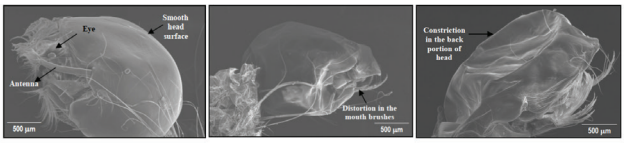
Various toxicity values, i.e. LC_{50} and LC_{90} of zinc sulphide-based hybrid nanoemulsion computed for 4th instar larvae of *Ae. aegypti* are given in Table 3. These values for LC_{50} and LC_{90} of zinc-based hybrid nanoemulsion were 7.63 and 9.22 ppm respectively against *Ae. aegypti* larvae as calculated by employing the computer programme POLO based on log-probit technique at 95% intervals (Finney 1971).

Morphological changes

Larvae exposed to effective concentration of ZnS-based hybrid nanoemulsions (9.5 ppm) were studied for morphological changes and compared with untreated larvae through the scanning electron micrographs. The modifications observed in the different body parts of the treated larvae have been described region wise as follows.

Head region – Control 4th instar larvae of *Ae. aegypti* were found to have smooth head surface, clearly visible eye and antennae (Figure 2a). After exposure to 9.5 ppm of ZnS-based hybrid nanoemulsion, distortion was observed in the mouth region, in particular the complete distortion of mouth brushes and constriction on the back of the head capsule was clearly visible (Figure 2b and 2c).

Abdominal region – Abdominal surface of control larvae was



(a) (b) (c)

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

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

Toxicity value (ppm)	Fiducial limits		Slope	χ2
	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

Table 2. Effect of different concentrations of stable zinc sulphide (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 ± S.D) (*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)

smooth as well as intact with no signs of disruption and cracking along with a clearly visible median spine (Figure 3a). Exposure of ZnS-based hybrid nanoemulsion led to reduction in thickness of exoskeleton, degradation of segmental cuticle, cracking and surface destruction of this region (Figure 3b and 3c).

Terminal region – In the case of control larvae, the siphon was found to have smooth surface with very distinct spiracular valves (Figure 4a). Treatment of *Ae. aegypti* larvae with ZnS-based hybrid nanoemulsions resulted in shrinkage of siphon as well as anal papillae (Figure 4b).

Histological changes

ZnS-based hybrid nanoemulsion treatment to *Ae. aegypti* 4th instar larvae resulted in destruction of various body parts/ organs present in various regions, so these histological changes have been described region wise.

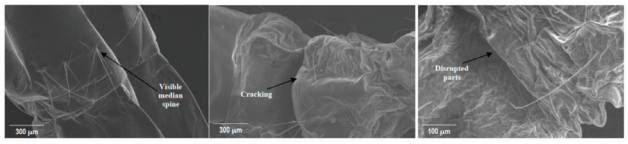
Head region – Control larvae had imaginal eyes (E), an imaginal pair of antennae buds (IBA), a pair of brush inner retractor muscle (IRB) and brush outer retractor muscle (ORB) and brain with a pair of optic lobes (OL), all these parts in the head region were found to have no damage (Figure 5a). Hybrid 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). Imaginal buds of antennae (IBA) were found to be intact, complete and normal in the control larva (Figure 6a) while ZnS-based hybrid nanoemulsion treatment induced stretching and elongation of the larval imaginal buds (Figure 6b).

Thorax area [Stomodeum or foregut part] – In the control larvae, cylindrical epithelial cells (EC), vesicles (V), nucleus (N), peritrophic membrane (PM), basement membrane (BM) and muscle fibres (MF) were clearly visible along with well-developed microvilli (MV) present in the gastric caeca located in thorax region (Figure 7a). ZnS-based hybrid nanoemulsion treatment showed slight changes in gastric caeca, as all cells were intact, nuclei were present and the appearance of epithelial cells was the same as that observed in the control larva except the occurrence of rifts in the peritrophic membrane and breakage of epithelium cells was also observed at certain areas (Figure 7b).

Abdomen – The control larva showed complete abdominal region with a clearly visible food channel showing lumen (L), muscle fibres (MF), and the gut lumen was filled with columnar cells of epithelium (Figure 8a) whereas in ZnS-based hybrid nanoemulsion-treated larvae signs of disruption in the alimentary canal were observed in the form of lesions as seen in Figure 8b.

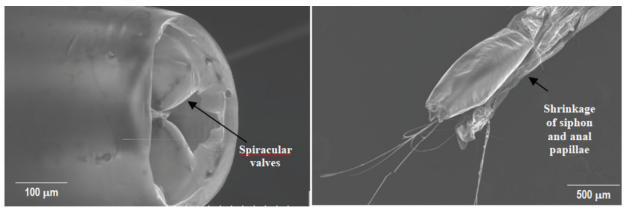
Mesenteron or midgut – The lumen content of the anterior midgut in the control larva showed well-developed fat body (FB) tissues (Figure 9a). However, much less distortion of fat bodies was observed in *Ae. aegypti* larvae treated with ZnS-based hybrid nanoemulsion (Figure 9b).

Proctodeum or hindgut – Intact epithelium layer in the hindgut region of the control larva with presence of food in the gut lumen was reported (Figure 10a). Disorganisation in the epithelial



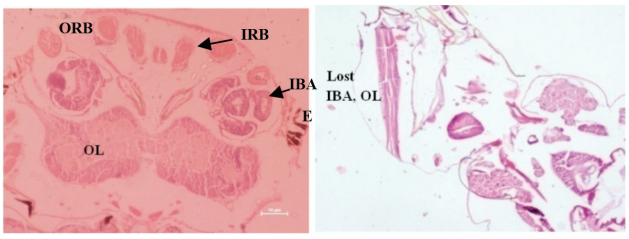
(a) (b) (c)

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



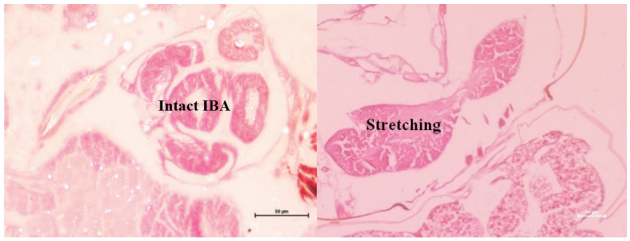
(a) (b)

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



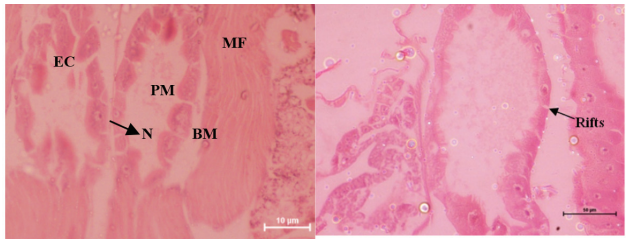
(a) (b)

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) (100×). (**b**) ZnS-based nanohybrid treated larva showing absence of OL and IBA (100×)



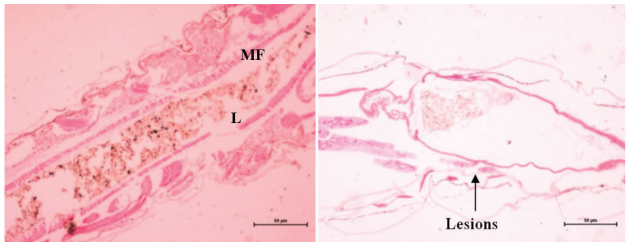
(a) (b)

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



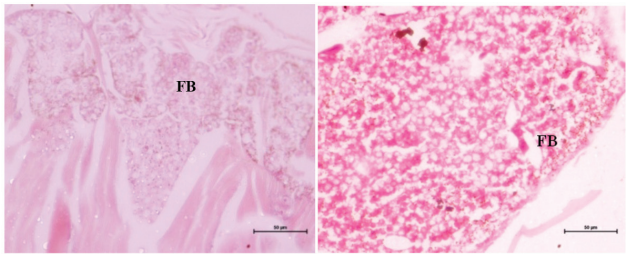
(a) (b)

Figure 7. Longitudinal section of thorax highlighting the gastric caeca of 4th instar *Ae. aegypti* larvae (400×). (**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)



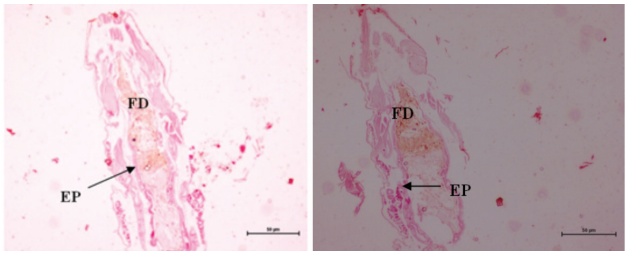
(a) (b)

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



(a) (b)

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



a) (b)

Figure 10. Longitudinal sections of terminal region highlighting hindgut of 4th instar *Ae. aegypti* larvae (400×). (**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)

layer was observed in the larvae treated with ZnS-based hybrid nanoemulsion (Figure 10b).

Changes in biochemical parameters

Exposure to effective ZnS-based hybrid nanoemulsion (9.5 ppm) showed variable effects on the amount of proteins and activity of different digestive enzymes of *Ae. aegypti* larvae which are described as below.

Protein content – The mean protein content was 24.38 ± 1.24 mg/g in control larvae, whereas exposure of ZnS-based hybrid nanoemulsion resulted in significant reduction in protein contents (F = 64.030, df = 2, $p \leq 0.05$) as it was observed to be 10.56 ± 2.48 mg/g (Table 4).

α-Amylase activity – The specific activity of α-amylase (F = 2680.627, df = 2, $p \leq 0.05$) was found to be 0.041 ± 0.08 nmol/min/mg protein in control larval homogenate, where as in treated group it was significantly decreased to 0.003±0.01 nmol/min/mg protein (Table 4).

Lipase activity – In the control larvae, the activity (F = 44.877, df = 2, $p \leq 0.05$) was found to be 0.0052 ± 0.002 µmol/min/mg of protein. On the other hand, ZnS-based hybrid nanoemulsion has no effect on this enzyme, as its activity was neither decreased nor increased as compared to control larvae (Table 4).

Protease activity – The activity of protease was found to decline statistically (F = 7.081, df = 2, $p \leq 0.05$) after the exposure of ZnS-based hybrid nanoemulsion as it was 0.0003 ± 0.07 µmol/min/mg protein, in comparison to that of control set (0.0032 ± 0.15 µmol/min/mg protein) as shown in Table 4.

Larvicidal potential of effective concentration of zinc sulphide-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 hours (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 hours respectively. No larval mortality was observed in control and vehicle-control sets (Table 5).

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 (F = 64.030, F = 2680.627, F = 44.877, F = 7.081, df = 2, $p \leq 0.05$) using Duncan Multiple Range Test.

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 the non-target organism studied during the present research.

DISCUSSION

Among the various prevailing approaches to mosquito control, the larvicidal approach has been shown to be one of the useful and target-specific tool (Killeen et al. 2002) and helpful in reducing adult mosquito population. Many workers have evaluated synthetic formulations of herbal based larvicidal agents. Wangrawa et al. (2018) tested essential oil from *Lantana camara* leaves against VK7 and Kisumu strains of *Anopheles gambiae* and found a higher sensitivity (LC_{50}: 7.73 µg/ml) of Kisumu strain to the tested oil compared with VK7 strain (LC_{50} : 25.63 µg/ml). Muturi et al. (2018) isolated essential oils from *Allium sativum*, *Ferula asafetida* to evaluate their larvicidal activity against *Culex pipiens* and *Cu. restuans* and calculated EC50 as 2.7 and 7.5 µg/ml, respectively, indicating high larvicidal potential of *A. sativum* oil. Andrade et al. (2018) investigated the larvicidal activity of essential oils from seven plants (*Cuminum cyminum, Citrus aurantifolia, Cinnamomum verum, Syzygium aromaticum, Laurus nobilis, Lippia berlandieri and Pimpinella anisum*) against *Cu. quinquefasciatus* larvae. In 2015, Brazilian researchers developed a nanoemulsion against 4th instar larvae of *Ae. aegypti* with *Rosmarinus officinalis* EO as an active component and mortality recorded after treatment with its 250 ppm was 80 and 90% after 24 and 48 hours respectively (Duarte et al. 2015). Previously, Prajapati et al. (2005) showed that non-emulsified *R. officinalis* EO had LD95 of 408 ppm after 24 hours of exposure which, in contrast, suggests the greater larvicidal efficacy of the nanoemulsion. Recently, Iranian researchers have published a work on inhibiting *Anopheles stephensi*, a major malaria vector in their country, through the action of *Artemisia dracunculus* (Asteraceae) derived nanoemulsion in EO. A nanoemulsion bioassay with LC_{50} or LC_{90} of 11.36 and 17.54 ppm was performed on 3rd and 4th larvae. P-allylanisole was reported as the main constituent of EO (Osanloo et al. 2017). Major nanoparticles synthesised by plant extracts are gold, silver, copper, copper oxide, palladium, platinum, titanium dioxide, zinc oxide, indium oxide, iron oxide, lead and selenium nanoparticles (Shah et al. 2015). Silver nanoparticles synthesis has been achieved using Kiwifruit juice, *Rumex hymenosepalus* extract, *Annona squamosa* leaf extract,

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

ZnS-based hybrid nanoemulsions concentration (ppm)	Per cent mortality (Mean ± S.D) (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 hours
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's multiple range test

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

ZnS-based hybrid nanoemulsions concentration (ppm)	Per cent mortality (Mean ± S.D) (n = 20)					Range of mortality (within hours)
	24 hr	48 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 Duncan's multiple range test

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

Podophyllum hexandrum leaf extract, extracts of *Acalypha indica Linn*., *Hibisicus cannabinus* leaf extract, *Macrotyloma uniflorum* seed extract (Vidhu et al. 2011). Zinc sulphide (ZnS) nanoparticles, synthesised by sono-chemical route employing zinc chloride and sodium sulphide, displayed significant anti-fungal property against the pathogenic yeast *Candida albicans* (MTCC 227) at a minimum fungicidal concentration of 300 µg/ml (Suyana et al. 2014). Supraja et al. (2016) have also reported antimicrobial efficacy of *Boswellia ovalifoliolata* stem bark-extract-mediated ZnO NPs. Magro et al. (2019) synthesised a novel photosensitizing hybrid nanomaterial based on chlorine-e6 and surface-active maghemite nanoparticles (SAMNs) with water self-assembly (SAMN@chlorin). A well-defined crystalline structure characterises the magnetic core, consisting of stoichiometric maghemite (π-Fe2O3). The most prominent feature of this nanomaterial is the ability to form stable colloidal suspensions in water without organic or inorganic coating to avoid aggregation and to reversibly and precisely bind organic molecules that form hybrid nanomaterials to biotechnology applications and has a photocidal potential against *Ae. aegypti*.

Chaithong et al. (2006) studied the morphological alterations by SEM in treated and control larvae of *Ae. aegypti* after exposure of ethanolic extracts of *Piper longum*. They observed that larvae become shrunken and anal papillae were damaged, probably led to their dysfunction and intrinsically related to death of larvae; similar observations have also been recorded in our present study. Kala et al. (2019) evaluated effect of cashew nut shell liquid (CSNL) nanoemulsion on morphological characters by scanning electron microscopy (SEM) in larvae of *Anopheles culicifacies* which revealed the adherence of nanodroplets to larval body resulting in complete larval deformation.

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, Mahmoud et al. (2019) studied larvicidal potential and histopathological changes induced after treatment of ethanolic extracts of *Piper nigrum*. The most characteristic disordered signs visualised in the midgut region of treated larvae were elongation and vacuolisation of epithelial cells, rupturing of the peritrophic membrane, detachment of basement membrane, disorganisation of microvilli, and furthermore, degeneration of muscle layer. Silva et al. (2008) reported histological changes in midgut of *Bacillus thuringiensis* var.*israelensis* (Bti) infected *Ae. albopictus* larvae, where disorganisation in intestinal cells, dilation and disintegration in rough endoplasmic reticulum with intense cytoplasmic vacuolisation was observed. Mishra et al. (2018) treated *Cx. tritaeniorhynchus* larvae 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. Kala et al. (2019) revealed 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.

Mishra et al. (2018) prepared permethrin nano-formulation against *Cx. tritaeniorhynchus* larvae and studied its effect on biochemical profiles. Glutathione S-transferase (GST), acetylcholine esterase (AChE) and acid and alkaline phosphatase assays were performed and decrease in cellular biomolecules and biomarker enzyme activity in the nanopermethrin-treated larvae as compared to bulk and control larvae was reported. Their results were substantiated with our results as activity of

digestive enzymes decreased which led to death of the larvae. In another study by Mishra et al. (2018) prepared neem-laced urea nanoemulsion (NUNE) and larvicidal potency was studied against *Ae. aegypti* and *Cx. tritaeniorhynchus* along with few biochemical components such as total protein, lipid, carbohydrate and biomarker enzymes like GST, acid and alkaline phosphatase activity. Treated larval samples were found to have decreased activity and larvicidal toxicity tests were corroborative with biochemical profiling obtained through biochemical analysis of treated samples.

Mdoe et al. (2014) tested the larvicidal efficacy of oil extracted from leaves of *Cryptomeria japonica* (Japanese cedar) against *An. gambiae* and observed that the rate of mortality increased with increasing dosages from 12.5 to 200 µg/ml. They also observed 31.75 to 100% mortality of larvae under laboratory, whereas in semi field conditions the per cent mortality was 17.75 to 99.5. The LC_{50} and LC_{90} values of *J. procera* against larvae of *An. arabiensis* were 14.42 and 24.65 mg/l under laboratory conditions and 24.51 and 34.21 mg/l under semi-field or simulated conditions, respectively (Karunamoorthi et al. 2014).

Govindarajan & Benelli (2016) demonstrated lvandulyl acetate cytotoxicity on three non-target mosquito predators, *Anisops bouvieri, Diplonychus indicus* and *Gambusia affinis* and its cytotoxicity was found to be 206, 336 and 534 µg/ml, respectively. Benelli et al.(2018) has investigated the effect of essential oil extracted from *Syzygium lanceolatum* leaves on non-target species such as *A.bouvieri*, *D.indicus, G.affinis* and *Poecilia reticulata*, the LC_{50} ranging from 4148 to 15762 µg/ml. Nanohybrid material based on chlorine-e6 and surface-active maghemite nanoparticles (SAMNs) with water self-assembly (SAMN@ chlorin) showed a great photocidal activity against *Ae. aegypti* and no adverse effects was observed in *Daphnia magna*. Hence this nanohybrid reduced environmental concerns and may be used as a safer alternative to conventional insecticides (Magro et al. 2019). Therefore, biosafety assessment of the formulated ZnS-based hybrid nanoemulsion against non-target species depicted minimal or no toxicity proves nontoxic behaviour of applied nanohybrid which portrays its environmental benevolence as an efficient and biosafe larvicidal agent.

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