

An investigation on behavioural, morphological and developmental changes induced by eucalyptus oil nanohybrid emulsions in larvae of *Aedes aegypti* (Linnaeus)

Komalpreet Kaur Sandhu^{1*} and Nisha Vashishat²

¹Department of Zoology, Akal University, Talwandi Sabo 151302, Punjab, India.

²Department of Zoology, Punjab Agricultural University, Ludhiana 141004, Punjab, India.

Email: komalpreetkaur903@gmail.com*

ABSTRACT: Zinc and copper sulphide-based hybrid nanoemulsions were synthesized using eucalyptus oil and lethal dosage of nanohybrids exposed to larvae of *Aedes aegypti*, to assess their effects on its development, behaviour and morphological features. The larvae showed retardation in development and late emergence of adult after treatment with metal sulphide hybrid nanoemulsions. Morphologically, intense damage to cuticle, pigmentation of gut, contraction and shrunken body was noted visually under light microscope. The larvae showed modified behaviour such as crankiness, restlessness, agitation and aggressiveness. These behavioural and developmental studies are of great significance in management of *Aedes* mosquito. © 2026 Association for Advancement of Entomology

KEY WORDS: Mosquito, metal sulfides, essential oil, nanoemulsions, vectors

INTRODUCTION

Mosquitoes (Diptera, Culicidae) cause a serious threat to humans throughout the world as they play a role of vectors which carry a number of devastating pathogens and parasites (Mishra *et al.*, 2017). *Aedes aegypti* (Linnaeus), commonly known as yellow fever mosquito, is most common vector that transmits several diseases such as Zika, Yellow fever, dengue and chikungunya (Sandhu *et al.*, 2023a). The prevalence of dengue has amplified greater than eight-fold during the last twenty years. In 2000, a total of 505,430 reported cases of dengue; this number increased to 2.4 million approximately by 2010 and reached 52 lacs by 2019 (WHO, 2023). Application of various synthetic insecticides such as Organophosphates (OPs),

Pyrethroids and treated nets etc. is the primary mosquito control method nowadays and is the only way to combat with mosquito-borne diseases (Dulacha *et al.*, 2022). These chemical measures posed mosquito resistance, resurgence, ecological problems as well as severe impacts on survival of non-target organisms (Sandhu *et al.*, 2023a). In context to this, plethora of scientific reports showed that there is a wide competence in botanical pesticides against mosquito vectors (Pavela, 2015). Plants contain aromatic and volatile components called essential oils and can be easily extracted. Moreover, due to their less toxic nature to non-target organisms make them an efficient control measure against broad range of insect pests (Elango *et al.*, 2009). *Eucalyptus* spp. belongs to family Myrtaceae had shown tremendous repellent effects to larvae

* Author for correspondence

of *Aedes* spp. (Cheng *et al.*, 2009). To improve the efficacy and stability of essential oils, Research in developing nations is mostly concentrated on green nanotechnologies (Kojom Foko *et al.*, 2021). Researchers also had shown a great deal of interest in metallic nanoparticles made using various metals that exhibit uncommon and wider chemical and physical properties in recent years (Sabri *et al.*, 2016). Therefore, in order to create metallic nanoparticles, several metals including iron, zinc, silver, gold, platinum, and titanium have been used. Out of these, silver nanoparticles have been studied more thoroughly and shown to have the ability to be more lethal to mosquito larvae, fungus, and bacteria (Ga'al *et al.*, 2018). Copper and zinc oxide nanoparticles prepared through green synthesis methods exhibited larvicidal efficacy against *Ae. aegypti* and also showed toxicity in cotton leafworm, *Spodoptera littorals* larvae and pupae (Shaker *et al.*, 2016; Selvan *et al.*, 2018). Apart from metal oxide nanoparticles, metal sulfides are considered as non-toxic types of metals (Wang *et al.*, 2013). Larvicidal potential of Copper sulfide (NCuS) and Zinc sulfide nanoparticles (NZnS) was evaluated against *Ae. aegypti*, with LC_{50} of 4.42 and 4.49ppm after 24 hours (Sandhu *et al.*, 2022; 2023a). To improve the efficacy and synergistic toxicity against *Ae. aegypti*, larvicidal potential of copper and zinc sulfide nanohybrids has also been prepared and revealed respective LC_{50} values of 5.00 and 7.63ppm after 24 hours of treatment (Sandhu *et al.*, 2023a, c). A study was undertaken with the synthesized eucalyptus oil emulsions and for their larvicidal efficiency (Sandhu and Vashishat, 2022). The effect of these nano composites on the behaviour of *Ae. aegypti* 4th instar was also studied.

MATERIALS AND METHODS

The research was conducted using effective copper and zinc sulfide-based hybrid nanoemulsions prepared from copper sulphide (CuS) and zinc sulphide (ZnS) nanoparticles in combination with eucalyptus oil nanoemulsion against *Ae. aegypti* larvae. The larvae were exposed to effective median lethal dosages of hybrid nanocomposite for twenty-four hours. The toxic impact of nanohybrids

was thoroughly studied. The larvae of *Ae. aegypti* were collected from different residential water collections from urban region of the District Ludhiana.

Aqueous nanoemulsions with non-polar oil, Tween and water were obtained after ultrasonication for 30 minutes. The emulsions having six percent non-polar eucalyptus oil and Tween 20 in different ratios (1:3, 1:2 and 1:1) prepared and checked for thermodynamic stability tests and larvicidal toxicity (Sandhu and Vashishat 2022). Stock solutions of metal sulfide nanoparticles i.e. NCuS and NZnS were prepared in 35.8 and 48.69ppm concentrations (Sandhu *et al.*, 2022; Sandhu *et al.*, 2023b). Afterwards, effective Copper sulphide nanoparticles (CuSNPs) and Zinc sulphide nanoparticles (ZnSNPs) were mixed with most stable eucalyptus oil nanoemulsion and whole formulation was sonicated to make metal nanohybrid or hybrid nanoemulsions (Sandhu *et al.*, 2023a, c). Different fractions of eucalyptus oil nanoemulsions and Zinc and Copper sulphide nanoparticles (1:1, 1:2, 1:3, 1:4, 1:5) were prepared from stock solutions and tested for stability tests.

The previous research has revealed the LC_{50} of aqueous eucalyptus oil nanoemulsion formulated with copper sulfide (NCuS) and zinc sulfide nanoparticles (NZnS) known as hybrid nanoemulsions (Sandhu *et al.*, 2023a, c). The calculated LC_{50} and LC_{90} values were obtained after treating with CuS based hybrid nanoemulsions in *Ae. aegypti* larvae were recorded as 5.00 and 5.81ppm, and with ZnS based hybrid nanoemulsions exposure were computed to be 7.63 and 9.22ppm. An experiment was conducted with 25 larvae and effective concentrations of nanohybrids for 24 hours. The larvae which survived were monitored until adult emergence. The developmental period and adult emergence were evaluated the underlying outcomes of prepared hybrid nanoemulsions. The experiment was performed in three replicates with each concentration. Simultaneously, larvae were also treated to median lethal dosages of CuS and ZnS based hybrid nanoemulsions for 24 hours in three replicates having 25 larvae each. The dead larvae were observed vigilantly under light

microscope for various morphological aberrations. Notable changes in head, thorax, abdomen and in siphon, antennae, eyes and mouth brushes were observed and compared with control larvae. The photomicrographs were taken using digital camera (Nikon DS-R11) attached to the microscope.

Effect of eucalyptus oil-based hybrid nanoemulsions on larval behavioral response:

The larvae were carefully monitored at specific time intervals followed by final observations at 24 hours. The changes in behavioural pattern i.e. wriggling speed, horizontal and vertical movements, aggression, aggregation, excitation, inability to rise up to the water surface, abnormal positions, convulsions and self-biting activity were noted. The larval behaviour symptoms were photographed with Nikon DSLR Digital Camera (Nikon D5200 with 18-55mm lens). In the same way, control larvae were also observed and compared with treated larvae.

RESULTS AND DISCUSSION

The prepared nanoemulsions were screened initially on the basis of certain parameters and various stability characteristics (Tables 1, 2). The most stable nanoemulsions were selected and larvicidal assays were performed visual appearance of synthesized oil nanoemulsions and hybrid nanoemulsions. The larvae exposed to LC_{50} and LC_{90} concentrations of CuS and ZnS based hybrid nanoemulsions, exhibited delayed development and reduced emergence of adult and it was dose-dependent. The development period was 5 days which increased significantly by 8.00 and 10.61 percent, 7.00 and 9.12 percent due to exposure of LC_{50} and LC_{90} dosages of CuS and ZnS nanoemulsions respectively. The adult emergence was 96-98 per cent in control, but it was only 10.21-12.66 percent at exposure of LC_{90} of CuS and ZnS nanoemulsions. Moreover, other increasing dosages affected the larval development significantly but more affect was noted in CuS based hybrid nanoemulsions.

Morphological observations of the larvae exposed with lethal concentrations of CuS and ZnS nanohybrids revealed ruptures in cuticle,

pigmentation, darkening or blackening of alimentary canal (Fig. 1d, 40X). However, in control larvae there was no alterations in larval external organs like head, eyes, antennae, thorax (Fig. 1a, b, 400X), abdominal scales, siphon with subventral or hair tuft (Fig. 1c, 400X) when observed under light microscope.

Essential oils extracted from various plants in combination with metallic nanoforms are considered to be safe and effective against mosquito larvae (Benelli and Mehlhorn, 2016). The previous findings showed that killing potential of ZnS and CuS-based hybrid nanoemulsions against *Ae. aegypti* larvae with a $LC_{50, 90}$ of 7.63, 9.22ppm and 5.00 and 5.81ppm respectively (Sandhu *et al.*, 2023a, c). The present investigation demonstrated the latent effects of ZnS and CuS nanohybrids of eucalyptus oil exposure resulted in dose-dependent delayed larval development and lessened adult emergence of treated larvae. Comparing CuS and ZnS based nanohybrids, the former had an enhanced and stronger effect. When *Ae. vagus* and *Ae. albopictus* larvae are treated with zinc oxide nanoparticles, resulting in slowed larval growth, indicating the prolonged effects of the nanoparticles (Gunathilaka *et al.*, 2021) and they also observed an increased duration of 13 days (at LC_{25}), 14.67 days (at LC_{50}), 16.67 days (at LC_{75}), and 17 days (at LC_{100}) in comparison to the control trial of developmental study (8 days), demonstrating the concentration-dependent impacts in present study (Gunathilaka *et al.*, 2021). In other studies, by Amrutha and John (2025) reported the effect of basil and turmeric essential oil nanoemulsions was quite toxic against eggs and adults of pests of rice.

ZnS and CuS-based hybrid nanoemulsions treated larvae showed behavioural changes as compared to the control sets. The control larvae displayed normal behaviour (Fig. 2a) and within one hour of hybrid nanoemulsion exposure, the larvae showed aggressive and restless activity with increased exposure times, i.e., an altered, excitable and abnormal movements, biting of own anal papillae, mouth parts preceding to the rounded or circular structures (Fig. 2b). It was found that these persistent actions and symptoms of the treated

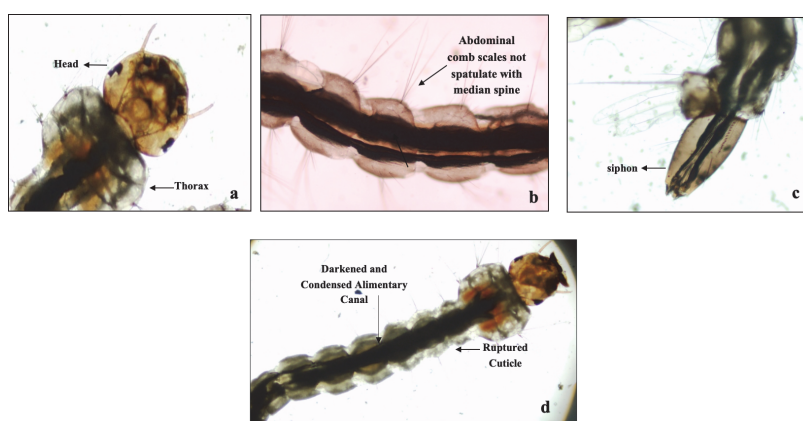


Fig. 1 Effect of copper and zinc sulphide nano hybrids on morphology of *Ae. aegypti* larvae

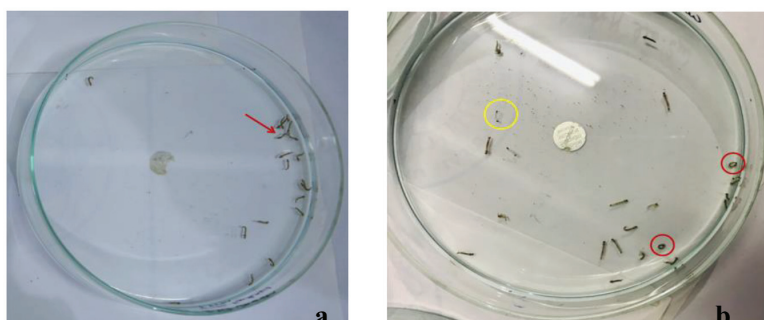


Fig. 2 Effect of copper and zinc sulfide nano hybrids on behaviour of *Ae. aegypti* larvae – a) Arrow showing control larvae showing normal movement and behaviour; b) Treated larvae - the red and yellow circles indicate aggressive and gill-biting behaviour and disappearance of the gut

larvae became more agitated and observed their twisting motions and various larvae showed vibration and paralytic movements. Moreover, cuticle layer of the copper-based hybrid nanoemulsion treated larvae got completely damaged as compared to zinc sulphide based nano hybrid. Despite the fact that several studies have shown that exposure to plant extracts causes behavioural changes in mosquito larvae, including agitated and excited behaviour, circular movements making ring shape structure, the impact of metal sulphide-based hybrid nanoemulsions as behaviour modifiers has not yet been evaluated. *Aedes aegypti* and *Cx. quinquefasciatus* larvae showed similar vibrating and paralytic symptoms after treatment of fungal isolates and appeared as neurobehavioral toxicant (Ragavendran *et al.*, 2019) which is similar with current study. The results are in contrast to previous reports on the larval behaviour of *Ae. aegypti* after treatment with *Achyranthes aspera* silver nanocomposites which reported that there were no noticeable behavioural changes in larvae except the induced mortality after

24 hours (Sharma *et al.*, 2022). The present study was in consistent with earlier findings of how extracts from *A. aspera* leaves and stems affect the behaviour of *Ae. aegypti* larvae (Sharma *et al.*, 2016), causing paralysis, convulsions, and aggression.

The structural analysis of *Ae. aegypti* larvae treated with CuS and ZnS nano hybrids formulated with eucalyptus oil nanoemulsions, revealed apparent effects, including gut darkening and disorganization, body shrinkage, ruptures in cuticle, contraction of anal papillae and a slight contraction of the internal membrane of anal papillae. These effects represented contact and systemic toxicity of nano hybrids affecting both larval cuticle and gut. Some studies have demonstrated that the toxic effect of green nanoparticles on the survival of *Ae. aegypti* larvae which is similar to the present study and previous reports (Sharma *et al.*, 2017, 2020). The coloured or pigmented gut content brought on by CuS and ZnS based nano hybrids exposure supports the theory that a broken gut may hinder

Table 1. Parameters for testing prepared aqua nanoemulsions of non-polar eucalyptus oil

Oil: Tween 20	Thermodynamic Stability	Optical transparency	Appearance	Phase separation
1:1	-	Poor	Milky	Yes
1:2	Most stable	Highest	Clear	No
1:3	-	Moderate	Turbid	Yes

Table 2. Parameters for testing prepared eucalyptus oil and copper sulphide and zinc sulphide based hybrid nanoemulsions

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

*Appearance of zinc sulfide based nanohybrids was dense to slight dense (1:1 to 1:4)

the survival of larvae. Some researchers have documented similar impacts of ZnO nanocomposites made by *Lobelia leschenaultiana* on the exterior structure of *Aedes aegypti* larvae (Banumathi *et al.*, 2017). While comparing with the current study, they also noted the larvae's significantly diminished abdomen area, as well as the removal of their mouth brushes and antennae. These behavioural and developmental related investigations are quite significant for controlling in management of *Aedes* mosquito. These hybrid nanoemulsions can be employed as a safe as well as efficient measure in controlling mosquitoes.

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