INTRODUCTION
Mosquitoes are key vectors for dissemination of various parasites and pathogens as they are haem feeders, thus drawing attention due to their medical importance. *Aedes aegypti* (Linnaeus) is one of the most significant species in the world. This mosquito can be identified by the lyre form marking on the upper surface of its thorax and white colouration on its legs. It originated in Africa and extensively dispersed in tropical and subtropical zones (Powell et al. 2018). This species is a carrier of Dengue Fever Virus (DENV) causing dengue fever, chikungunya fever, and dengue haemorrhagic fever (Yang et al. 2009). Dengue is an emerging disease which infects millions of people in the world (Goncalvez et al. 2007; WHO 2015). In over 128 nations, more than 3.9 billion people are at risk of dengue, with about 96 million cases annually (WHO 2017). This disease is endemic in all the states of India and major cause of hospitalisation (Kumar et al. 2018).

Therefore, the control of vector mosquito is an important public health concern (Magro et al. 2019) for ending epidemics in the tropical areas. Generally, synthetic insecticides like malathion and permethrin are sprayed to control the adult mosquitoes (Nwabor 2019). However, these chemicals have adverse effects on the non-target species, environment and human health (Margulis-Goshen et al. 2013). Further, a number of mosquito species have become resistant towards them (Wattanachai & Tintanon 1999). Control strategies at the larval stages are the most pertinent concept as far as their practical eradication is concerned. The control of larval stages is a strategy to tackle the resurgence in vectors, in more effective way (Wilke & Marrelli 2012). Moreover, environmental safety is considered most important. In order to be acceptable, an insecticide should also show its potential not to affect non-target species (WHO 2018) rather than only showing mortality of target species (Ghosh et al. 2012).

Nanotechnology has developed remarkably in the last decade and many innovative materials were created with a wide range of applications in medical, pharmaceutical, industrial, biotechnological and scientific fields. In recent years, there has been emphasis on the application of nanotechnology in insect pest management (Prabhakar et al. 2017). Nanoparticles were considered as a useful tool loaded with herbicides, fungicides, larvicides and targeting specific tissues in desired plants or insects to release their charge to desired part of the target animal to achieve desired results (Duhan et al. 2017). Furthermore, nanopesticide-based formulations have a potential and bright future for the development of more effective and safer biopesticide/pesticides (Deka et al. 2021).

On the nanotechnological front, studies on copper nanoparticles (Cu NPs) and copper oxide nanoparticles (CuO NPs) have been reported for their larvicidal, antibacterial, antifungal and antimicrobial properties. Valodkar et al. (2011) synthesised nanoparticles of 5–10 nm of silver and copper using latex from Euphorbiaceae. These nanoparticles have excellent antibacterial activity towards Gram positive and Gram negative bacteria. Ramyadevi et al. (2011) conducted a study on synthesis of copper nanoparticles by polyol process and showed their anti-larval and anti-parasitic properties.

Sulphide analogues of transition metals are hypothesised as one of the most detoxified forms of these metals (Wang et al. 2013; Sidhu et al. 2017). Various transition metals applied in various forms were detoxified in the environment by sulphidation. The eminent bio efficacy and detoxified nature of metal sulphide nanoparticles are drawing the attention of scientists for their use against vector control programmes.

**Larvicidal potential of copper sulphide nano aqua dispersions against *Aedes aegypti* (Linnaeus)**

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**African Entomology** is the journal of the Entomology Society of Southern Africa

**Nanotechnology has emerged as promising field in insect pest management. Aedes aegypti (Linnaeus) a well-known vector of dengue, chikungunya, and dengue haemorrhagic fever has no commercial management practice for their eradication at the larval stage. In the present study, copper sulphide one of the most detoxified form of copper with biopotent properties was synthesised by standard methodology using sonochemical irradiation method and was evaluated for their larvicidal potential against Ae. aegypti. Treated larvae were observed for various morphological changes as compared to control. Larvae were most susceptible to CuSNPs at 7 ppm showing 100% mortality within 24 h. LC50 and LC90 values calculated with the help of POLO software were 4.42 and 5.73 ppm. The epithelium layer of treated larvae was damaged as compared to control. Remarkable results of copper sulphide nanoformulations at low dosage against Ae. aegypti larvae advocates their further exploration for vector control programmes.**
a variety of environmental menaces. Copper sulphide (CuS) is one of the most detoxified forms of copper (Baek 2017; Back & Kim 2017; Guo et al. 2013; Wang et al. 2013), natural, thermally stable (Rao et al. 2016), water insoluble (Guo et al. 2013) still retaining the biopotential of copper (Chakraborty et al. 2016; Prabhavathi et al. 2015). It is quite safe and non-toxic to humans (Guo et al. 2013) and therefore may be used in the environment against vector mosquitoes without causing harm to non-target species. There is limited literature on synthesis and biopotential of water-dispersed CuS nanoparticles which prompted us to prepare the novel surface protected aqueous nano formulations of copper sulphide for the evaluation of larvicidal potential against Ae. aegypti. The possible morphological alterations of larvae by NCuS are also given in the paper.

MATERIALS and METHODS

Synthesis of copper sulphide nano-aqua dispersions (NCuS)

Copper nitrate trihydrate (Cu(NO_3)_2.3H_2O) solution (0.001 M, 30 ml), prepared in ethylene glycol, was added dropwise to 30 ml of sodium sulphide aqueous solution (0.001 M), during sonication, along with a pinch of cetyltrimethyl ammonium bromide (CTAB) (surfactant) (Sidhu et al. 2017). This reaction mixture was subjected to ultrasonic irradiation at temperature 40 ± 25 °C having pulse of 05 on and 01 off and amplitude of 60%. The solution was irradiated with microwaves for 30 sec and allowed to cool down at room temperature. An amount of 0.3 g of capping agent, polyvinyl pyrrolidone (PVP) dissolved in 20 ml distilled water was added to the above prepared solution during sonication, which was continued for another 15 min, to get surface stabilised copper sulphide nanoparticles (NCuS). The prepared NCuS (0.0005 M, 35.85 µg/ml) were retained as such as stock solution, which was further diluted by adding autoclaved water, as and when required.

Morphological characterisation of prepared NCuS solution

The morphology and size of nanoparticles were recorded in a Hitachi Transmission Electron Microscope Hi-7650 at an accelerated voltage of 200 kV by casting a drop of particle solution onto a 200-mesh carbon coated copper grid from EMN Laboratory, Punjab Agricultural University, Ludhiana.

Mosquito larvae collection

Water samples were collected in the monsoon season from different peri-domestic fresh water collections like desert coolers, earthen pots, fire buckets, water tanks, plates under pots and roadside ditches in different zones of Punjab Agricultural University Ludhiana district (30.9010 °N and 75.8573 °E) using plastic dippers from June to August 2019. From the various types of mosquito larvae present in the collected water samples, Ae. aegypti larvae were recognised from the other types of mosquito larvae (if present) on the basis of their morphological features by following the standard keys given by Becker et al. (2010) and Bar & Andrew (2013). All mosquito larvae were adequately fed with a mixture of dog biscuits and yeast ground in the ratio of 3:1 (2 mg/100 ml) as used by Mavundza et al. (2013).

Larvicidal bioassay

Varying concentrations of the NCuS (0.0005 M, 35.85 µg/ml) at 5, 6, 7 and 8 ppm were prepared by diluting it with de-chlorinated water. Twenty 4th-instar Ae. aegypti larvae were exposed to these concentrations in triplicate. In addition, a vehicle-control and a control set with PVP and only dechlorinated water respectively were run in triplicate. The experimental sets were kept in a Bio-Oxygen Demand incubator at 26 ± 2 °C. Mortality of larvae was recorded in copper sulphide nano-aqua dispersions treated, vehicle control and control sets after 3, 6, 12 and 24 h. Treated larvae were observed under a microscope for various outer morphological changes.

Statistical analysis

Statistical analysis was performed by comparing the mortality data recorded from NCuS treated sets with control and vehicle control sets by using ANOVA (Duncan multiple range test) with the help of SPSS software version 16.0. LC₅₀ and LC₉₀ values after 24 h post-exposure, were worked out by log probit technique (Finney 1952) employing the POLO programme (Robertson et al. 1980).

RESULTS

Surface protected copper sulphide nanoparticles (NCuS) in aqua-dispersed form were synthesised by sonochemical irradiation method. Cu²⁺ ions from Cu(NO_3)_2 in ethylene glycol (HOCH₂CH₂OH) solution were made to combine with S²⁻ from sodium sulphide (Na₂S) solution, while sonication, forming copper sulphide nanoparticles, which were stabilised using stabilising agent PVP.

Characterisation of copper sulphide nano-aqua dispersions

The topo-morphological characters and particles size of synthesised surface protected copper sulphide nanoparticles (CuS NPs) in aqua-dispersed form by Transmission Electron Microscopy (TEM). Shape analogy indicated from TEM morphograph showed distorted spherical to oval shapes of CuS nanoparticles with slight aggregations. The average particle size was 16 nm (Figure 1a) and range of CuS nanoparticles size is from 1 to 29 nm (Figure 1b).

Figure 1. a) Particle size of NCuS analysed by TEM, b) Histogram showing NCuS particles size range calculated by line intercepts varying from 1 to 29 nm
Copper sulphide nanoparticles were tested for their larvicidal potential against *Ae. aegypti* at 5, 6, 7 and 8 ppm to assess their larvicidal potential. With the exposure of 5 ppm of copper sulphide nanoparticles (NCuS), the percent mortality was observed as 20.00 ± 5.00 after 3 h and 36.67 ± 2.89, 45.00 ± 5.00, 75.00 ± 5.00, 96.67 ± 2.89 after 6, 12, 24 and 48 h, respectively.

Whereas at 6 ppm percent mortality increased, i.e. 40.00 ± 5.00, 45.00 ± 5.00, 80.00 ± 5.00, 90.00 ± 5.00 and 100.00 ± 0.00 after 3, 6, 12, 24 and 48 h respectively (Table 1) which is statistically significant. With the treatment of 7 and 8 ppm of NCuS 100% mortality was observed within 24 h while 50.00 ± 0.00, 65.00 ± 5.00 and 80.00 ± 5.00 and 55.00 ± 5.00, 70.00 ± 5.00 and 91.67 ± 2.89% mortality was observed after 3, 6 and 12 h respectively. Statistically there is no relationship between percent mortality at 7 and 8 ppm of NCuS. No larval mortality was recorded in the untreated control and vehicle control sets. During the present study, larvae were most susceptible to CuS NPs at 7 ppm and it was found to be the most effective concentration out of the four tested concentrations, as it resulted into 100% larval mortality within 24 h or before their conversion to the next developmental stage, i.e. pupae. No mortality was recorded in control and vehicle-control sets.

The values for LC$_{50}$ and LC$_{90}$ of copper sulphide nanoparticles after 24 h of post-exposure against fourth instar larvae of *Ae. aegypti* was calculated to be 4.42 ppm and 5.73 ppm (Table 2) respectively by the log probit technique. Exposure to effective copper sulphide nanoparticles resulted in destruction of the outer epithelial layer of a larva while an untreated larva was intact and normal (Figure 2). There was disappearance of the gut in treated larvae while it is present in the control larva.

**DISCUSSION**

Copper nanoparticles (CuNPs) are considered to have antifungal, antibacterial and antiparasomal potential; so we intend to evaluate if they could be applied as mosquito larvicidal agents. Previous studies demonstrated that bulk copper particles were less efficient as compared to CuNPs against fungal and bacterial infections (Rai et al. 2018). To the best of the authors’ knowledge, it is the first report on the use of copper sulphide nanoparticles as larvicidal agents against *Ae. aegypti*. No previous report has been documented about copper sulphide nano aqua dispersions as larvicidal agents against any of the mosquito species, but several nanoparticles such as silver (Yadav & Prakash 2016), gold (Soni & Prakash 2012), palladium (Minal & Prakash 2018), copper (Ramyadevi et al. 2011), zinc (Vijayakumar et al. 2015), silica and carbon (Murugan et al. 2016) nanoparticles, were exemplified to be the best candidates for mosquito control.

The appreciable effective larvicidal concentration is 7 ppm in our results which is much lower than the previously reported concentration for other metal nanoparticles against *Ae. aegypti*. No previous report has it is the first report on the use of copper sulphide nanoparticles against 4th instar larvae of *Ae. aegypti*. After 24 h of post-exposure against fourth instar larvae of *Ae. aegypti*, the percent mortality was calculated by line intercepts varying from 1 to 29 nm (Figure 1). The appreciable effective larvicidal concentration is 7 ppm in our results which is much lower than the previously reported concentration for other metal nanoparticles against *Ae. aegypti*. No previous report has it is the first report on the use of copper sulphide nanoparticles against 4th instar larvae of *Ae. aegypti*.

**Table 1.** Effect of different concentrations of copper sulphide nano-aqua dispersions on mortality of 4th instar larvae of *Ae. aegypti*.

| CuS NPs concentration (ppm) | 3 h       | 6 h       | 12 h      | 24 h      | 48 h      | Range of mortality (within hours) |
|-----------------------------|-----------|-----------|-----------|-----------|-----------|----------------------------------|
| 5                           | 20.00 ± 5.00$^a$ (3–5) | 36.67 ± 2.89$^b$ (7–8) | 45.00 ± 5.00$^c$ (8–10) | 75.00 ± 5.00$^c$ (14–16) | 96.67 ± 2.89$^b$ (19–20) | 3–48                             |
| 6                           | 40.00 ± 5.00$^a$ (7–9)  | 45.00 ± 5.00$^a$ (8–10) | 80.00 ± 5.00$^a$ (15–17) | 90.00 ± 5.00$^a$ (17–19) | 100.00 ± 0.00$^b$ (20) | 3–48                             |
| 7                           | 50.00 ± 0.00$^b$ (12–14) | 65.00 ± 5.00$^b$ (12–14) | 80.00 ± 5.00$^b$ (15–17) | 100.00 ± 0.00$^b$ (20) | – | 3–24                             |
| 8                           | 55.00 ± 5.00$^b$ (10–12) | 70.00 ± 5.00$^b$ (13–15) | 91.67 ± 2.89$^b$ (18–19) | 100.00 ± 0.00$^b$ (20) | – | 3–24                             |
| 0 (Control)                 | 0.00 ± 0.00$^d$ (0) | 0.00 ± 0.00$^d$ (0) | 0.00 ± 0.00$^d$ (0) | 0.00 ± 0.00$^d$ (0) | 0.00 ± 0.00$^d$ (0) | –                                |
| 0 (Vehicle-control)         | 0.00 ± 0.00$^d$ (0) | 0.00 ± 0.00$^d$ (0) | 0.00 ± 0.00$^d$ (0) | 0.00 ± 0.00$^d$ (0) | 0.00 ± 0.00$^d$ (0) | –                                |

$^a$n represents number of larvae taken.

Figures followed with different superscripts indicate significant difference (P < 0.05) with respect to control and vehicle-control sets by using Duncan multiple range test.

![Figure 2. Effect of copper sulphide nano-aqua dispersions treatment on the outer morphology of *Ae. aegypti* larva. a) Control larva, b) treated larva (20×).](image-url)

![Table 2. Toxicity values of copper sulphide nano-aqua dispersions against 4th instar larvae of *Ae. aegypti.*](image-url)

| Toxicity value (ppm) | Fiducial limits | $\chi^2$ |
|----------------------|----------------|---------|
| LC$_{50}$ = 4.42     | 2.78 – 4.92    | 2.089   |
| LC$_{90}$ = 5.73     | 5.26 – 6.73    | 2.089   |

Figures in parenthesis indicate the range in number of dead larvae from the start of experiment till that period.
studies like silver doped copper oxide nanosheets had mortality effects at 300 ppm concentration against larvae of *Spodoptera littoralis* (Atwa et al. 2017). In another study, Shaker et al. (2016) synthesised copper oxide nanoparticles used in control and management of cotton leafworm (*S. littorals*) as an example for the pest which attacks large scale of crops and vegetables by applying six different concentrations 1000, 500, 250, 125, 62.5 and 31.25 mg/L. Treated larvae showed 100% mortality with LC₅₀ of 62.5 mg/l which is different from our results and they reported significant impact on the biological features of the insect as well. Minal & Prakash (2019) tested the larvicidal efficacy of *Azadirachta indica* leaf extract mediated bimetallic nanoparticles. They concluded that monometallic forms of nanoparticles, i.e. copper and zinc alone showed less larvicidal efficacy as compared to copper-zinc bimetallic nanoparticles against *Culex quinquefasciatus*. Copper oxide nanoparticles were synthesised using seed of *Artocarpus heterophyllus* and proved them as an anti-larval application (Mishra et al. 2015). Selvan et al. (2018) also synthesised copper oxide nanoparticles using a leaf extract of *Tridax procumbens* and proved them to have larvicidal properties against *Ae. aegypti*. Suresh et al. (2020) demonstrated that metal oxide nanoparticles act as potential mosquitoicidal agents and also reported by Gayathri et al. (2018) that magnesium oxide (MgO) nanoparticles had efficient larvicidal potential against *Ae. aegypti*. Anopheles stephensi and *Culex quinquefasciatus*. 

Sidhu et al. (2017) reported in vitro anti-fungal evaluation at 7 μg/ml of copper sulphide nanoformulations against *Alternaria alternata*, *Drechslera oryzae* and *Curvularia lunata*. They found the average particle size was 12 nm and range of size of CuS NPs was 10–30 nm which was at par with of with our results. Analogous studies were carried out by Naik et al. (2014) in which copper oxide nanoparticles were synthesised using a leaf extract of *Gloriosa superba*. Scanning electron microscopic (SEM) images indicate that they have particle size in the range of 5–10 nm which is smaller than observed in the present study. In another study, Lee et al. (2013) synthesised Cu nanoparticles of 37–110 nm using *Magnolia kobus* leaf extract which was greater in size as compared to present study and have shown antibacterial activity against *Escherichia coli*. 

The nanoparticles entered the larval body through the tracheal system. In this study, various morphological alterations such as damaged epithelium and gut disappearance were observed. These were analogous to the findings of Kaur et al (2019) in which nanomulsion of *E. globulus* affected the cuticle layer of the abdomen of larvae of *Ae. aegypti*. Such morphological changes in the form of distortion and damage in various body parts of larvae treated with copper sulphide nano-formulations results in larval mortality and could be used as part of a mosquito control programme.

Copper sulphide is natural as well as non-toxic and has been reported to be metabolised in the human body to cure various ailments like cancer and atherosclerosis (Guo et al. 2013). Moreover, copper and sulphur belong to essential micro- and macro-nutrients for the living kingdom which presents that these copper sulphide nanoformulations are somewhat nontoxic. The remarkable mortality of *Ae. aegypti* larvae at a low dosage of CuS NPs in the present study, advocates their possible use in the vector control strategies and further research of CuS NPs in the field along with biosafety tests could lead to a very promising avenue for vector control.

**ACKNOWLEDGEMENTS**

The authors are grateful to the Head, Department of Zoology, for providing all the necessary facilities, and Department of Science and Technology, Government of India, New Delhi, for providing infrastructural facilities under FIST grant.

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