**Single-step novel biosynthesis of silver nanoparticles: A potent and eco-friendly mosquitocides**

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**ABSTRACT**

Mosquito-borne diseases such as malaria, dengue, chikungunya, filariasis, and Japanese encephalitis cause thousands of deaths per year. Mosquito control is to enhance the health and quality of life of county residents and visitors through the reduction of mosquito populations. Mosquito control is of serious concern in developing countries like India due to the lack of general awareness, development of resistance, and socioeconomic reasons. Insecticides of botanical origin may serve as suitable alternative biocontrol techniques in the future. In view of the recently increased interest in developing plant origin insecticides as an alternative to chemical insecticide, in this research, we biosynthesized silver nanoparticles (AgNPs) using the *Sida acuta* leaf extract as reducing and stabilizing agent. The biosynthesis of AgNP was confirmed analyzing the excitation of surface plasmon resonance using ultraviolet-visible spectrophotometry. Scanning electron microscopy and transmission electron microscopy showed the clustered and irregular shapes of AgNP. The presence of silver was confirmed by energy-dispersive X-ray spectroscopy. Fourier transform infrared spectroscopy investigated the identity of secondary metabolites, which may also act as AgNP capping agents. The acute toxicity of *S. acuta* leaf extract and biosynthesized AgNP was evaluated against larvae of *Aedes aegypti*, *Anopheles stephensi*, and *Culex quinquefasciatus*. Compared to the leaf aqueous extract, biosynthesized AgNP showed higher toxicity against *A. aegypti*, *A. stephensi*, and *C. quinquefasciatus* with the LD₅₀ values of 35.12, 39.53, and 41.44 µg/mL, respectively. This result suggests that the leaf extract has the potential to be used as an ideal eco-friendly approach for the control of vector mosquitoes.

**KEY WORDS:** Green synthesis, Mosquito adulticide, nanoparticles, scanning electron microscopy, *Sida acuta*, transmission electron microscopy

**INTRODUCTION**

Mosquitoes constitute a major public health problem as vectors of serious human diseases malaria, filariasis, Japanese encephalitis, dengue fever, chikungunya, and yellow fever that cause substantial mortality and morbidity among people living in tropical and subtropical zones (Jang *et al.*, 2002). Essential oil, nanoparticles, and aqueous extracts from plants may be alternative sources of mosquito control agents because they constitute a rich source of bioactive compounds that are biodegradable into nontoxic products and potentially suitable for use to control mosquitoes. Insecticides of botanical origin may serve as suitable alternative bio control techniques in the future. Mosquito control is being strengthened in many areas, but there are significant challenges, including an increasing mosquito resistance to insecticides and a lack of alternative, cost-effective, and safe insecticides. Increasing insecticide resistance requires the development of strategies for prolonging the use of highly effective vector control compounds (Govindarajan *et al.*, 2008). The use of combinations of multiple insecticides and phytochemicals is one such strategy that may be suitable for mosquito control. Thus, attempts to develop novel materials as mosquitocides are still necessary. With the progress of nanotechnology, many laboratories around the world have investigated silver nanoparticle (AgNP) production (Murugan *et al.*, 2012).

Among 53 anopheline species present in India, 9 are vectors of malaria. In India, malaria is still the most important
cause of morbidity and mortality with approximately two to three million new cases arising every year (Sharma et al., 2009). Malaria mortality rates have fallen by 47% globally since 2000 and by 54% in the African region. Most deaths occur among children living in Africa, where a child dies every minute from malaria. Malaria is caused by Plasmodium parasites, vectored to people through the bites of infected Anopheles mosquitoes, which bite mainly between dusk and dawn (Jensen and Mehlhorn, 2009).

Aedes mosquitoes are painful and persistent biters. Aedes aegypti is responsible for spreading dengue and chikungunya. Dengue is prevalent throughout the tropics and subtropics. The World Health Organization estimates that around 2.5 billion people are at risk of dengue. Infections have dramatically increased in recent decades due to increased urbanization, trade, and travel (WHO, 2014). Culex is a genus of mosquito which serves as vectors of important diseases, such as West Nile virus, filariasis, Japanese encephalitis, St. Louis encephalitis, and avian malaria. Culex quinquefasciatus is responsible for transmitting the filarial nematode, Wuchereria bancrofti. More than 1.3 billion people in 72 countries worldwide are threatened by lymphatic filariasis, commonly known as elephantiasis. Over 120 million people are currently infected, with about 40 million disfigured and incapacitated by the disease (WHO, 2012).

Recently, a growing number of plant-borne compounds have been reported as excellent toxics against mosquitoes, acting as adulticidal, larvicidal, ovicidal, growth and/or reproduction inhibitors, and/or adult repellents (e.g., Govindarajan and Sivakumar, 2014; Veerakumar et al., 2013; 2014a). In recent years, the biosynthesis method using plant extracts has received more attention than chemical and physical methods and even more than the use of microbes, for the nanoscale metal synthesis, due to the absence of any requirement to maintain an aseptic environment. Nanoparticles have attracted considerable attention because of their various applications. Use of plant extract for the synthesis of nanoparticles could be advantageous over other environmentally benign biological processes because it eliminates the elaborate process of maintaining cell cultures. Recently, green AgNPs have been synthesized using various natural products like Feronia elephantum (Veerakumar et al., 2014b).

The larvicidal and repellent properties of essential oils from various parts of four plant species Cymbopogon citrates, Cinnamomum zeylanicum, Rosmarinus officinalis, and Zingiber officinalis against Culex tritaeniorhynchus and Anopheles subpictus (Govindarajan et al., 2011). The larvicidal activities of myco synthesized AgNPs against vectors and responsible for diseases of public health importance have been evaluated (Salunkhe et al., 2011). The toxicity of mosquito larvicidal activity of leaf essential oil and their major chemical constituents from Ocimum basilicum were evaluated against C. tritaeniorhynchus, Aedes albopictus, and An. subpictus (Govindarajan et al., 2013).

The larvicidal activity of AgNPs synthesized using F. elephantum plant leaf extract against late third instar larvae of Anopheles stephensi, A. aegypti, and C. quinquefasciatus (Veerakumar et al., 2014b). The adulticidal and repellent activities of crude hexane, ethyl acetate, benzene, chloroform, and methanol extracts of leaf of Eclipta alba and Acalypha paniculata were assayed for their toxicity against two important vector mosquitoes, viz., C. quinquefasciatus and A. aegypti (Govindarajan and Sivakumar 2012). The larvicidal efficacy of the crude leaf extracts of Ficus benghalensis, with three different solvents such as methanol, benzene, and acetone, were tested against the early second, third, and fourth instar larvae of C. quinquefasciatus, A. aegypti, and A. stephensi (Govindarajan, 2010).

The adulticidal activity of AgNPs synthesized using Heliotropium indicum plant leaf extract against adults of A. stephensi, A. aegypti, and C. quinquefasciatus (Veerakumar et al., 2014c). Sida is an erect perennial shrub found throughout the hotter parts of India and Nepal. The genus name Sida is from the Greek for “pomegranate or water lily,” Tamil name Vatta thiruthi. Carl Linnaeus adopted the name from the writings of Theophrastus. Many Sida are attractive to butterflies and moths. Arrow leaf sida, for example, is a larval host for the Tropical Checkered Skipper (Pyrgus oileus). These are annual or perennial herbs or shrubs growing 20 cm (7.9 in) to two meters (6 ft 7 in) tall. Most species have hairy herbage. The leaf blades are usually unlobed with serrated edges, but may be divided into lobes.

They are borne on petioles and have stipules. Flowers are solitary or arranged in inflorescences of various forms. There are many stamens and a style divided into several branches. The fruit is a disc-shaped schizocarp up to two centimeters (0.79 in) wide which is divided into five to 12 sections, each containing a seed. It is used for various medicinal purposes such as liver disorders, diuretic and abortifacient, in ayurvedic preparations, asthma, fever, headache (migraine), cough, cold, ulcer, anthelmintic, snake bite, urinary diseases, female disorders, antifertility agents, and sedative (Kirtikar et al., 1993). In this research, we reported a method to biosynthesize AgNPs using the
aqueous leaf extract of the Sida acuta, a cheap and eco-
friendly material acting as reducing and stabilizing agent.
AgNPs were characterized by ultraviolet-visible (UV-Vis)
spectrophotometry, Fourier transform infrared (FT-IR)
spectroscopy, scanning electron microscopy (SEM),
energy-dispersive X-ray analysis (EDX), and transmission
electron microscopy (TEM). In the research evaluated,
the aqueous extract of S. acuta and the biosynthesized
AgNPs were tested for their adulticidal potential against
the malaria vector A. stephensi, the dengue vector A. aegypti
and the Japanese encephalitis vector C. quinquefasciatus.

MATERIALS AND METHODS

Materials

Silver nitrate was procured from Merck, India. The
glassware was acid-washed thoroughly and then rinsed
with Millipore Milli-Q water. Healthy and fresh leaves of
S. acuta (Figure 1) were collected from Annamalai Nagar
area, Tamil Nadu State, India. The identity was confirmed
at the Department of Botany, Annamalai University,
Annamalai Nagar, Tamil Nadu. Voucher specimens were
numbered and kept in our laboratory and were available
on request.

Preparation of Plant Extract

Leaves of S. acuta were dried in the shade and ground to
fine powder in an electric grinder. The aqueous extract was
prepared by mixing 50 g of dried leaf powder with 500 mL
of water (boiled and cooled distilled water) with constant
stirring on a magnetic stirrer. The suspension of dried
leaf powder in water was left for 3 h and filtered through
Whatman No. 1 filter paper, and the filtrate was stored
in an amber-colored airtight bottle at 10°C temperature
until testing (Veerakumar et al., 2013).

Figure 1: Sida acuta

Biosynthesis and Characterization of AgNPs

The broth solution of fresh leaves was prepared by taking
10 g of thoroughly washed and finely cut leaves in a 300 mL
Erlenmeyer flask along with 100 mL of sterilized double-
distilled water and then boiling the mixture for 5 min
before finally decanting it. The extract was filtered with
Whatman filter paper No. 1, stored at −15°C and tested
within a week. The filtrate was treated with aqueous 1 mM
AgNO₃ (21.2 mg of AgNO₃ in 125 mL of Milli-Q water)
solution in an Erlenmeyer flask and incubated at room
temperature. 88 mm of an aqueous solution of 1 mM
silver nitrate was reduced using 12 mL of leaf extract at
room temperature for 10 min, resulting in a brown-yellow
solution indicating the formation of AgNP.

The bioreduction of Ag⁺ ions was monitored using
UV-visible spectrophotometer (UV-160v, Shimadzu,
Japan). Analysis of size, morphology, and composition of
AgNP was performed by SEM (Hitachi S3000 H SEM),
TEM (TEM Technite 10 Philips) and EDX. The purified
AgNP was examined for the presence of biomolecules
using FT-IR spectrum (Thermo Scientific Nicolet 380 FT-
IR Spectrometer) KBr pellets.

Mosquito Rearing

Laboratory-bred pathogen-free strains of mosquitoes were
reared in the vector control laboratory, Department of
Zoology, Annamalai University. At the time of adult feeding,
these mosquitoes were 3-4 days old after emergences
(maintained on raisins and water) and were starved for
12 h before feeding. Each time, 500 mosquitoes per cage
were fed on blood using a feeding unit fitted with Parafilm
as membrane for 4 h. A. aegypti feeding was done from
12 noon to 4.00 p.m. and A. stephensi and C. quinquefasciatus
were fed during 6.00 p.m. to 10.00 p.m. A membrane
feeder with the bottom end fitted with Parafilm was placed
with 2.0 ml of the blood sample (obtained from a slaughter
house by collecting in a heparinized vial and stored at 4°C)
and kept over a netted cage of mosquitoes. The blood was
stirred continuously using an automated stirring device,
and a constant temperature of 37°C were maintained
using a water jacket circulating system. After feeding, the
fully engorged females were separated and maintained on
raisins. Mosquitoes were held at 28°C ± 2°C, 70-85%
relative humidity, with a photoperiod of 12 h light and
12 h dark.

Adulticidal Experiment

Adulticidal bioassay was performed by the WHO method
(1981). Based on the wide range and narrow range tests,
the aqueous crude extract was tested at 40, 80, 120, 160, and 200 μg/mL concentrations and AgNPs were tested at 8, 16, 24, 32, and 40 μg/mL concentrations. Aqueous crude extract and AgNPs were applied on Whatman no. 1 filter papers (size 12 cm × 15 cm). Control papers were treated with silver nitrate and distilled water. Twenty female mosquitoes were collected and gently transferred into a plastic holding tube. The mosquitoes were allowed to acclimatize in the holding tube for 1 h and then exposed to test paper for 1 h. At the end of the exposure period, the mosquitoes were transferred back to the holding tube and kept 24 h for the recovery period. A pad of cotton soaked with 10% glucose solution was placed on the mesh screen. Each test included a set control groups (silver nitrate and distilled water) with five replicates for each individual concentration.

Data Analysis

Mortality data were subjected to probit analysis. LD_{50} and LD_{90} were calculated using the method by Finney (1971). All data were analyzed using the SPSS Statistical Software Package version 16.0. A probability level of P < 0.05 was used for the significance of differences between values.

RESULTS AND DISCUSSION

Biosynthesis and Characterization of AgNPs

While silver nitrate was added to the *S. acuta* leaf extract, the formation of AgNP occurred and color changed from yellowish to dark brown (Figure 2a). The intensity of color was directly proportional to the formation of AgNP. The color change was rapid, and as soon as the two solutions were mixed, the solution turned brown within 10 min. This color change was due to the reduction of Ag^+ to Ag^0 by various biomolecules present in the leaf extract. The UV-Vis absorption spectrum was reported in Figure 2b; an intense, broad absorption peak was observed at 449 nm because of surface plasmon resonance (SPR). SPR peak is sensitive to the size and shape of the nanoparticles, amount of extract, silver nitrate concentration, and the type of biomolecules present in the leaf extract (Singh et al., 2010; Zargar et al., 2011). The AgNP was observed as stable in solution and also showed little aggregation. Besides, the plasmon bands were broadened with an absorption tail in longer wavelengths; this could be related to the size distribution of nanoparticles (Ahmad et al., 2003). The FT-IR spectrum of biosynthesized AgNP by using *S. acuta* leaf extract is shown in Figure 3. It showed main bands at O-H group (1269.92/cm), C=N stretch (1486.57), -NH$_2$ (1636.98), =NH (2332.25), -H stretch (2358.27), and O-H stretch (3345.57).

In particular, the band at 3345/cm corresponded to O-H, as also the H-bonded alcohols and phenols. Shanmugam et al. (2014) suggested that these bonds could be due to the stretching of -OH in proteins, enzymes or polysaccharides present in the extract. The peak at 2358/cm indicated carboxylic acid (Li et al., 2007). Shoulder peaks at 1636/cm indicated that the amide I and amide II arise due to carbonyl and -NH stretch vibrations in the amide linkages of the proteins. The band at 1269/cm corresponded to C=C stretching of the aromatic amine. Overall, the immediate reduction of silver ions in the present investigation seems linked with the presence of water-soluble phytochemicals such as flavones, quinones, and organic acids present in the leaves of *S. acuta*.

A representative SEM micrograph (Figure 4a) of AgNP showed that nanoparticles were mostly spherical or with cubic structures. We also noted that “capped” AgNPs were stable in solution for at least 8 weeks. The EDX analysis provided information on the chemical analysis of the fields being investigated or the composition at specific locations (spot EDX). Figure 4b shows a representative profile of
the spot EDX analysis, obtained by focusing on AgNP. As a general trend, the particle shape of plant-mediated AgNP was spherical, with the exception of some neem-synthesized AgNP. They are polydisperse, with spherical or flat, plate-like, morphology, and mean size range of 5-35 nm in size (Shankar et al., 2004). Furthermore, SEM images of AgNP fabricated using Emblica officinalis were also predominantly spherical with an average size of 16.8 nm ranging from 7.5 to 25 nm (Ankamwar et al., 2005).

Moreover, Figure 5 shows the TEM of AgNP synthesized using S. acuta leaf extract. Among shapes, spheres, triangle, truncated triangles, and decahedral morphologies dominated and ranged from 32 to 45 nm with an average size of 38 nm. Most of the AgNP was roughly circular in shape with smooth edges. In agreement with these findings, AgNP from Annona squamosa leaf extract were spherical in shape with an average size ranging from 20 to 100 nm (Vivek et al., 2012) while Thirunavokkarasu et al. (2013) reported spherical nanoparticles with size ranging from 8 to 90 nm using Desmodium gangeticum as reducing agent. Our TEM images also showed that the surfaces of the AgNPs were surrounded by a black thin layer of some material, which might be due to the capping organic constituents of the plant broth as previously highlighted by Rafiuddin (2013).

**Adulticidal Potential against Mosquito Vectors**

In laboratory conditions, the S. acuta aqueous leaf extract showed adulticidal properties against A. stephensi, A. aegypti, and C. quinquefasciatus; LC50 values were 157.88, 164.89 and 177.48 μg/ml, for A. stephensi, A. aegypti, and C. quinquefasciatus, respectively (Table 1). Recently, a growing number of plant extracts have been found effective against C. quinquefasciatus larvae (e.g. Govindarajan et al., 2013; Veerakumar et al., 2014; 2013; Sivakumar and Govindarajan, 2013).

Furthermore, the S. acuta-synthesized AgNPs were highly toxic against A. stephensi, A. aegypti, and C. quinquefasciatus adults; LC50 values were 35.12, 39.53, and 41.44 μg/ml, A. stephensi, A. aegypti, and C. quinquefasciatus, respectively (Table 2). In latest years, a growing number of plant-synthesized AgNP has been studied for their excellent adulticidal activity against important mosquito vectors (Veerakumar et al., 2014c). For instance, the larvicidal activity of AgNP biosynthesized using S. acuta plant leaf extract was tested against III instar larvae of C. quinquefasciatus (LC50 = 26.13 μg/mL), A. stephensi (LC50 = 21.92 μg/mL), and A. aegypti (LC50 = 23.96 μg/mL) (Veerakumar et al., 2013). The mortality effect evoked by AgNP on mosquito larvae and pupae may be due by the small size of the AgNP, which allows their passage through the insect cuticle and into individual cells, where they interfere with molting and other physiological processes.

**CONCLUSIONS**

Overall, we biosynthesized AgNPs using a cheap aqueous extract of S. acuta leaves as reducing and stabilizing agent. Our AgNPs were mostly spherical in shape, crystalline in nature, with face-centered cubic geometry, and their mean size was 28-35 nm. This research highlighted that S. acuta -synthesized AgNPs are easy to produce, stable over time, and can be employed at low dosages to strongly reduce populations of vectors mosquitoes.
Table 1: Adulticidal activity of *S. acuta* aqueous leaf extract against the mosquito vectors *A. stephensi*, *A. aegypti*, and *C. quinquefasciatus*

<table>
<thead>
<tr>
<th>Mosquitoes</th>
<th>Concentration</th>
<th>24 h mortality (%) ± SD</th>
<th>LD_{50} (μg/mL) (LCL-UCL)</th>
<th>LD_{90} (μg/mL) (LCL-UCL)</th>
<th>χ²</th>
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</thead>
<tbody>
<tr>
<td><em>A. stephensi</em></td>
<td>Control</td>
<td>0.0 ± 0.0</td>
<td>157.88 (109.89-203.33)</td>
<td>287.44 (234.84-401.57)</td>
<td>21.933*</td>
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<td></td>
<td>70</td>
<td>31.7 ± 2.0</td>
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<td>140</td>
<td>42.3 ± 1.4</td>
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<td>210</td>
<td>65.6 ± 1.8</td>
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<td>280</td>
<td>84.3 ± 0.6</td>
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<td>350</td>
<td>100.0 ± 0.0</td>
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<td><em>A. aegypti</em></td>
<td>Control</td>
<td>0.0 ± 0.0</td>
<td>164.89 (121.20-207.16)</td>
<td>299.01 (248.05-402.42)</td>
<td>18.493*</td>
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<td>70</td>
<td>29.7 ± 0.4</td>
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<td>140</td>
<td>40.3 ± 1.6</td>
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<td>63.2 ± 1.9</td>
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<td>280</td>
<td>82.4 ± 0.8</td>
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<td>350</td>
<td>98.6 ± 1.2</td>
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<td><em>C. quinquefasciatus</em></td>
<td>Control</td>
<td>0.0 ± 0.0</td>
<td>177.48 (135.60-220.02)</td>
<td>321.05 (268.06-427.43)</td>
<td>16.743*</td>
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<td>70</td>
<td>26.4 ± 0.8</td>
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<td>140</td>
<td>39.2 ± 1.9</td>
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<td>210</td>
<td>58.6 ± 1.3</td>
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<td>280</td>
<td>76.2 ± 0.4</td>
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<td>350</td>
<td>96.7 ± 1.5</td>
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</table>

Values are mean ± SD of five replicates. No mortality was observed in the control, SD: Standard deviation, LD_{50}: Lethal concentration that kills 50% of the exposed organisms, LD_{90}: Lethal concentration that kills 90% of the exposed organisms, UCL: 95% upper confidence limit, LCL: 95% lower confidence limit, χ²: Chi-square, *Significant at P < 0.05 level, A. stephensi: Anopheles stephensi, A. aegypti: Aedes aegypti, C. quinquefasciatus: Culex quinquefasciatus, S. acuta: Sida acuta

Table 2: Adulticidal activity of silver nanoparticles biosynthesized using the *S. acuta* leaf extract against the mosquito vectors *A. stephensi*, *A. aegypti*, and *C. quinquefasciatus*

<table>
<thead>
<tr>
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<th>LD_{90} (μg/mL) (LCL-UCL)</th>
<th>χ²</th>
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</thead>
<tbody>
<tr>
<td><em>A. stephensi</em></td>
<td>Control</td>
<td>0.0 ± 0.0</td>
<td>35.12 (25.94-44.94)</td>
<td>62.60 (51.99-84.17)</td>
<td>18.945*</td>
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<td>70</td>
<td>27.1 ± 0.2</td>
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<td>140</td>
<td>43.4 ± 1.3</td>
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<td>210</td>
<td>61.7 ± 1.6</td>
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<td>280</td>
<td>83.3 ± 0.8</td>
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<td>350</td>
<td>100.0 ± 0.0</td>
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<tr>
<td><em>A. aegypti</em></td>
<td>Control</td>
<td>0.0 ± 0.0</td>
<td>39.53 (30.08-49.27)</td>
<td>70.94 (58.92-95.98)</td>
<td>17.763*</td>
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<td>70</td>
<td>25.4 ± 0.6</td>
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<td>140</td>
<td>36.0 ± 1.6</td>
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<td>57.3 ± 0.2</td>
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<td>280</td>
<td>72.6 ± 1.3</td>
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<td>350</td>
<td>96.2 ± 0.2</td>
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<tr>
<td><em>C. quinquefasciatus</em></td>
<td>Control</td>
<td>0.0 ± 0.0</td>
<td>41.44 (32.25-51.26)</td>
<td>73.94 (61.62-99.46)</td>
<td>16.597*</td>
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<td>24.1 ± 0.8</td>
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<td>140</td>
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<td>54.2 ± 0.5</td>
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<td>69.7 ± 1.5</td>
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Values are mean ± SD of five replicates. No mortality was observed in the control, SD: Standard deviation, LD_{50}: Lethal concentration that kills 50% of the exposed organisms, LD_{90}: Lethal concentration that kills 90% of the exposed organisms, UCL: 95% upper confidence limit, LCL: 95% lower confidence limit, χ²: Chi-square, *Significant at P < 0.05 level, A. stephensi: Anopheles stephensi, A. aegypti: Aedes aegypti, C. quinquefasciatus: Culex quinquefasciatus, S. acuta: Sida acuta

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