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Insecticidal activity of silver nanoparticles synthesised from aqueous leaf extract of *Eucalyptus tereticornis* on horn flies

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Abstract

An insecticidal efficacy of silver nanoparticles (AgNps) synthesised from aqueous leaf extract of *Eucalyptus tereticornis,* was carried out against adults, larval and pupal stages of *Haematobia* flies. In *in vitro* adulticidal and larvicidal assays the mortality ranged between 30.0 to 67.0 and 5.0 to 75.0 per cent in treated groups after 24 hours at different concentrations, respectively. The inhibition of pupation ranged between 5.0 to 30.0 per cent after 24 hours by dipping method. The lethal concentration values were calculated for all the assays. The probit analysis revealed the significant ($p<0.05$) effect at different concentrations of AgNPs synthesised from aqueous leaf extract of *E. tereticornis* on adults, larvae and pupal mortality.

Key Words: *Haematobia* flies, silver nanoparticles, *in vitro* assays

Haematobia species grouped under biting muscid flies, are commonly known as horn flies are of great importance in medical, veterinary, forensic and agricultural fields. They are the smallest blood-sucking obligate feeders especially found on cattle, buffaloes and aberrant hosts *viz.,* horses and other large mammals. They play a major role as biological vectors in the transmission of stephanofilariosis in cattle leading to anaemia, intense irritation, decreased production and lesions on the skin that attract other myiasis-producing flies. The estimated economic loss to cattle production in Mexico was approximately 231.67 million dollars for *Haematobia* flies during the year 2013 (Rodriguez *et al.,* 2017).

The control of horn flies is conventionally done by the application of chemical insecticides which could result in the development of tolerance or resistance, ecological imbalances, and harm to non-target organisms (Srinivasan *et al.,* 2008). Because of these limitations, there is a necessity to explore novel approaches such as synthetic insecticides. Recently, the green synthesised nanoparticles (NPs) have gained attention over other chemical and physical methods as

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they were cost-effective, environmental-friendly and could be easily scaled up. Various plant extracts have been studied for the biosynthesis of NPs and it was observed that the primary and secondary bioactive molecules present in these plants mainly act as reducing, stabilising and capping agents during the synthesis of nanoparticles. The nano characterisation has to be carried out to confirm the structural and morphological properties of the green synthesised nanoparticles (Benelli, 2016).

The nanomaterials are considered suitable for biological, biomedical and environmental applications due to their huge surface volume ratio (Das *et al.,* 2017). Further, the nanotechnology has a wide assay of revolutionised applications which would potentially uplift the human and animal welfare. Various researchers have documented the broad spectrum insecticidal or acaricidal activity of the plant-derived metallic nanoparticles (silver, gold, copper, zinc, nickel, titanium dioxide) in the control of vectors of medical and veterinary importance. Among different metallic nanoparticles, AgNPs have been receiving much attention because of their distinctive broad-spectrum insecticidal activity, more colloidal stability and biodegradability (Gul *et al.,* 2016; Singh *et al.,* 2018). Previous studies have shown the insecticidal and acaricidal efficacy of green synthesised silver nanoparticles in India and abroad (Kamaraj *et al.,* 2012; Gul *et al.,* 2016; Avinash *et al.,* 2017). Therefore, the study was conducted to explore the insecticidal efficacy of AgNPs synthesised from aqueous leaf extract of *E. teriticornis* against the developmental stages of horn flies.

Material and methods

Collection and In vitro rearing of flies

The flies were collected from the cattle dairy farm at the Department of Livestock Farm Complex (LFC), Veterinary College, KVAFSU, Hebbal, Bengaluru using a sweep net and were transported to the laboratory for further *in vitro* studies. The flies were morphologically identified using the standard keys as described by Walker (1994); Kettle (1995) and Mullen and Durden (2019).

The flies were reared in the laboratory by releasing them into crafted polyethene (plastic) box cages and fed cattle whole blood with sodium citrate (2mg/ml) and *ad libitum* water under *in vitro* conditions (Asha, 2013). Further, for oviposition, the moist tissue paper towel was placed (Holderman *et al.,* 2020) and the rearing cages were maintained in the laboratory at a temperature of 25 ± 2° C and relative humidity of 40.0 to 80.0 per cent with a photoperiod of 12:12.

Collection of eggs, larvae and pupae for in vitro assays

To obtain the larvae and pupae for the *in vitro* assays, the eggs were transferred into fresh cattle dung collected directly from the rectum. Later, the developed third larval stages and pupae were collected for *in vitro* insecticidal assays.

Collection of Eucalyptus tereticornis plant leaves

The leaves of *E. tereticornis* were collected from in and around the Veterinary College, Hebbal, Bengaluru and brought to the laboratory. The plant leaves were identified by the botanist in the Department of Forestry and Environmental Sciences at the University of Agricultural Sciences, GKVK, Bengaluru (Fig. 1).

Synthesis and characterisation of E. tereticornis silver nanoparticles

In the present study, the synthesis of green silver nanoparticles was carried out by using one millimolar silver nitrate (1 mM $AgNO_3$) and leaf extracts of *E. tereticornis* (Packialakshmi and Nazia, 2014; Shankar *et al.,* 2004). The colour change from light yellow to dark brown indicated the green synthesis of AgNPs. Later, the solution was subjected to three cycles of refrigerated centrifugation (4ºC) at 10000 rpm (7826 g) for 15 minutes each. The obtained filtrate and the sediment were stored at 4°C and -20 ºC for further *in vitro* assays and nano characterisation, respectively.

Nano characterisation of plant-based synthesised silver nanoparticles

The characterisation of synthesised silver nanoparticles was carried out by UV-visible spectroscopy, zeta potential analysis, dynamic light scattering (DLS), scanning electron microscopy (SEM) and Fourier transform

Fig. 1: *E. tereticornis* lance shaped to curved matured leaves

infrared spectroscopy (FTIR).

Evaluation of insecticidal efficacy of green synthesised AgNPs by in vitro assays

The insecticidal efficacy of the green synthesised AgNPs was evaluated on the third-stage larvae (L3), pupae and adult flies of *Haematobia* spp. by *in vitro* assays *viz.,* Whatman filter paper impregnation method, diet incorporation method and dipping method at different concentrations as per the insecticidal testing protocols (Wright, 1971; Velayutham *et al.,* 2012; El-Monairy *et al.,* 2020).

The filter paper impregnation method was carried out with different concentrations of the synthesised AgNO3 *viz*., 1.25, 2.5, 5, 10 and 20 mg per litre on adult flies. Approximately, ten adult flies were introduced onto each of the jars which contained the impregnated filter paper. In control groups, the filter paper treated with double distilled water was placed in the jar. All the jars were maintained at a temperature of 25 ± 2 ºC and 70 to 80 per cent relative humidity. The per cent mortalities and the average mean± SD were recorded after 24 hours post-exposure in both treated and control groups.

The diet incorporation method was used to evaluate the larvicidal efficacy of the plant-based AgNPs as per the procedure described by El-Monairy *et al.* (2020) in duplicates. The larvicidal efficacy of AgNPs was carried out against the third instar larvae of *Haematobia* flies at different concentrations *viz*., 1.25, 2.5, 5.0, 10.0 and 20.0 mg per litre. The artificial larval diet included cattle faeces, double distilled water and peanut shell pellets in the ratio of 2:2:1 (Lohmeyer and Kammlah, 2006). The diet was prepared by adding 20 g of the cattle dung sample and 10 g of peanut shell pellets. Further, 3 g of the diet and 2 ml of the different concentrations AgNps *viz.,* 1.25, 2.5, 5.0, 10.0 and 20.0 mg per litre were added to each insect breeding jar. For each concentration, 10 larvae were transferred into an insect breeding jar. The control group was provided with 3 g of the diet and 2 ml of distilled water. The mortality percentage was recorded after 24 and 48 hours of exposure in both treated and control groups.

The dipping method was evaluated in duplicates as per the procedure described by Rabab (2018) with slight modifications. The dark reddish brown matured pupae were collected for the study. Ten pupae were submerged in each of the different concentrations of AgNPs *viz.*, 0.5, 1, 2, 4 and 8 mg per ml for 10 minutes. In the control group, pupae were dipped in deionized water and the experiment was conducted in duplicates. Later, the pupae were transferred into small Petri plates and kept at a temperature of 25 ± 2 ºC and 70.0 to 80.0 per cent of relative humidity under laboratory conditions. Further, the number of emerged adults was counted after 24 hours of exposure in both control and treated groups.

Statistical analysis

The control mortalities were corrected by using Abott's formula (Abott, 1925). The number of dead adults/larvae/pupae was counted in treated groups and the per cent mortality was calculated from an average of the duplicates or triplicates. The mortality percentage was subjected to the probit analysis to calculate Lethal Concentration$_{25}$, LC$_{50}$ and LC$_{90}$ (Finney, 1971). The SPSS version 16.0 software was used. The Chi-square (X^2) test was used to test for goodness of fit of the probit model. The average mean mortalities and standard deviation were calculated by GraphPad Prism 5. The results were considered to be statistically significant at p -0.05.

Results and discussion

Identification of muscid flies

An attempt was made in the present study to know the adulticidal, larvicidal and pupicidal efficacy of AgNPs synthesised from aqueous leaf extract of *E. tereticornis* against horn flies. The collected flies were identified based on the morphological characteristics of mouthparts, thorax, abdomen and wing venation pattern (Fig. 2a, b, c).

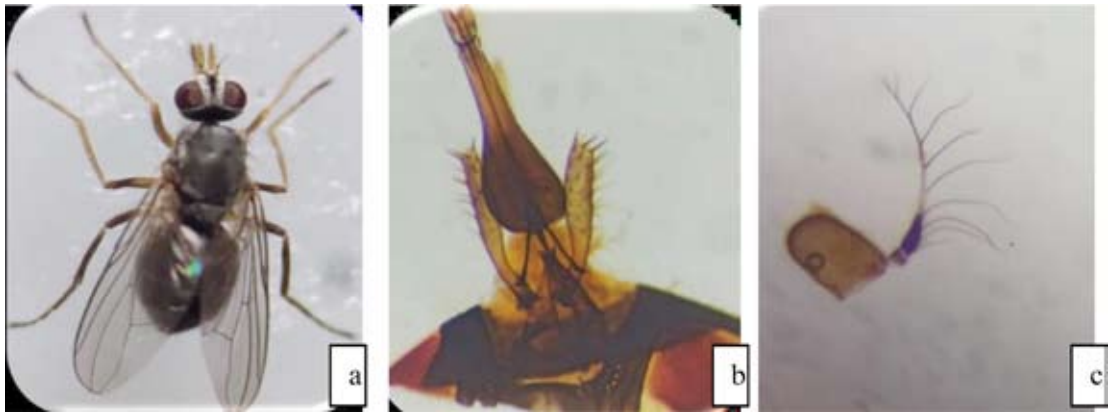

Fig. 2: a. *Haematobia* adult female fly; b: Labella with prestomal teeth; c: Arista plumed on dorsal side only with last plume longer than the bare tip

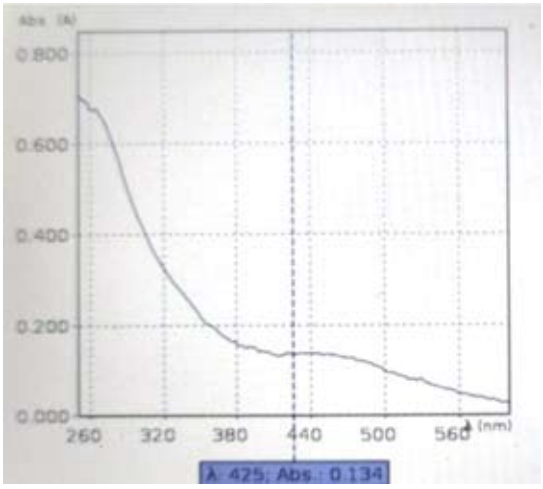

Fig. 3: UV-visible spectra of AgNPs

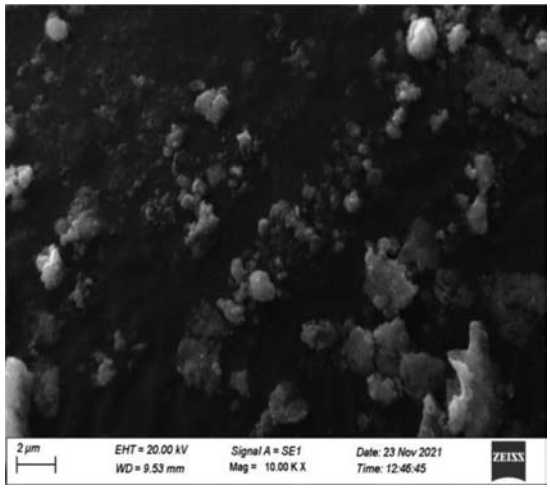

Fig. 4: Scanning electron microscopic images higher magnification (2 µm)

Characterisation of synthesised silver nanoparticles

The maximum absorbance was found at 425 nm by UV-visible spectra for AgNPs synthesised from aqueous leaf extracts of both *E. tereticornis* due to excitation of surface Plasmon resonance (Fig. 3). The electric charge on the surface of dispersed AgNPs was found to be -46.1 mV and -34.5, mV respectively. DLS revealed a particle size of 142.8 nm with a diameter of 50.8 nm. The synthesised AgNPs obtained revealed spherical shaped particles by SEM (Fig. 4).

The FTIR spectra showed a sharp and strong absorption band at 3510 cm^{-1} corresponding to O-H and N-H groups, CH_2 at 2925 cm^{-1}, C-H at 2854 cm^{-1}, carbonyl and aldehydes at 1744 cm^{-1}, aliphatic and alkaline C-H bend at 1016 and 673 cm^{-1}, respectively. The overall nano characterisation indicated the presence of terpenoids, flavonoids, phenols, proteins and carbohydrates in aqueous leaf extracts and confirmed that the proteins probably acted as capping agents on nanoparticles preventing agglomeration thereby stabilising the medium (Rajan *et al.,* 2015).

Evaluation of insecticidal efficacy of AgNPs derived from aqueous leaf extracts

Adulticidal, larvicidal and pupicidal efficacy of AgNPs

During this study, the mortality percentage of adult flies ranged from 30.0 to 67.0 per cent in treated groups. The AgNPs synthesised from aqueous leaf extracts of *E. tereticornis* were significantly toxic at 20 mg concentration per litre against the adult flies compared to 1.25, 2.5, 5.0, and 10.0 mg per litre after 24 hours of exposure. In treated groups, significant mortality up to 67.0 per cent were recorded. The mean ± SD and per cent mortalities in both treated and control groups were recorded. Probit analysis revealed the significant (p=0.004) effect with different concentrations of AgNPs synthesised from aqueous leaf extract of *E. tereticornis* on the mortality of flies. The LC_{25}, LC_{50}, LC_{90}, upper confidence limit (UCL), lower confidence limit (LCL) and X^2 values of all three assays are presented in Table 1. However, various researchers have reported the adulticidal activity of AgNPs synthesised from different

Table 1: Insecticidal efficacy of AgNPs synthesised from aqueous leaf extract of *E. teriticornis*

Sl. No.	Horn flies	LC_{25} (mg/ litre)	LC_{50} (mg/ litre)	LC_{90} (mg/ litre)	95 % confidence limit (LC_{50})		X^2 (df=13)
					LCL	UCL	
1	Adulticidal	1.013	8.736	523.49	4.509	41.171	3.618
2	Larvicidal	1.528	5.239	54.485	3.083	9.198	1.641
3	Pupicidal	4.20	30.386	62.32	7.90	2.176	1.940

LC_{25}: 25 % Lethal concentration LCL: Lower confidence limit LC_{50}: 50 % Lethal concentration UCL: Upper confidence limit

Table 2: Regression correlation values of adulticidal, larvicidal and pupicidal efficacy of AgNPs

Variables	Adulticidal	Larvicidal	Pupicidal
Intercept (SE)	-0.679±0.206 (0.001)	-0.906±0.260 (0.001)	-1.164±0.785 (0.000)
Concentration	0.721±0.249 (0.004)	1.260±0.322 (0.000)	0.785±0.364 (0.031)

aqueous leaf extracts against other non-biting muscid flies and adult mosquitoes (Veerakumar *et al.,* 2014; Subramaniam *et al.,* 2015). The mortality of adult flies in treated groups might be due to the death of insects due to the absorption of AgNPs into the cuticular lipids causing physical damage (Barik *et al.,* 2008), introduction of oxidative stress (Nair *et al.,* 2013) and morphological and histological abnormalities due to accumulation of NPs in the thorax and abdomen of insect (Banumathi *et al.,* 2017).

In treated groups, the larvicidal efficacy of AgNPs synthesised from aqueous leaf extract of *E. tereticornis* by diet incorporation method revealed larval mortality up to 75.0 and 80.0 per cent after 24 and 48 hours of exposure at 20 mg per litre, respectively. Similarly, LC_{50} values of 2.03 and 6.41 mg per ml concentration for Ag and zinc oxide (ZnO) nanoparticles synthesised from *Moringa oleifera* leaf extract against first instar larvae of *M. domestica* were reported (Rabab, 2018). However, maximum mortality was reported by Gnanadesigan *et al*. (2011); Elumalai *et al*. (2016) and Soni and Dhiman (2020) after 24 and 48 hours of exposure to titanium dioxide (TiO_2) and ZnO NPs synthesised from *Cuscuta reflexa* stem extract against larval stages of mosquitoes.

The highest per cent inhibition of 30.0 was observed against pupae of horn flies at 8 mg per ml concentration of AgNPs after 24 hours of dipping. The regression co-relation values are shown in Table 2. Rabab (2018) and Nagarajan and Vijayaranjan (2019) indicated comparatively lower LC_{50} values for Ag and ZnO nanoparticles synthesised from aqueous leaf extract of *M. oleifera* against pupae of *M. domestica* and *An. stephensi,* respectively.

In contrast to the present findings, Kalimuthu *et al.* (2013) and Murugan *et al.* (2016) observed significantly higher mortality against pupae of *Culex quinquefasciatus.* The larvicidal and pupicidal toxicity observed during this study could be probably attributed to the penetration of the nanoparticles through the larval membrane and pupal case which further binds to proteins or DNA in the intracellular space leading to denaturation of organelles and enzymes (Raj and Viswanathan, 2009). Subsequently, decreased membrane permeability may cause loss of cellular function leading to death (Sareen *et al.,* 2012). However, the pupicidal efficacy of AgNPs indicated 30.0 to 45.0 per cent inhibition of pupae which could be probably due to the presence of puparium within the highly sclerotised pupal case and further interference with penetration of the nanoparticles through cuticle (Soulsby, 1982).

Conclusion

To conclude, the present study is the first report on the evaluation of insecticidal efficacy of AgNPs synthesised from aqueous leaf extracts of *E. tereticornis* on horn flies in India, particularly in Karnataka state. In future, the AgNPs synthesised from aqueous leaf extracts of *E. tereticornis* can be a potential candidate in the production of biofabricated molecules commercially for use in the integrated pest management of *Haematobia* spp. which are of economic importance in the veterinary field. However, a further trial assay of synthesised AgNPs should be explored at the field level for their suitable application.

Conflict of Interest

All the authors declare that they have no conflict of interest.

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