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In vitro rearing and evaluation of insecticidal activity of silver nanoparticles synthesized from *Eucalyptus tereticornis* and *Pongamia pinnata* on *Stomoxys calcitrans*

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Abstract
An insecticidal efficacy of silver nanoparticles (AgNPs) synthesized from aqueous dried leaf extract of *E. tereticornis* and *P. pinnata* were evaluated against adult flies, larvae and pupae of *S. calcitrans* by *in vitro* assays at different concentrations. The flies collected from in and around cattle dairy farm at Livestock Farm Complex, Veterinary College, Hebbal, Bengaluru were identified based on their morphology and further reared in the laboratory in customized insect cages. The synthesized AgNPs were Nano characterised by UV-Visible spectroscopy, Zeta potential analysis, Dynamic light scattering, Scanning electron microscopy and Fourier transform infrared spectroscopy before *in vitro* assays. An adulticidal assay by filter paper impregnation method in triplicates showed the highest mortality of 70.0 percent against adult flies of *S. calcitrans* at 20 mg per litre after 24 hours exposure. The LC_{25}, LC_{50} and LC_{90} values were 1.073, 9.218 and 546.86, respectively. The larvicidal assay by diet incorporation method indicated 100.0 percent larval mortality at 20 mg concentration per litre after 48 hours exposure and the LC_{25} (0.0873), LC_{50} (2.294) and LC_{90} (14.375) values were observed. The pupicidal assay showed the highest percent inhibition of adults emergence (45.0%) from pupae at 8 mg per ml concentration after 24 hours of dipping. The LC_{25}, LC_{50} and LC_{90} values observed were 0.412, 1.921, and 35.801, respectively. The probit analysis revealed the significant effect with different concentrations of AgNPs synthesized from aqueous dried leaf extract. However, further field trial assays should be explored for their suitable application.

Keywords: *Eucalyptus tereticornis*, *Pongamia pinnata*, green synthesized nanoparticles, insecticidal efficacy, stable flies

Introduction
The arthropods are dangerous vectors due to their vector potentiality in the increasing world population of humans and animals [1]. Among arthropods, *S. calcitrans* are of great importance in medical, veterinary, forensic and agricultural sciences causing economic losses in terms of reduced weight gain, decreased milk and meat production. The haematophagous activity of the fly causes painful bites on animal body and also acts as a vector for transmission of parasitic infections.

Since many decades, the control of *S. calcitrans* is mainly achieved by use of chemical insecticides which have shown development of tolerance or resistance, ecological imbalances and also harm to non-target organisms [2]. Hence, there is necessity for the search of novel approaches which may replace these synthetic insecticides. However, recently the use of plant derived nanoparticles (NPs) are attractive and advantageous over chemical and physical methods since it is single-step, cost effective, environmental-friendly and easily scaled up. In the recent years a number of plant extracts have been studied for the biosynthesis of nanoparticles and observed that the primary and secondary bioactive molecules present in the plants (proteins, carbohydrates, enzymes, vitamins and flavonoids, terpenoids, alkaloids, polyphenols) acts as reducing, stabilizing and capping agents during the synthesis of nanoparticles.

During the 21st century, the nanotechnology has potentially revolutionized with wide assay of applications in several fields which would directly up lift the human and animal welfare.

Several studies have shown insecticidal and acaricidal efficacy of plant based silver nanoparticles in abroad and India [3-6]. Therefore, the present study is undertaken to evaluate the insecticidal efficacy of biosynthesized nanoparticles in control of arthropod pests.

Materials and Methods

Study area and collection of flies

The *S. calcitrans* flies were collected from in and around the cattle dairy farm at Department of Livestock Farm Complex (LFC), Veterinary College, KVAFSU Regional Campus, Hebbal, Bengaluru by using sweep net during the year from August 2021 to January 2022 and flies were transported to the laboratory for further *in vitro* studies.

Identification and *In vitro* rearing of *S. calcitrans*

In the laboratory, the collected flies were subjected to refrigeration (-4 °C) for about 60 seconds to immobilize them and were immediately identified based on the morphological characters *viz.*, mouth parts, thorax, abdomen and wing venation pattern [7-9]. The flies were separated based on the genus characters for further *in vitro* rearing.

In the laboratory, the *S. calcitrans* adult flies were reared by releasing them into crafted polyethylene (plastic) (36 L×16 H×24 W cm) /thermocoal (31 L×15 H×20 W cm) box cages. *S. calcitrans* flies were fed with whole blood collected from cattle with sodium citrate (2 mg/ml) as an anti-coagulant and *ad libitum* water under *in vitro* conditions [10]. Further, for oviposition the petriplates containing the horse and cattle dung was kept inside the insect rearing cages [11, 12]. The insect cages were maintained in the laboratory at temperature 25±2 °C and relative humidity of 40.0 to 80.0 percent with a photoperiod of 12:12 (Light: Dark). To obtain larvae and pupae for *in vitro* assays, the petriplates containing the eggs of *S. calcitrans*, were shifted to rectangular boxes which contained soil for further development of larval instars and pupae. Later, the developed third larval stages and pupae were collected for *in vitro* insecticidal assays.

Collection of *E. tereticornis* and *P. pinnata* plant leaves

During this study, the leaves of *E. tereticornis* and *P. pinnata* plants were collected from in and around the Veterinary College, Hebbal, Bengaluru and brought to the laboratory. Before further processing, the plant leaves were identified based on the shape and fruits by the Botanist in the Department of Forestry and Environmental Sciences at the University of Agricultural Sciences, GKVK, Bengaluru. Later, only disease free healthy leaves were separated in the laboratory and subjected for synthesis of plant derived nanoparticles.

Synthesis and characterisation of *E. teriticornis* and *P. pinnata* silver nanoparticles

In the present study, the synthesis of green silver nanoparticles was carried out by using silver nitrate ($AgNO_3$) and leaf extracts of *E. teriticornis* and *P. pinnata*.

Preparation of aqueous leaf extract

For the preparation of aqueous leaf extract, the separated disease free healthy leaves were washed in the laboratory with the tap water and dried under shade for 8 to 10 days and later subjected for electric blender to obtain the powder. About 20 g of *E. tereticornis* and 10 g of *P. pinnata* leaves powder was taken separately into two 500 ml Erlenmeyer flask and 100 ml of sterile double distilled water was added to each of the flask. The suspension was mixed thoroughly and kept at 60 ºC in water bath for 5 minutes as per the previous procedure [13]. Then the suspension was taken out from the water bath and was allowed to cool. Later, the suspension was filtered through Whatman No.1 filter paper (11µ) and the obtained aqueous leaf extract was further used for synthesis of silver nanoparticles.

Preparation of 1 Milli molar (1 Mm) silver nitrate solution

In the present study, 1 Mm silver nitrate solution was prepared by dissolving 0.017 g of molecular grade silver nitrate (Himedia, Mumbai) in 100 ml of double distilled water and stored at 4 ºC for further synthesis of silver nanoparticles [14].

Synthesis of *E. tereticornis* silver nanoparticles

The silver nanoparticles of *E. tereticornis* were synthesized from aqueous leaf extract as per the procedure [13]. An approximately about 90 ml of 1 Mm silver nitrate solution was taken into the 250 ml conical flask and 10 ml of aqueous leaf extract was added. Initially, about 5 ml of the leaf extract was added and stirred continuously to obtain uniform mixing with the silver nitrate solution. Later, the remaining 5 ml was added and stirred continuously. The pH of the solution was adjusted between 7.0 to 7.4 and the solution was incubated at room temperature (25 to 27 ºC) for about 24 hours in a dark room. The colour change from light yellow to dark brown indicates the synthesis of silver nanoparticles. Later, the solution was subjected to refrigerated centrifugation (4ºC) at 10000 rpm (7826 g) for 15 minutes and three cycles of washing was carried out for an obtained sediment with double distilled water at 10000 rpm (7826 g) for 15 minutes each. The obtained filtrate and the sediment was stored at 4 °C and -20 ºC for further use, respectively.

Synthesis of *P. pinnata* silver nanoparticles

In the present study, the silver nanoparticles of *P. pinnata* were synthesized with slight modifications [13]. An approximately, about 90 ml of 1 Mm silver nitrate solution was taken into the 250 ml conical flask and 10 ml of aqueous leaf extract was added and the above procedure was repeated to obtain the uniform mixing of silver nitrate solution and leaf extract. The pH was adjusted between 7.0 to 7.4 and the solution was kept at 60 ºC in water bath for two hours. The colour change from light yellow to dark brown indicates the synthesis of silver nanoparticles. Later, the solution was cooled and subjected to refrigerated centrifugation (4 ºC) at 10000 rpm (7826 g) for 15 minutes and three cycles of washing was carried out as described above.

Characterisation of plant based synthesized silver nanoparticles

The characterisation of synthesized silver nanoparticles was carried out by UV-Visible Spectroscopy, Zeta potential analysis, Dynamic Light Scattering, Scanning Electron Microscopy and Fourier Transform Infrared Spectroscopy.

Evaluation of insecticidal efficacy of plant synthesized silver nanoparticles by *in vitro* assays

During this study, an insecticidal efficacy of plant synthesized silver nanoparticles were evaluated on the third stage la rvae

(L_3), pupae and adult flies of *M. domestica*, *S. calcitrans* and *H. irritans irritans* by *in vitro* assays such as Whatman filter paper impregnation method, diet incorporation method and dipping method at different concentrations as per the insecticidal testing protocols [15-18].

Insecticidal efficacy of AgNPs synthesized from aqueous leaf extracts of *E. tereticornis* and *P. pinnata*

The stock solution was prepared by adding 10 ml of the filtrate to 90 ml of the double distilled water and from the stock solution different concentrations were prepared.

Adulticidal activity by Filter paper impregnation method

The filter paper impregnation method was carried out with different concentrations of synthesized silver nanoparticles *viz*., 1.25, 2.5, 5, 10 and 20 mg per litre of *E. tereticornis* and *P. pinnata* on adult flies of *S. calcitrans* in triplicates [15, 17]. The Whatman filter paper No. 1 of 9x cm diameter was impregnated with different concentrations of synthesized silver nanoparticles and air dried under room temperature for 2 to 3 hours. Later, the dried impregnated filter papers were introduced into circular insect breeding jars of size 10 cm in diameter. An approximately, ten adult flies were introduced onto each of the jars which contained impregnated filter paper. In control groups, filter paper treated with double distilled water was placed in the jars. All the jars were maintained at temperature of 25±2 ºC and relative humidity between 70 to 80 percent.

Larvicidal efficacy by Diet incorporation method

During this study, the diet incorporation method was used to evaluate the larvicidal efficacy of plant based AgNPs as per the procedure in duplicates [18]. Larvicidal efficacy of AgNPs synthesized from *E. tereticornis* and *P. pinnata* was carried out against third instar larvae of *S. calcitrans* at different concentrations *viz*., 1.25, 2.5, 5.0, 10.0 and 20.0 mg per litre. An artificial diet (38 g of wheat bran and 2 g of milk powder) which was used for oviposition and larval rearing was used as a diet for the third larval stages of *S. calcitrans* with slight modifications [19]. Further, 4 g of an artificial diet was transferred into an individual circular insect breeding jars and 2 ml of distilled water and 2 ml of the different concentration of synthesized plant based AgNPs was added to each of the jars. Later, an approximately ten larvae were transferred into each of the concentration. The control groups were treated with 4 g of artificial diet with 4 ml of distilled water. The present study was carried out under laboratory conditions at 25±2 ºC and 70.0 to 80.0 percent of temperature and relative humidity, respectively. The larval mortality in each concentration and control groups were recorded at 24 and 48 hours after exposure.

Pupicidal activity by dipping method

The dipping method was used to evaluate the insecticidal efficacy of *E. tereticornis* and *P. pinnata* AgNPs against pupae of *S. calcitrans* as per the procedure with slight modifications [17]. The dark reddish brown matured pupae were collected for the study. Ten pupae were submerged in each of the different concentrations *viz*., 0.5, 1, 2, 4 and 8 mg per ml for 10 minutes. In control groups, pupae were dipped in deionized water only and the experiment was conducted in duplicates. Later, the pupae were transferred into small petriplates and kept at temperature 25±2 ºC and 70.0 to 80.0 h of relative humidity under laboratory conditions. Further, the number of emerged adults were counted after 24 hours exposure in both control and treated groups.

Statistical analysis

The number of dead adults/larvae/pupae were counted in treated groups and the percent mortality was calculated from an average of the duplicates or triplicates by the formula:

$$\text{Percent mortality} = \frac{\text{Number of dead adults/larvae/pupae}}{\text{Total number of adults/larvae/pupae}} \times 100$$

The control mortalities were corrected by using Abott's formula [20]

$$\text{Corrected mortality} = \frac{\text{Observed mortality in treatment - Observed mortality in control}}{\text{100 - Control mortality}} \times 100$$

The mortality percentage from each method was subjected to the probit analysis to calculate LC_{25}, LC_{50} and LC_{90} [21]. The SPSS version 16.0 software was used. The Chi-square (X2) 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

Identification and Rearing of *S. calcitrans*

In the present study, the adult male fly measured about 6 to 6.5 mm in length and female measured 7.0 to 8.0 mm and greyish black in colour. In mouth parts, the maxillary palps were short and half the length of the proboscis, arista was plumed only on dorsal side and the last plume was smaller than the bare tip. Thorax was greyish in colour with four longitudinal stripes, lateral pair stripes were narrow and do not reach the end of scutum. The abdomen was shorter and broader than that of *M. domestica* with three dark spots on each of the third and fourth segments which showed checker board appearance. In wings, the M_{1+2} veins were curved gently and forwarded to form an open apical cell. The eggs of *S. calcitrans* were dirty white in colour with a longitudinal groove on one side. The fully grown larvae were similar to *M. domestica* but in anterior spiracles about six projections were observed. In posterior spiracles, stigmal plates were far apart triangular in shape and each has 3 'S' shaped sinuous slits. The larval period varied between 3 to 5 days. The pupa was oval in shape, measured about 4 mm in length and dark brown to blackish brown in colour. The pupal stage period lasts for 5 days. The entire developmental period lasts for about 15 to 20 days.

Synthesis of silver nanoparticles from *E. tereticornis* and *P. pinnata*

In the present study, the synthesis of AgNPs was indicated visually based on the change in the colour intensity. The reaction mixture of *E. tereticornis* changed from light yellow to dark brown after 24 hours of incubation whereas *P. pinnata* changed from light yellowish brown to dark brown after incubation in the water bath at 60 ºC for about 2 hours (Figure 1a and b). Further, the significant colour change was not observed in both the reaction mixtures.

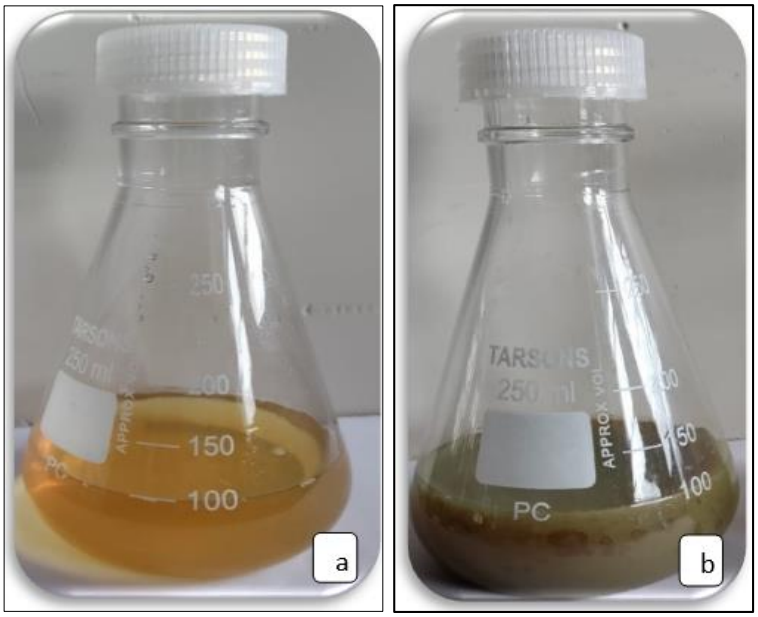

Fig 1: Photograph showing change in colour after adding silver nitrate solution
A and B: Before and after reaction with leaf extract of *E. tereticornis*

Characterisation of plant based synthesized silver nanoparticles

The UV-Visible spectra of the synthesized AgNPs showed the maximum absorbance at 425 nm for AgNPs synthesized from aqueous leaf extracts of both *E. tereticornis* and *P. pinnata* due to excitation of surface plasmon resonance. In zeta potential analysis, the electric charge on the surface of dispersed AgNPs was found to be -46.1 mV for *E. tereticornis* and -34.5, mV for *P. pinnata*, respectively. DLS revealed that the particle size was 142.8 and 184.2 nm with a diameter of 50.8 and 45.6 nm for *E. tereticornis* and *P. pinnata*, respectively. An image obtained using SEM to determine the shape of synthesized nanoparticles are shown (Figures 2a and 2b). The synthesized AgNPs obtained from aqueous leaf extracts of *E. tereticornis* and *P. pinnata* were spherical in shape. The FTIR spectrum of AgNPs synthesized from *E. tereticornis* exhibited prominent peaks at 3510, 2925, 2854, 1744, 1016 and 673 cm-1 (Figure 3a). The spectra showed sharp and strong absorption band at 3510 corresponding to O-H and N-H groups, CH_2 at 2925, C-H at 2854, carbonyl and aldehydes at 1744, aliphatic and alkaline C-H bend at 1016 and 673, respectively. In FTIR spectrum of AgNPs synthesized from *P. pinnata* showed prominent peaks at 3402, 3328, 2856, 1639 and 652 (Figure 3b). The absorbance bands at 3402 corresponds to O-H stretch, 3328 to alkaline C-H stretch, 2856 to C-H aliphatic compounds, 1639 to C=C stretch and 652 to alkaline C-H bend.

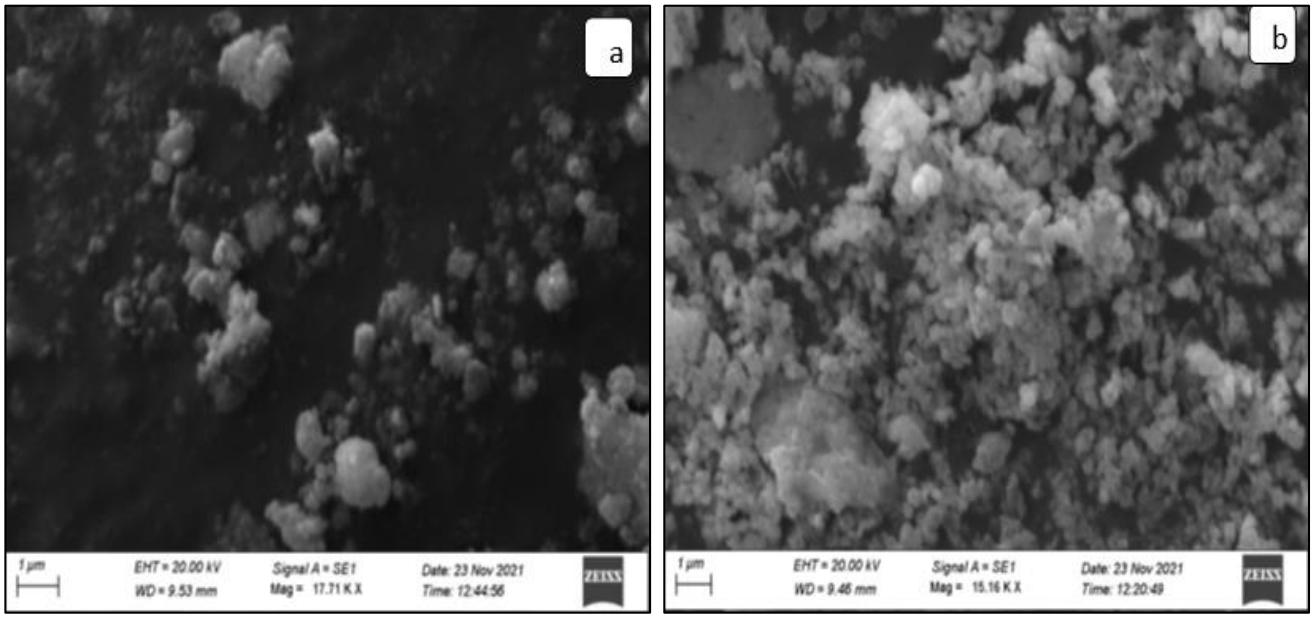

Fig 2 a and b: Electron microscopic images of synthesized silver nanoparticles from aqueous leaf extract of *E. tereticornis* and *P. pinnata* in lower magnification (1 µm)

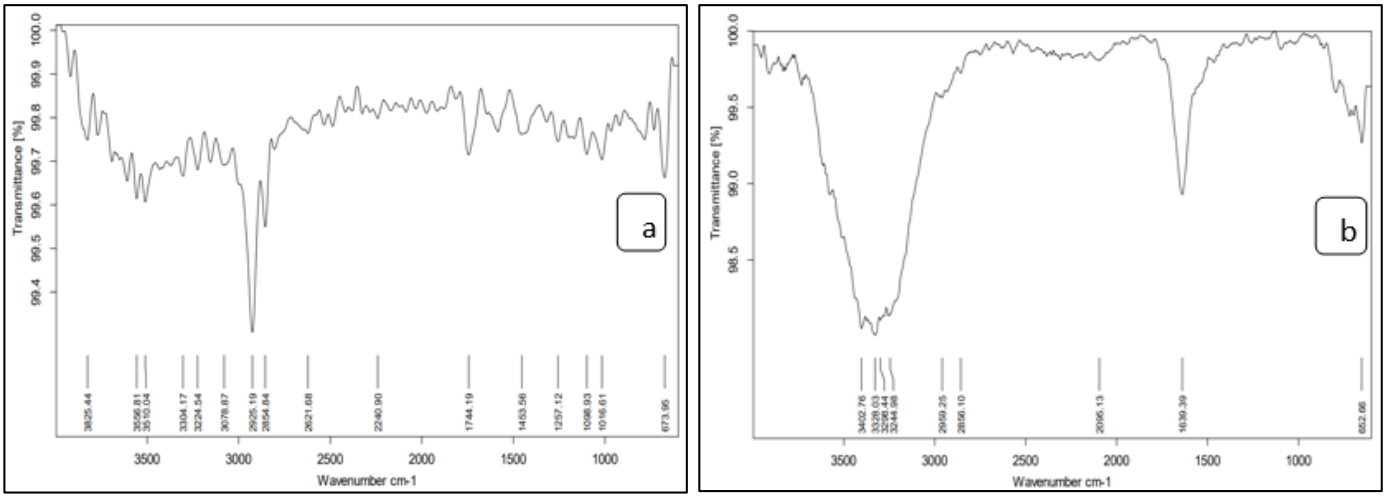

Fig 3a-b: FTIR spectrum of synthesized silver nanoparticles by reacting silver nitrate with aqueous leaf extract of *E. tereticornis* and *P. pinnata*

Adulticidal efficacy of AgNPs synthesized from aqueous leaf extracts of *E. tereticornis* and *P. pinnata*

During this study, the adulticidal efficacy was evaluated against adult flies of *S. calcitrans* at five different concentrations *viz*., 1.25, 2.5, 5.0, 10.0 and 20.0 mg per litre in triplicates by filter paper impregnation method. The average mean ± SD and percent mortality in both treated and control groups are presented in Table-1. The adulticidal efficacy of AgNPs synthesized from aqueous leaf extract of *E. tereticornis* and *P. pinnata* are presented in terms of LC_{25}, LC_{50} and LC_{90} along with upper and lower confidence limit values (LC_{50}) and regression correlation are shown. The chi-square values (x^2) was statistically non-significant at $p \leq 0.05$. There was no LCL and UCL for *S. calcitran* with in groups treated with *P. pinnata*, since the regression values were non-significant. The x^2 value of 4.036 for *S. calcitrans* was found to be statistically non-significant at $p \leq 0.05$.

Table 1: Adulticidal efficacy of AgNPs synthesized from aqueous leaf extracts

Sl. No.	Aqueous leaf extracts	LC_{25} (mg/ litre)	LC_{50} (mg/ litre)	LC_{90} (mg/ litre)	95% confidence limit (LC_{50})		X^2 (DF=13)
					LCL	UCL	
1	*E. teriticornis*	1.073	9.218	546.86	4.780	47.322	11.489
2	*P. pinnata*	0.203	6.482	467.26	0.000	0.000	4.036

LC_{25}: 25% Lethal concentration LCL: Lower confidence limit
LC_{50}: 50% Lethal concentration UCL: Upper confidence limit
LC_{90}: 90% Lethal concentration DF: Degrees of freedom

The larvicidal efficacy of AgNPs synthesized from aqueous leaf extract of *E. tereticornis*

The larvicidal efficacy of AgNPs synthesized from *E. tereticornis* and *P. pinnata* was evaluated against third larval stages of *S. calcitrans* by diet incorporation method in duplicates. The percentage mortality of third stage larvae of *S. calcitrans* at five different concentrations *viz*., 1.25, 2.5, 5.0, 10.0, 20.0 are recorded after 48 hours exposure. However, during this study, the percent mortalities were recorded after 24 and 48 hours of exposure in treated groups. The larval mortality was found to be 20.0 to 80.0 percent after 24 hours exposure. After 48 hours of exposure, the mortality percentage ranged from 50.0 to 100.0 percent for *S. calcitrans*. The LC_{25}, LC_{50} and LC_{90} values along with LCL and UCL including x^2, regression values are presented below (Table 2 and 3). In all the groups, the x^2 values were statistically non-significant at $p \leq 0.05$.

Table 2: Larvicidal efficacy of AgNPs synthesized from aqueous leaf extracts

SL. No.	Aqueous leaf extracts	LC_{25} (mg/ litre)	LC_{50} (mg/ litre)	LC_{90} (mg/ litre)	95% confidence limit (LC_{50})		X^2 (DF=8)
					LCL	UCL	
1	*E. territicornis*	0.0873	2.294	14.375	1.141	3.531	11.473
2	*P. pinnata*	1.166	3.508	28.455	1.979	5.447	6.214

LC_{25}: 25% Lethal concentration LCL: Lower confidence limit
LC_{50}: 50% Lethal concentration UCL: Upper confidence limit
LC_{90}: 90% Lethal concentration DF: Degrees of freedom

The pupicidal efficacy of AgNPs synthesized from aqueous leaf extract *E. tereticornis* and *P. pinnata*

The pupicidal efficacy of AgNPs synthesized from *E. tereticornis* were evaluated against pupae of *S. calcitrans* at different concentrations *viz*., 0.5, 1.0, 2.0, 4.0 and 8.0 mg per ml in duplicates. The LC_{25}, LC_{50} and LC_{90} values of 1.714, 6.270 and 73.675 mg per ml were recorded. The upper and lower confidence limits (LC_{50}) and regression correlations are described in Table 3. There was no LCL and UCL for *S. calcitrans*, since the regression values were non-significant. The x^2 values were found to be statistically non-significant at $p \leq 0.05$. The LC_{25}, LC_{50} and LC_{90} values along with LCL and UCL including chi-square (x^2) values for *M. domestica*, *S. calcitrans* and *H. irritans irritans* are presented in Table 3. In

M. domestica treated groups, the LC_{25}, LC_{50} and LC_{90} values were 1.908, 28.229 and 4.719 mg per ml respectively, for *S. calcitrans* and *H. irritans irritans*, the values observed were 3.00 and 4.757 (LC_{25}), 19.955 and 17.599 (LC_{50}) and 730.52 and 211.367 (LC_{90}), respectively. There was no LCL and UCL for *M. domestica*, since the regression values were non-significant. The x^2 values were found to be statistically non-significant at $p \leq 0.05$.

Table 3: Pupicidal efficacy of AgNPs synthesized from aqueous leaf extracts

Sl. No.	Aqueous leaf extracts	LC_{25} (mg/ ml)	LC_{50} (mg/ ml)	LC_{90} (mg/ ml)	95% confidence limit (LC_{50})		Regression equation		X^2 (DF=8)
					LCL	UCL	Intercept (P Value)	Concentration (P Value)	
1	*E. teriticornis*	0.412	1.921	35.801	0.000	0.000	0.697±0.206 (0.001)	0.722±0.249 (0.004)	25.161
2	*P. pinnata*	3.00	19.955	730.52	6.451	686675.639	0.364±0.199 (0.067)	0.448±0.243 (0.065)	0.848

LC_{25}: 25% Lethal concentration LCL: Lower confidence limit
LC_{50}: 50% Lethal concentration UCL: Upper confidence limit
LC_{90}: 90% Lethal concentration DF: Degrees of freedom

In the present study, an overall significant mortality of 83.0 percent was observed in adult flies of *H. irritans irritans* and *M. domestica* (70.0%) for AgNPs synthesized from aqueous leaf extract of *P. pinnata* compared to *E. tereticornis*. In larvicidal activity, maximum larval mortality was observed for *S. calcitrans* (100.0%) treated with AgNPs synthesized from *E. tereticornis* than *P. pinnata* (90.0%) followed by both *M. domestica* and *H. irritans irritans* (85.0%) larvae treated with AgNPs synthesized from *P. pinnata*. However, significant differences were not observed in pupicidal activity for AgNPs synthesized from both *E. tereticornis* and *P. pinnata* for all the three pupae.

Discussion

Stomoxys calcitrans is an important haematophagus fly which cause painful bites and feeds on the blood of their animal hosts causing productivity losses. Although most of the damage is caused by blood feeding activity, but they are also an important vectors in the transmission of livestock pathogens. Since many decades, control of these flies is mainly achieved by use of chemical insecticides. However, it is a great challenge to have an effective control method. Presently, an alternative methods such as application of plant synthesized nanoparticles are gaining much importance in the control of flies of both veterinary and medical importance. Therefore, an attempt has been made during this study to know the adulticidal, larvicidal and pupicidal efficacy of AgNPs synthesized from aqueous leaf extract of *E. teriticornis* and *P. pinnata* against *S. calcitrans*. The present study is the first report on evaluation of insecticidal efficacy of AgNPs synthesized from aqueous leaf extracts of *E. teriticornis* and *P. pinnata* on *S. calcitrans* in India and in particular in Karnataka state. During this study, *S. calcitrans* adult flies were reared under *invitro* conditions at temperature 25±2 ºC and 70.0 to 80.0 percent of relative humidity. Oviposition was observed in both fresh cattle and horse dung. However, the first stage larvae after hatching did not survive in the cattle dung under laboratory conditions. Since, the first stage larvae requires nutritional niche provided in the habitat of horse dung for further development of larva to pupa [22].

The morphometric findings of the adult flies, larval instars and pupae are in accordance with the previous studies [23, 9] who have described the body length of adult *S. calcitrans* around 5 to 7 mm. The *S. calcitrans* flies were fed with the whole blood collected from cattle with sodium citrate (2 mg/ml) as an anticoagulant and along with *ad libitum* water. Although, sodium citrate is the most common anticoagulant used for *invitro* feeding but the concentration or percent varies with the earlier studies [24, 25].

Synthesis and characterisation of plant derived *E. territicornis* and *P. pinnata* silver nano particles

During the present study, 10 g of *P. pinnata* dried leaf powder was used to prepare aqueous leaf extract, since 20 g of powder resulted in thick suspension and further was not able to filter through Whatman No. 1 filter paper. The dried leaf powder was used for preparation of aqueous leaf extracts due to availability of effective phytochemical compounds present especially in leaves such as ketones, aldehydes, flavonoids, amides, terpenoids, carboxylic acids, phenols and ascorbic acids which are capable of reducing metal salts into metallic nanoparticles [26].

Characterisation of the synthesized AgNPs

The maximum absorbance was found at 425 nm in UV-vis for AgNPs synthesized from aqueous leaf extracts of *E. teriticornis* and *P. pinnata* due to excitation of surface plasmon resonance which could be attributed to the free conduction of electron induced by an interacting electromagnetic field [27]. However, the absorption peak varies with the reaction time and concentration of metal ions [1].

Adulticidal, lavicidal and pupicidal efficacy of AgNPs synthesized from aqueous leaf extract of *E. teriticornis* and *P. pinnata*

In the present study, the AgNPs synthesized from aqueous leaf extracts of *E. teriticornis* and *P. pinnata* were found to be significantly toxic at 20 mg concentration per litre against adult flies of compared to 1.25, 2.5, 5.0, 10.0 mg per litre. The significant mortality of 70.0 and 63.0 percent was recorded in groups treated with AgNPs synthesized from *E. teriticornis* and *P. pinnata* at 20 mg concentration per litre after 24 hours exposure, respectively. Since, there is a lack of literature on insecticidal efficacy of plant derived nanoparticles on *S. calcitrans* as such, hence the present study results are discussed in correlation to reports of other muscid and nematocerran flies (mosquitoes). In contrast to the present findings, the adulticidal efficacy of AgNPs synthesized from aqueous leaf extract of *Manilkara zapota* against adult flies of *M. domestica* indicated significantly higher mortality of 72.0, 89.0 and 100.0 percent in 1, 2 and 3 hours exposure at concentration of 10 mg per ml [3]. The higher mortality of 71.67, 91.66 and 100.0 percent was reported in 1, 2 and 3 hours of exposure at 10 mg per ml compared to 4 and 8 mg per ml against *M. domestica* adult flies treated with AgNPs

synthesized from fresh melon [6]. However, various researchers have reported adulticidal activity of AgNPs synthesized from different aqueous leaf extracts against adult mosquitoes *viz*., an adulticidal activity of AgNPs synthesized from aqueous leaf extract of *Heleiotropium indicum* against adults of *An. stephensi, Ae. aegypti* and *C. Quinquefasciatus* showed maximum efficacy against *An. stephensi* followed by *Ae. aegypti* and *C. quinquefaciatus*[28]. The LC_{50} values of 13.7 ppm against *An. stephensi* and 14.7 ppm were indicated against *An. albopictus* for AgNPs derived from *Mimusops elengi* [29].

During this study, the mortality of adult flies in treated groups could be attributed to the death of insects due to absorption of AgNPs into the cuticular lipids causing physical damage[30], introduction of oxidative stress[31] and morphological and histological abnormalities due to accumulation of NPs in the thorax and abdomen of insect [32].

The larvicidal efficacy was carried out against third larval stages and the larval mortality was recorded after 24 and 48 hours of exposure. In treated groups, 80.0 and 55.0 percent mortality was observed after 24 hours of exposure at 20 mg per litre. However, the highest mortality of 100.0 and 90.0 percent was observed at 20 mg per litre concentration for AgNPs synthesized from aqueous leaf extract of *E. teriticornis* after 48 hours of exposure. The similar findings were reported for Ag and Zno nanoparticles synthesized from *Moringa oleifera* leaf extract against first instar larvae of *M. domestica* with LC_{50} values of 2.03 and 6.41 mg per ml concentration [17]. The maximum mortality (100.0%) against fourth instar larvae of *Ae. aegypti* and *C. quinquefasciatus* mosquitoes was reported with AgNPs synthesized from leaf extract of *Rhizophora mucronata* at 10 mg per litre after 24 hours of exposure [33]. The mortality of 100.0 percent was observed against the fourth instar larvae of *An. stephensi, Ae. aegypti* and *C. quinquefasciatus* treated with AgNPs synthesized from leaf extract of *Achyranthes aspera* at 10 mg per ml after 24 hours of exposure and also reported the LC_{50} and LC_{90} values of 2.48 and 8.14 mg per ml for *C. quinquefasciatus*, 3.68 and 8.92 for *Ae. aegypti* and 3.89 and 10.78 mg per ml for *An. stephensi*, respectively[34]. The mortality of 92.0, 96.0 and 100.0 percent was observed in first, second and third instar larvae of *An. stephensi* and 80.0, 88.0 and 100.0 percent against *Ae. aegypti* respectively, for AgNPs synthesized from aqueous leaf extract of *Artemisia nilagirica* at 0.25 percent concentration. The LC_{50} values of 0.343, 0.169 and 0.198 were indicated for *An. Stephensi*, whereas, 0.460, 0.352 and 0.331 were reported for *Ae. aegypti.*

However, the larvicidal activity of Zno NPs synthesized from *Lagenaria siceraria* aqueous peel extract was showed against fourth larval instar of *An. stephensi* with LC_{50} and LC_{90} values of 3.491 and 7.834 ppm, respectively [36]. Similarly, the high susceptibility of *An. stephensi* third instar larvae was reported to Tio2 and Zno NPs synthesized from *Cuscuta reflexa* stem extract after 48 hours exposure at 250 ppm with LC_{50} values of 50±0.12 and 25±0.12, respectively.

The pupicidal efficacy of AgNPs synthesized from *E. teriticornis* and *P. pinnata* were evaluated against pupae and the highest percent inhibition of 45.0 and 40.0 were observed in adult emergence at 8 mg per ml concentration after 24 hours of dipping, respectively. However, previous studies indicated comparatively lower LC_{50} values of 9.604 and 17.10 mg per ml for Ag and Zno nanoparticles synthesized from aqueous leaf extract of *M. oleifera* against pupae of *M. domestica*, respectively [17]. The LC_{50} and LC_{90} values of 2.328 and 6.501 ppm were indicated for Zno NPs synthesized from aqueous peel extract of *Lagenaria siceraria* against pupae of *An. stephensi*, respectively [36].

In contrast to the present findings, the higher pupal mortality of 68.8±1.3 and 71.4±1.0 with LC50 values of 25.27 and 23.83 at 50 ppm was observed for AgNPs synthesized from leaf extract of *Cadaba indica lam* against *An. stephensi* and *C. quiquefaciatus*, respectively [38]. The significantly higher LC_{50} and LC_{90} values of 18.676 and 38.077 ppm were indicated for AgNPs synthesized from aqueous leaf extract of *M. oleifera* against pupae of *C. quinquefasciatus* respectively. The lower and upper confidence limits of both LC_{50} were 16.897 and 20.890 and LC_{90} values were 33.244 and 46.032 ppm, respectively [39].

The larvicidal and pupicidal toxicity observed during this study could be probably attributed to the penetration of the nanoparticles through larval membrane and pupal case which further binds to proteins or DNA in the intracellular space leading to denaturation of organelles and enzymes [40]. Subsequently, decreased membrane permeability may cause loss of cellular function leading to death [41]. However, during this study the pupicidal efficacy of AgNPs synthesized from both the aqueous leaf extracts indicated 30.0 to 45.0 percent inhibition of pupae which could be probably due to the presence of puparium within the highly sclerotized pupal case and further interference with penetration of the nanoparticles through cuticle [22]. Secondly, the lower efficacy observed on pupae with an adult emergence could also be due to use of only ten pupae per concentration during all the experiments. To conclude, the Probit analysis revealed the significant effect with different concentration of AgNPs synthesized from aqueous leaf extract of *P. pinnata* than *E. teriticornis.*

In future, the AgNPs synthesized from aqueous leaf extracts of *E. teriticornis* and *P. pinnata* can be a potential candidate in production of biofabricated molecules commercially for use in integrated pest management of biting flies especially, *S. calcitrans* which are of economic importance in the field of veterinary science. However, further trial assay of synthesized AgNPs should be explored at the field level for their suitable application.

Significance of the study

What's already known about the topic?

The green synthesis of nanoparticles from various plants has been has been studied by various authors. Further, many reports in abroad and India has focussed on evaluation of insectidical efficiacy of plant synthesized nanoparticles especially on mosquitoes and ticks.

What does this study add?

The present study is the first report on evaluation of insecticidal efficacy of silver nanoparticles synthesized from aqueous leaf extracts of *E. tereticornis* and *P. pinnata* on *S. calcitrans* in India and in particular in Karnataka. To conclude, the AgNPs synthesized from aqueous leaf extract of *E. tereticornis* and *P. pinnata* showed significant larvicidal, adulticidal and pupicidal activity against *S. calcitrans.* Further, the synthesized AgNPs may be considered as a potential bio insecticide candidate in integrated pest management of stable flies (*S. calcitrans*).

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