THINK INDIA JOURNAL

ISSN: 0971-1260

Vol-22-Issue-17-September-2019

Efficacy of *Eucalyptus globulus* mediated bio-nanoparticles against Malarial vector

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Abstract

Mosquitoes are the principal vectors of severe diseases like malaria, dengue, Japanese encephalitis, chikungunya, filariasis, yellow fever etc. The three genera *Anopheles stephensi*, *Aedes aegypti* and *Culex quinquefasciatus* are responsible for transferring the above mentioned diseases. For mosquito control, the synthetic insecticides were used but their continuous application leads to the development of resistance in mosquitoes, hazardous effects on environment and toxic to non- target organisms including humans. It creates a big challenge in front of researchers that they synthesized eco-friendly and non-toxic insecticides by using natural products against mosquito vectors. In the present study, the larvicidal activity of silver nanoparticles (AgNPs) engineered by using leaf extract of *Eucalyptus globulus* against the third instar larvae of *Cx. quinquefasciatus.* The bio-engineered AgNPs were characterized by UV-visible spectroscopy and Fourier transform infrared (FTIR). The surface plasmon resonance (SPR) was observed at 420nm in UV-vis spectrophotometer. The presence of different functional groups in compounds was studied by FTIR. The different concentrations (25, 50, 75, 100 and 125ppm) of plant extract, silver nitrate ($AgNO_3$) and AgNPs were tested against 3rd instar larvae of *Cx. quinquefasciatus.* The synthesized AgNPs were shown more effect than plant extract against *Cx. quinquefasciatus.* The LC_{50} and LC_{90} values of synthesized AgNPs were 83 and 90ppm. There was no mortality in the control. This result suggests that the synthesized AgNPs from *E. globules* can be safer, eco-friendly and novel insecticide against mosquito vectors.

Keywords: Bio-engineered AgNPs, *Eucalyptus globulus*, larvicidal activity, mosquito vectors, LC_{50} and LC_{90}.

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ISSN: 0971-1260

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Introduction

One of the real general worries in nations is the indirect transmission of diseases through vectors. The three dipteran major genus vectors are *Aedes, Anopheles* and *Culex* transmit genuine diseases; viz, malaria, filariasis, Japanese encephalitis, dengue, zika fever, and chikungunya are the real mosquito-vectors. In the present worldwide, various mosquitoes are especially expanding in simultaneousness with a high rate of dengue [1]. Chikungunya, Zika and dengue fever are highly caused by *Ae. Aegypti* [2]. As per the WHO report 2009, two-fifth of world population comes under the danger of dengue contamination and in 2010, 28,292 cases and 108 passing's announced in India, when compared to 2013 the number cases increased from 75,808 to 188,401 at 2017 [3-5].

An expected one hundred twenty million individuals in all regions of the globe are contaminated with filariasis disease; nearly twenty-five million males had genital sickness and just about 15 million, in ladies, have elephantiasis in the leg. Roughly 66 % are in danger of disease found in the southeastern Asia Region and 33 % of the Africa District [6]. Internationally, an expected twenty-five males suffer from sexual sickness whereas more than fifteen million are suffering from lymphedema [7]. The most imperative malarial vectors in India and other Asian nations are *An. stephensi* [8-9]. Malaria is caused by pathogens of the family Plasmodium. Almost three billion individuals are in danger of jungle fever around the world [10].

P. falciparum can cause serious maternal frailty. Around 25 million pregnant ladies in Africa infected by *P. falciparum* and every year 100, 000 newborn mortality was recorded [11-

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ISSN: 0971-1260

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12]. In the year 2013, 0.88 million cases had accounted, 53% caused by *P. falciparum* and 47% of the contaminations caused by *P. vivax.* In the Southeast Asia Region of India, the rate of malaria represented 58% of cases [7].

Mohali district has recorded the most elevated number of dengue and malaria cases out of 22 regions of Punjab, figures by the Punjab Health Department uncover in 2017. WHO reacted to little episodes of chikungunya in late 2015 in the city of Dakar, Senegal, and the territory of Punjab, India. For mosquito control, many insecticides have prepared that creates various natural issues, for example, the development of resistance in insect strains, biological irregularity, and toxic to non- target animals. Henceforth, there is a consistent requirement for eco-friendly larvicides, which reduce the risks to human and different life forms by reducing the collection of hazardous substances in the earth. Thus, natural products are favored for controlling mosquitoes given low destructive nature towards non-target living beings as well as because of their natural bio-degradability [13].

Plants are rich well source for inhibiting the metabolism of mosquitoes. They have bio-active compounds that are effective against particular target organisms because of which they are considered as environment-friendly. Generally, natural items are utilized for a long time for controlling insects. Some secondary metabolites are highly beneficial for the treatment of insect bite. Herbal pesticides are not harmful but they are eco-friendly and effortlessly bio-degradable [14]. There are various plants have an insecticidal properties, the phytochemical compounds like saponine, tannins, and steroids present in plants act as a larvicidal agent [15-18].

Presences of large number of properties like ovicidal, pupicidal and larvicidal, phytochemicals are presently considered as effective insecticide to replace chemically-synthesized insecticide. Numerous engineered insecticides appeared to impact ovipositor in mosquitoes [19].

The larvicidal potential of incorporated silver nanoparticles utilizing the aqueous leaf concentrate of *Tinospora cordifolia* have been accounted for against the fourth instar larvae of *An. subpictus* and *Cx. quinquefasciatus* [20]. Ovicidal and repellent properties of methanol leaf concentrate of *Ervatamiacoronaria* and *Caesalpiniapulcherrima* were assessed against *Cx.*

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ISSN: 0971-1260

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quinquefasciatus, *Ae. aegypti* and *An. stephensi* [21]. The larvae-killing and repellent properties of basic oils from totally different components of four plant species *Cymbopogan citrates*, *Cinnamomum zeylanicum*, Rosemary and Common Ginger against *Cx. tritaeniorhynchus* and *Ae. Subpictus* [22]. The larvicidal and ovicidal adequacy of assorted concentrates of *Cardiospermum halicacabum* against *Cx. quinquefasciatus* and *Ae.aegypti* was set [23].

Numerous organisms were utilized for synthesis of silver nanoparticles extracellularly and intracellularly, among that fungus sp., *Aspergillus niger*, *Beauveria bassiana*, *Isaria fumosorose*, *Fusarium oxysporum* [24-26] and bacteria sp., *Eubacteria licheniformis*, *Pseudomonas meridian*, *Bacillus thuringiensis* [27-29].

Conversely, the silica nanoparticles have been tried against the larvae and pupae of *An. stephensi*, *Cx. quinquefasciatus*, and *Ae. aegypti* [30]. Larvicidal and pupicidal activity of *Euphorbia hirta* synthesized AgNPs were tested against *An. stephensi* [31]. *Catharanthus roseus* and *Carica papaya* synthesized nanoparticles were successful against *Ae. aegypti* and *Cx. quinquefasciatus*, [32-33]. Recently, the new era was developed for reducing the population of mosquitoes by treating with biosynthesized AgNPs. Hence, this experiment aimed to review the efficacy of *Eucalyptus*- synthesized AgNPs against the third larvae of *Cx. quinquefasciatus.*

Materials and Methods

Collection and Preparation of plant extract

The *Eucalyptus globulus* leaves were collected from campus of lovely professional university, Phagwara, Punjab, India. The fresh and mature leaves were collected and washed by double distilled de- ionized water for removing unwanted dust particles and soil. The leaves were, then, dried at room temperature and cut into small pieces with the help of sharp knife. Take these small pieces in conical flask and add 100ml distilled water. Boil it for 10- 15 minutes. After boiling, leave the solution for cooling at room temperature. Then, filter it with whatman filter paper no. 1 and collect filtrate in another conical flask. Label the flask, stored at 4°Cand kept ready for using it in the experiment.

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ISSN: 0971-1260

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Synthesis of Silver nanoparticles (AgNPs)

The silver nitrate was brought from HiMedia Pvt. Ltd. Company. In conical flask 1mM silver nitrate solution was prepared. In order to prevent its photo- oxidation, wrap the flask with black paper and stored in dark conditions. For the synthesis of silver nanoparticles, 100ml plant extract and 900ml silver nitrate solution was mixed (1:9 ratio). During mixing, immediate color change was observed which indicated the formation of silver nanoparticles in solution. The solution was stored in the refrigerator and used for the formation of dry pellets which further used for other investigation like FTIR.

Characterization of Synthesized Silver Nanoparticles

The characterization of *E. globulus* synthesized AgNPs were carried through UV- Vis spectroscopy and Fourier transform infrared spectroscopy at Department of chemistry, Lovely Professional University, Phagwara, Punjab, India.

UV- Vis spectra of *Eucalyptus globulus* mediated AgNPs analysis

E. globulus synthesized AgNPs have been observed by UV-Vis spectroscopy. The 1ml AgNPs solution diluted with 2ml distilled water and estimated by the UV–Vis range at general intervals by utilizing a quartz cuvette with water as reference [34]. UV– Vis spectra of these aliquots were observed on Elico- Double beam SL 210 spectrophotometer, Japan in the 300– 700 nm range.

Purification of AgNPs

The preliminary identification of AgNPs was carried out by observing the color change in the mixture within few seconds at room temperature and after 5 minutes, color becomes dark. Further, the watch-glass was filled with AgNPs solution and subjected to dry at hot oven plate for 20-30 min to prepare nanopellets. The dried sample was scraped with the help of blade and collected in the falcon tube. The subsequent pellet was washed by pouring deionized water in the falcon tube and centrifuge at 15,000 rpm for 10 minutes. The supernatant was disposed of and the pellet was mixed with de-ionized water. This mixture kept in watch glass and dried under hot

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air oven at 30- 35 °C for 10-15 minutes. The dried AgNPs were scraped and stored in the eppendorf. An aliquot of these AgNPs was utilized for Fourier transform infrared (FTIR) investigation

FT-IR Spectroscopy

The association among phytochemical compounds and AgNPs was studied by FT-IR spectra analysis. To expel any extra compound i.e. not the part of nanoparticles, the AgNPs solution was centrifuged at 10,000 rpm for 20-30 minutes and the pure pellets were obtained. After it, the pellets were dried at hot oven air to obtain dry powder sample.

FT-IR spectra were obtained utilizing Perkin-Elmer Range on FTIR-84005 SHIMADZU, Japan in the range of 4000–500 cm−1 at resolution of 4 cm−1. The phytochemical compounds present in the extract act as reducing agent and are responsible for reduction Ag ions to Af atom (AgNPs). The reaction between plant extract and AgNO3 may be written as:-

Plant extract of Eucalyptus + 0.5 mM Silver nitrate ($AgNO_3$) —(Phytochemical compounds / Reducing agent)→ *Eucalyptus*-synthesized AgNPs

Optimization of Silver Nanoparticles

For studying the optimization of silver nanoparticles, different parameters were considered like concentration, pH and temperature of silver nitrate. The extract was prepared as for the same procedure mentioned above. Different $AgNO_3$ concentration, temperature and pH (like 0.5mM, 1 mMand 2 mM; below room temperature (16 °C), room temperature (28 °C) and above room temperature (35 °C); and acidic (pH 5), neutral (pH 7) and alkali (pH 9) were prepared in conical flask and mixed with plant extract in 1:9 ratio. The color change was noted and further characterized by UV-vis spectrophotometer.

Collection of Mosquito larvae

The mosquitoe larvae of *Cx. quinquefasciatus* accumulated from pond water, outside the university campus and rose in the research laboratory, Branch of Zoology, Lovely Professional University, Phagwara, Punjab, India. These larvae were identified by some morphological

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ISSN: 0971-1260

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features i.e. *Culex* larvae have long and light color siphon at the distal end. Their body is more hairy as compared to *Aedes* larvae (www.researchgate.net). These identified larvae kept in the plastic trays filled with water and supplemented with dog biscuits and-yeast powder (3:1) under laboratory conditions.

Mosquito Larvicidal activity

The Bioassay was conducted with *E. globulus* synthesized silver nanoparticles against the third instar larvae of *Cx. quinquefasciatus*. For bioassay, 10 hatchlings moved into disposable plastic cups (250ml capacity) containing 100ml distilled water. The three replicates (R1, R2 and R3) of 10 larvae each were maintained. All were treated with different concentrations like 25ppm, 50ppm, 75ppm, 100ppm and 125ppm of plant extract, $AgNO_3$ and *Eucalyptus*-AgNPs. The 1000ppm stock solution of plant extract, AgNO3 and *E. globulus* AgNPs were prepared and further working solutions of different concentrations (25ppm, 50ppm, 75ppm, 100ppm and 125ppm) formed.

$$\text{Percent mortality} = \frac{\text{No. of dead larvae}}{\text{No. of introduced larvae}} \times 100$$

Result

This experiment portrays the biological synthesis of AgNps using leaves of *Eucalyptus globulus* plant as shown in Fig 1. *Cx. quinquefasciatus* third instar larvae have been used as an experimental model.

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ISSN: 0971-1260

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Fig 1: Plant sample

Synthesis of AgNPs

The color change was observed by visually in the *E. globulus* leaf extract when treated with AgNO3 solution. There was no color changes noted at both positive and negative control (*Eucalyptus* leaves extract without $AgNO_3$+ $AgNO_3$) Fig 2.

Fig 2: (a) leaf extract, (b) AgNO3 solution and (c) *E. globulus* synthesized silver nanoparticles

Characterisation and Optimization of Silver nanoparticles

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ISSN: 0971-1260

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UV spectroscopy the wavelength, and spectral bandwidth related with a nanoparticle are subject to size, shape, and material association. The spectral response of silver nanoparticles is studied via surface plasmon resonance (SPR) peak. The UV- Vis spectra of E. eucalyptus leaf at various wavelengths going from 300 to 700 nm uncovered a crest at 420 nm (Fig 3).

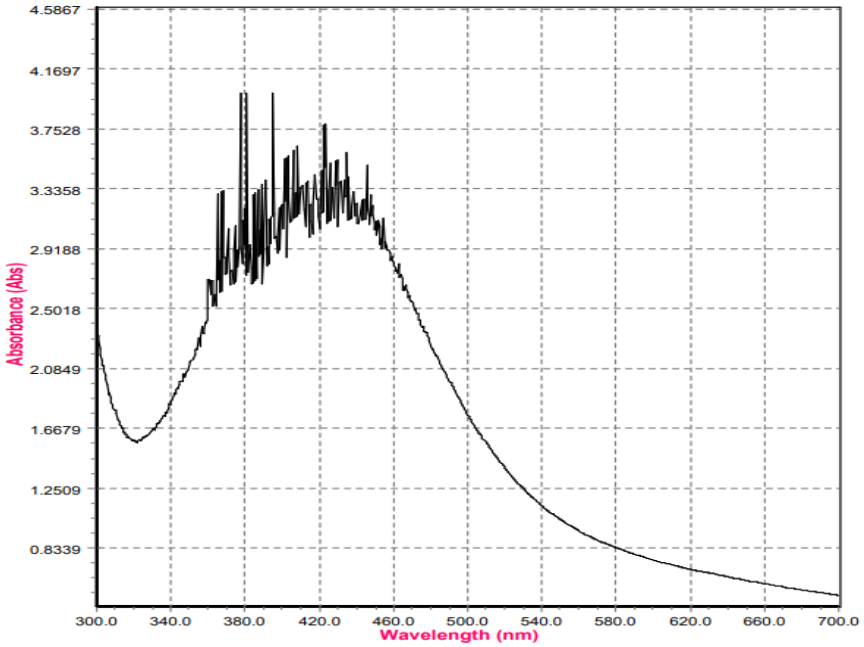

Fig. 3: UV-Vis spectra of *Eucalyptus* mediated AgNPs

FTIR analysis of *Eucalyptus*-AgNPs

FTIR spectroscopy was done to recognize the possible biomolecules responsible for capping and stabilization of the silver nanoparticles formed by *Eucalyptus* leaf extract. The FTIR spectral analysis confirmed the presence of O-H stretch strong peak, C-H stretch strong peak, C=C stretch variable peak, N-O stretch strong peak and C=C stretch medium-weak peak at 3258.84, 2922.25, 1637.62, 1523.82 and 1472.7 cm−1 respectively (Fig 4 and Table 1).

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ISSN: 0971-1260

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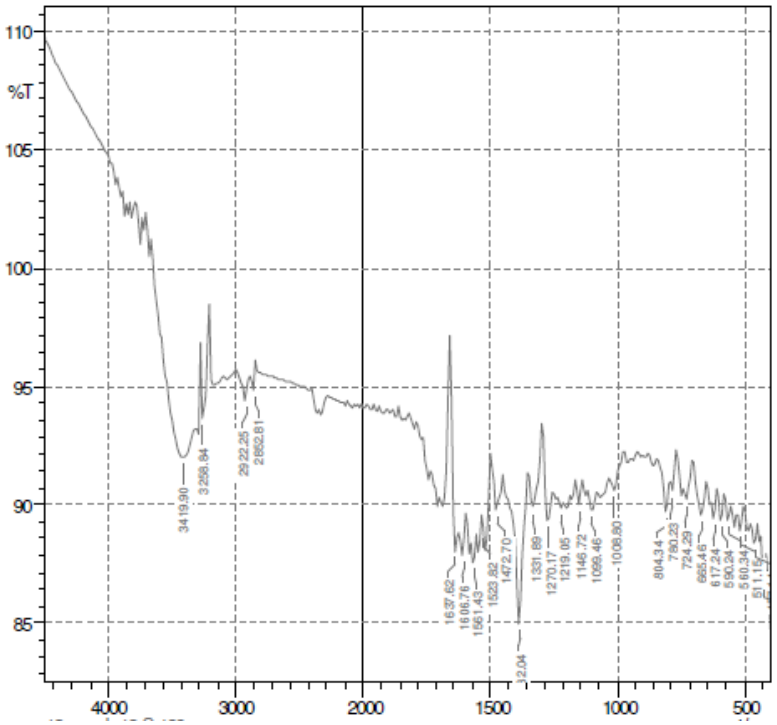

Fig. 4: FTIR spectra of *Eucalyptus*- AgNPs

Visible intensity	Functional group	Observed peak	Vibration type
Strong, broad	O-H (alcohol)	3258.84	Stretch, H-bonded
Strong	C-H (alkane)	2922.25	Stretch
Variable	C=C (alkene)	1637.62	Stretch
Strong, two bands	N-O (nitro)	1523.82	Stretch
Medium-weak	C=C (aromatic)	1472.7	Stretch

Table 1: FTIR spectra of Synthesized AgNPs.

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ISSN: 0971-1260

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Further, the filtrate was optimized at different parameters and results were shown in Table 2. The instant color change was observed in alkaline conditions. The concentrations (1mM), temperature at 28°C were optimum conditions for the synthesis of AgNPs.

Parameters	Optimized conditions
Concentration	1 mM
pH	9
Temperature	28 °C

Table 2: Optimized conditions for *Eucalyptus*- AgNPs.

Mosquito larvicidal assay

In-vitro studies showed the efficacy of leaf extract and *Eucalyptus*- synthesized AgNPs against the third instar larvae of *Cx. quinquefasciatus* at various concentrations like 25ppm, 50ppm, 75ppm, 100ppm and 125ppm. The mortality was observed after 24-h and 48-h exposure of plant extract and AgNPs as shown in Table 3 and 4. The mortality data of mosquito larvae through leaf extract was as follows: at 25ppm concentration, 10% mortality was recorded. At 50ppm concentration, 13% deaths were observed. 20% at 75ppm, 27% at 100ppm and 37% mortality at 125ppm concentration after 24-h exposure. After 48-h exposure, 27% mortality was observed at 25ppm, 33% at 50ppm, 43% at 75ppm, 57% at 100ppm and 80% at 125ppm concentration (Table 3 and Fig 6a). The *Eucalyptus*-nanoparticles shows mortality at lower concentration after 24-h exposure. By increasing the concentration, the percent mortality goes higher and 100% mortality was obtained after 48-h exposure. The mortality values were recorded till 48-h of exposure. After 24-h, 13% mortality recorded at 25 ppm. These values were increased by raising the concentration of AgNPs. 17% deaths were found at 50ppm, 27% at 75ppm, 30% at 100 ppm and 43% mortality was observed at 125 ppm. After 48-h, the mortality values were

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ISSN: 0971-1260

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33% at 25ppm, 40% at 50ppm, 53% at 75ppm, 60% at 100ppm and 100% at 125ppm (Table 4 and Fig 6b).

% Mortality		
concentration (ppm)	Day 1	Day 2
25	10±0.000	27±0.577
50	13±0.577	33±0.000
75	20±0.000	43±0.577
100	27±0.577	57±1.000
125	37±0.577	80±0.577
Control	**	**

% Percent mortality values are percent mortality ± SD; ** no mortality

Table 3:- Larvicidal activity of Leaf extract against *Culexquiquefasciatus.*

% Mortality		
Concentration (ppm)	Day 1	Day 2
25	13±0.577	33±0.000
50	17±0.577	40±0.577
75	27±0.577	53±0.577
100	30±0.000	60±1.000
125	43±0.577	100±0.577
Control	**	**

% Percent mortality values are percent mortality ± SD; ** no mortality

Table 4:- Larvicidal activity of Leaf extract against *Culexquiquefasciatus.*

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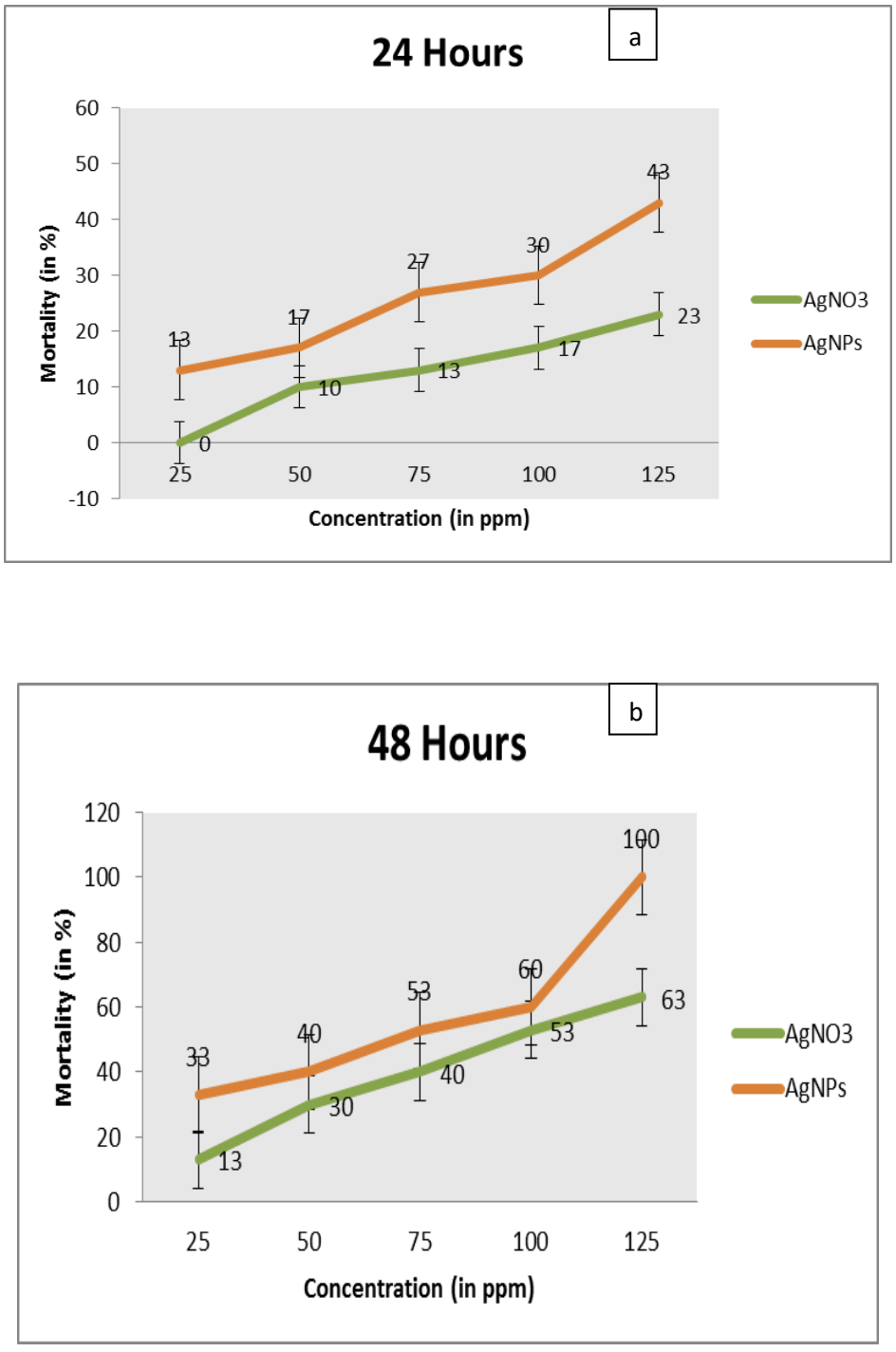

Fig 6: Larvicidalefficacy of Leaf extract and AgNPs against *Culex* larvae (a) after 24-h (b) 48-h exposure.

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ISSN: 0971-1260

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The LC_{50} and LC_{90} values for third instar larvae of *Cx.* were 65 and 110ppm of leaf extract whereas 58 and 113ppm of *Eucalyptus*- synthesized AgNPs after 24-h exposure. After 48-h exposure, LC_{50} and LC90 values were 60 and 120ppm of leaf extract & 83 and 90ppm of *Eucalyptus*- synthesized AgNPs as shown in Table 5 and 6.

Treatment	LC_{50} (Lower – Upper limit)	LC_{90} (Lower – Upper limit)	Chi- square
AgNO3	72 (55-160)	117 (80-320)	0.99*
Leaf extract	65 (49-143)	110 (80-320)	1.00*
Eucalyptus-AgNPs	58 (45-110)	113 (70-280)	1.00*

Table 5: LC_{50}, LC_{90} and Chi-square values after 24-h exposure.

Treatment	LC_{50} (Lower – Upper limit)	LC_{90} (Lower – Upper limit)	Chi- square
AgNO3	90 (67-220)	190 (120-590)	0.91*
Leaf extract	60 (46-180)	120 (84-500)	0.99*
Eucalyptus-AgNPs	83 (60-200)	90 (80-120)	1.00*

Table 6: LC_{50}, LC_{90} and Chi-square values after 48-h exposure.

Discussion

Phytochemicals may fill as appropriate other option to chemical based insecticide, later on, as these are generally protected, economical, and are promptly accessible in numerous zones of the world. Distinctive parts of plants contain a complex of chemicals with one of the

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ISSN: 0971-1260
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biological activity which is thought to be serving as mosquitocides because of toxic and secondary metabolites. *E. globulus* plant was selected to determine their potential for the production of nanoparticles. The leaf extract and their AgNPs were tested for larvicidal activity against mosquitoes. The present study demonstrated the synthesis of silver nanoparticles which was denoted by the gradual color change of leaf extract from yellow to dark brown color. It was due to the transformation of silver ions to silver atoms and excitation of Surface Plasmon Resonance (SPR) effect as compared with other results given by 28 and 34.

UV-Visible spectrum of synthesized silver nanoparticles was observed in the range 300 to 700 nm and the strong as well as broad SPR obtained at about 420 nm. This revealed the maximum synthesis of silver nanoparticles in aqueous solution. No peak was obtained in control. Many researchers obtained the absorption spectrum between 415 and 460 nm. In the same context, Veerakumar et al. [35] synthesized the silver nanoparticles using *Feronia elephantum* and observed the similar trend in UV spectroscopy. This shows that the above results resemble the literature. FTIR analysis was done to study the morphology of surface of AgNPs and also detect the presence of biomolecules which act as reducing agent for reduction of silver ions and capping agent for bio-reduced synthesized AgNPs.

Elumalai et al. [34] investigated the strong peak at 3433 cm−1 indicated the presence of O-H compounds. This was due to the strong association of water with surface of silver .At 2923 cm−1, medium peak observed due to C-H alkenes. From the present study, FTIR spectra of synthesized AgNPs showed similar results, very strong and broad peak was found at 3258.84 cm−1 (O-H stretch), stretch and variable peak at 1637.62 cm−1(C=C). Thus, FTIR results proved that there was no change in the phytochemicals compounds as an outcome of association with silver ions or AgNPs. This showed that the biomolecules may be responsible for the synthesis of AgNPs.

Many researchers worked on larvicidal activity of plant extract of *Mimosa pudica, Nelumbonucifera, Aloe vera, Polianthustuberosa, Feroniaelephantum, Achyranthesaspera* against mosquitoes but very researchers worked on *Eucalyptus* plant. There are 12 compounds were removed from two *Eucalyptus* species and tested against fourth instar of *Ae. aegypti* and

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ISSN: 0971-1260

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Ae. albopictus. Among them eucalyptol (1,8-cineole) and α- terpinyl acetic acid derivation were actively participated for controlling the mosquito larvae at the lower concentration (LC50 >50.0 mg L−1) [36]. Similarly *E. prostrate* synthesized AgNPs have been tested against *Cx. quinquefasciatus* (LC50= 27.49 and 4.56 mg/l; LC90=70.38 and 13.14 mg/l) [37].

The larvicidal efficacy of *Heliotropiumindicum* synthesized silver nanoparticles was studied against the mosquito vectors [38]. They got better results in the synthesized nanoparticles than the aqueous leaf extract. Mortality was observed after 24-h exposure of synthesized AgNPs. The LC50 and LC90 value against *Cx. quinquefasciatus* was 21.84 µg/mL and 38.10 µg/mL.In the current study, the *Eucalyptus* synthesized AgNPs showed very effective larvicidal activity at lower doses against *Cx. quinquefasciatus*. The better results were obtained with longer duration of exposure times. The LC50 and LC90 values against the third instar larvae of *Culex* larvae were 80 and 90ppm after 48-h exposure .Above data was not significantly comparable with present investigation data; 37% and 43% deaths were found at 125ppm concentration after 24-h exposure of leaf extract and AgNPs whereas 80% and 100% mortality found at 125ppm concentration of leaf extract and AgNPs after 48-h of exposure. The leaf extract was less effective than the synthesized AgNPs against third instar larvae of *Cx. quinquefasciatus.* Even, the AgNPs showed high larvicidal activity at lower doses and act as novel insecticide against mosquito larvae.

Conclusion

In conclusion, biological approach towards insect control proved to be more effective than chemically manufactured insecticides. Biological control also played a vital as well as safer role in reducing the insect population. Compared to the present report, the Eucalyptus synthesized AgNPs were quite effective in reducing the mosquito population. Generally, plants consists of secondary metabolites which maintain their larvicidal, bactericidal and adulticidal properties. Due to these properties, various plant parts i.e. leaves, fruits, seeds utilized for the synthesis of nanoparticles. Nanoparticles are responsible for controlling the growth and metabolism that leads to the death of insect. The plant- mediated silver nanoparticles have rapid

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ISSN: 0971-1260

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effect on mosquito population and thus concluded that the Eucalyptus- mediated AgNPs illustrates a major role in controlling mosquito population.

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