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Larvicidal activity of Silver Nanoparticle synthezed by the leaf extracts of *Azadirachta indica* against *Culex quinquefasciatus* (Say) (Diptera : Culicidae)

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Abstract
In the present investigation the larvicidal activity of Silver nanoparticle synthezed by the leaf extracts of *Azadirachta indica* against *Culex quinquefasciatus* was attempted in the laboratory conditions. The result of the present study indicated that the larval mortality of *C. quinquefasciatus* was high in 0.05% (500 ppm) and the lowest mortality was recorded in 0.01% (100 ppm). The LC_{90} and regression equation were 0.062, Y=40.914+11.388 X. The 95% lower and upper confidence limit of LC_{50}, LC_{90} (LCL-UCL) were (0.022 – 0.043) and (0.048-0.105) ppm respectively. The chi –square value 0.001 was significant at $p<0.05$ level (Chi: Pearson Goodness of Fit test 19.437 Df 4 P 0.001). The percentage of adult emergence inhibition were 22.0±1.8%, 35.5±2.5%, 48.0±3.0%, 68.0±3.4% and 90.0±2.2% at different concentration viz., 25.0ppm, 50.0ppm, 75.0ppm, 100.0ppm and 125.0ppm against the larvae of *C. quinquefasciatus.* The LC_{50} value was 515.11 ppm and the LC_{90} value was 954.24 ppm. The regression equation was Y=11.15+0.416X. The 95% lower and upper confidence limit of LC_{50}, LC_{90} (LCL-UCL) were (470.22-625.20) and (880.11-1252.15) ppm respectively. The chi –square value 0.04.35 was significant at $p<0.05$ level. The result revealed that the *A. indica* leaf extract was safe for non-target organisms as no adverse effects or mortality was recorded. This shows that the extract if used in the field where these organisms co-inhabit with the mosquito larvae will only kill the larvae and not harm other organisms.

Keywords: Larvicidal activity, silver nanoparticles, leaf extracts, *Azadirachta indica*, *Culex quinquefasciatus*

1. Introduction
Biological control is slow but can be long lasting, inexpensive, and harmless to living organisms and ecosystem; it neither eliminates pathogen nor disease, but brings them into natural balance [1]. Mosquitoes which transmit a number of diseases such as malaria (*Anopheles*), filariasis (*Culex*, *Mansonia*), and dengue (*Aedes aegypti*) etc., causing millions of deaths every year all around the world, are the most important group of insects in terms of public health. Lymphatic filariasis, whose vector is *Culex quinquefasciatus*, is a widely distributed tropical disease with around 120 million people infected worldwide and 44 million people having common chronic manifestation [2]. Hence, to prevent mosquito borne diseases especially lymphatic filariasis, it is necessary to control the *C. quinquefasciatus* mosquito population.

Nanoparticles have attracted considerable attention, owing to their various applications particularly silver nanoparticles, which are reported to possess antifungal, anti-inflammatory and anti-viral activity [3]. Additionally, AgNPs' larvicidal activity was proved [4]. Due to the above mentioned reasons, the use of biologically synthesized silver nanoparticles for the control of *Culex* mosquito larvae will fulfil the disadvantages of the synthetic insecticides. Nanotechnology is mainly concerned with synthesis of nanoparticles of variable size, shapes, chemical composition and controlled dispersity for human benefits.

The most predominantly study about nanoparticles today is those made from noble metals, in particular Ag, Pt, Au and Pd. Among the four metals, silver nanoparticles lay a significant role in the field of biology and medicine. There is a growing need to develop clean, nontoxic and environmentally friendly (green chemistry) procedures or synthesis and assembly of nanoparticles, biosynthesis of silver nanoparticles using plants, bacteria, fungi and yeast are known to reduce silver ions into silver nanoparticles by both extra and intra cellular. In the present study, the evaluation of larvicidal activity was carried out by applying Silver Nanoparticle synthezed by the leaf extracts of *Azadirachta indica* against *Culex quinquefasciatus* larvae.

Commonly, synthetic insecticides have created a number of ecological problems, ecological imbalance, harm to human and animals, environment ill effect, non-target organisms being affected in addition to the physiological resistance of vectors to synthetic insecticides [5]. Plant products have been used traditionally by human communities [6] and application of easily degradable plant compounds is considered to be one of the safest methods of control of insect pests and vectors [7]. Many medicinally important plant extracts have been studied for their efficacy as mosquito agent against different species of vector mosquitoes [8]. Not only can medicinal plant extracts be effective but also they may greatly reduce the risk of adverse ecological effects and do not induce pesticide resistance in mosquitoes.

In the present day the chemical based substances affect food chain. This has produced serious repercussions such as pest resistance, mammalian toxicity; bioaccumulation and environment food chain and pollution of the environment. In larval mosquito control application of insecticide in ponds, well and other water bodies may cause health hazards to human and larvivorus fishes. Constant application of organophosphates such as temephos and fenthion and insect growth regulators such as diflubenzuron and methoprene are generally used for the control of mosquito larvae [9]. Mosquito repellent using people complained of ill health effect and some time required medical

treatment [10]. These problems have highlighted the need for the development of new strategies for selective mosquito control.
The leaf extracts are considered to be a potential alternative approach against various stages and species of mosquitoes due to their excellent properties like environmental safety nature and month presence of bioactive ingredients/phytocompounds which can act as ovicidal, larvicidal, insect growth regulators, repellent, oviposition deterrence and reduction of fecundity and fertility [11]. Malaria causes substantial losses to national economies in terms of last productivity, costs of treatment, school and work absenteeism, and funeral costs [12]. Human malaria is transmitted only by females of the genus Anopheles, only by females of the genus *Anopheles*, only 30-40 transmit malaria in nature. The mosquito *Anopheles gambiae* is the principal vector of malaria in Africa. Control measures used against mosquitoes include elimination of breeding sites, application of surface films of oil to clog the breathing tubes of wrigglers and the use of larvicides. Many strains of the mosquito are resistance to conventional insecticides. Keeping the above mentioned points in view, it is very clear that the larvicidal activity of Silver Nanoparticle Synthezed by the leaf extracts of *A. indica* against *C. quinquefasciatus* is essentially unstudied in Musiri area. Hence, the present study was carried out to evaluate the larvicidal activity of crud extract of *A. indica* along with mixture of Silver nanoparticle against the larvae of *C. quinquefasciatus*.

2. Materials and methods

2.1 Cleaning of glassware

In the present study clean Borosil glasswares soaked in tap water for a few minutes and then thoroughly washed in tap water and then soaked in dichromate solution for a few hours to remove tough residues. Finally they all were washed in distilled water. All types of glassware such as conical flask, petri plates, test tubes, pipettes, centrifuge tubes, tip boxes, saline bottles etc., were sterilized at 121°C for 15 min in an autoclave.

2.2 Collection of mosquito larvae

The mosquito larvae were collected from stagnant water samples from roadside ditches, irrigation canals, drainage canals, temporary water pools and ponds around Musiri area (N11° 03.845'; E078° 41.007'), Tiruchirappalli district, Tamil Nadu, India, using sterile wide mouth container. The identified *Culex* sp. mosquito larvae were kept in plastic and enamel trays containing tap water, maintained and reared in laboratory [13].

2.3 Collection of plant materials

The plant *A. indica* leaves were collected from Musiri area and was taxonomically identified at the Department of Botany, Nehru Memorial College, Puthanampatti and the voucher specimen was preserved.

2.4 Preparation of plant extract

The leaves of *A. indica* were carefully examined and old leaf, insect damaged, and fungus infected leaves were carefully removed. The fresh and healthy leaves were washed with tap water and shade dried at room temperature (27-31ºC) for 5 to 10 days or until they broke easily by hand. Once completely dry, plant material (1.0 kg) was ground to a fine powder using electrical blender. The methanol was used for the extraction of 1.0 kg in the Soxhlet apparatus followed by the standard procedure [14]. The plant material was loaded in the inner tube of the Soxhlet apparatus and then fitted into a round bottomed flask containing ethanol. The solvent was boiled gently (40ºC) over a heating mantle using the adjustable rheostat. The extraction was continued until complete extraction was effected (8 hrs) and the solvent was removed at the reduced pressure with the help of rotary vaccum evaporator to yield a viscous dark green residue (12.5 g) of leaf extracts.

2.5 Preparation of silver nitrate solution

For preparation of 1mm silver nitrate solution (21.2mg of silver nitrate solution powder in 125ml milliQ-water) solution in an erylen meyer flask and incubated in room temperature.

2.6 Preparation of silver nano particle

Added 88ml of 1mm silver nitrate solution with 12ml of leaf extract and incubate the solution under dark room and use for testing the larvicidal activity.

2.7 Test for larvicidal activity

Testing of the plant extracts along with silver nanoparticle for larvicidal activity was carried out at different concentration by preparing the required stock solution by using the standard procedure [15]. The desired concentration of the test solution was achieved by adding 1.0 ml of an appropriate stock solution to 249 ml of dechlorinated water. Six replicates for each concentration were maintained. Six test tubes were taken and 30µl (0.01%), 50 µl (0.02%), 100 µl (0.03%), 200 µl (0.04%) and 250 µl (0.05%) of plant leaf extract (0.001g of leaf extract mixed with 5 ml of distilled water). Then 5 ml of silver nanoparticle were introduced in each tube. Then twenty numbers of late third larvae were introduced into the beaker were obtained from the laboratory colony. Acetone was used as control. The larval mortality in both treated and control was recorded after 24 hrs and the percentage of mortality was calculated using Abbott's formula [16].

$$\% \text{ Mortality} = \frac{\text{Mortality at treatment-Mortality at control}}{100 - \text{Mortality at control}} \times 100$$

2.8 Statistical analysis

The statistical evaluation of LC50v, LC90, regression equation and 95% confidence limit LCL and UCL were calculated from the data, which was carried out by Probit analysis [17] by using SPSS package version16.

3. Results & Discussion

The larval mortality of *Culex quinquefasciatus* was high in 0.05% (500 ppm) and the lowest mortality was recorded in 0.01% (100 ppm) Table 1.

Table 1: Larvicidal activity of Silver Nanoparticle synthezed by the leaf extracts of *Azadirachta indica* against *Culex quinquefasciatus* in 24 hrs.

% solution	Mortality of larva in 24 hrs (Replica N=5)					Average	% Mortality
	1	2	3	4	5		
0.01% (100ppm)	03	05	05	06	08	5.4	27%
0.02% (200ppm)	07	06	07	09	09	7.6	38%
0.03% (300ppm)	10	09	10	11	13	10.6	53%
0.04% (400ppm)	12	10	13	13	13	12.2	61%
0.05% (500ppm)	14	13	15	15	16	14.6	73%
Control	0	0	0	0	0	0	0%

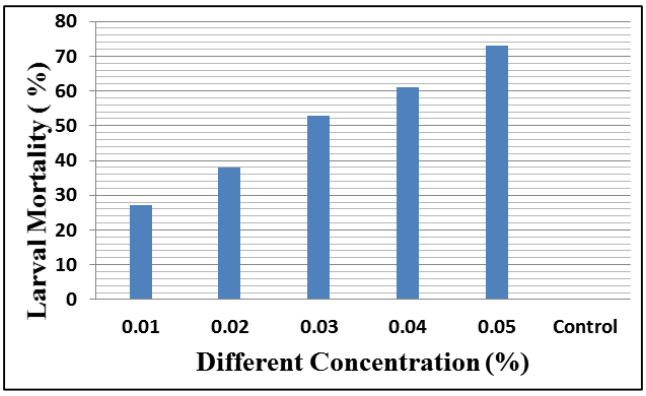

Fig 1: Larvicidal activity of Silver Nanoparticle synthezed by the leaf extracts of *Azadirachta indica* against *Culex quinquefasciatus* in 24 hrs.

The LC_{50} values of the methanolic leaf extract of *Azadirachta indica* along with silver nanoparticle was 0.031 ppm against late third larva *C. quinquefasciatus* (Table 2). The LC_{90} and regression equation were 0.062, Y=40.914+11.388 X. The 95% lower and upper confidence limit of LC50, LC90 (LCL-UCL) were (0.022 – 0.043) and (0.048-0.105) ppm respectively. The chi –square value 0.001 was significant at p<0.05 level (Chi: Pearson Goodness of Fit test 19.437 Df 4; P 0.001). In the present study *A. indica* leaf was chosen with ethanol extract along with silver nanoparticles. However, LC_{50} value of crude obtained by soxhlet extraction showed higher larval mortality. The soxhlet method to be more effective for extraction of larvicidal components. Some time the Hot extraction does not show any appreciable mortality even at 250 ppm concentration. In many parts of the world, plant-derived natural products have traditionally been used against mosquitoes. the screening of locally available medicinal plants for mosquito control would reduce dependence on expensive imported products and stimulate local efforts to enhance public health and crude extracts of many plants showed larvicidal activity against *C. quinquefasciatus*[18].

Table 2: Larvicidal activity of Silver Nanoparticle synthezed by the leaf extracts of *Azadirachta indica* against *Culex quinquefasciatus* in 24 hrs.

Concentration (%)	Larval mortality (%)±SD	LC_{50} (ppm)	LC_{90} (ppm)	Regression equation	Chi square
0.01% (100ppm)	27±3.4	0.031 (0.022-0.043)	0.062 (0.048-0.105)	Y=40.914+11.388X	0.001*
0.02% (200ppm)	38±2.8				
0.03% (300ppm)	53±3.7				
0.04% (400ppm)	61±3.0				
0.05% (500ppm)	73±2.5				
Control	0±0.0				

*significant at $P<0.05$

Values in parenthesis represent 95% confidence interval

(Chi: 19.437 Pearso Goodness of Fit test; df 4 ; P= 0.001)

The methanolic leaf extract of *A. indica* along with silver nanoparticle was significantly inhibits the adult emergence. The percentage of adult emergence inhibition were 22.0±1.8%, 35.5±2.5%, 48.0±3.0%, 68.0±3.4% and 90.0±2.2% at different concentration viz., 25.0ppm, 50.0ppm, 75.0ppm, 100.0ppm and 125.0ppm against the larvae of *C.quinquefasciatus* (Table 3, Fig 2). The LI_{50} value was 515.11 ppm and the LI_{90} value was 954.24 ppm. The regression equation was Y=11.15+0.416X. The 95% lower and upper confidence limit of LC_{50}, LC_{90} (LCL-UCL) were (470.22-625.20) and (880.11-1252.15) ppm respectively. The chi –square value 0.04.35 was significant at p<0.05 level (Table 3). The results of present study are comparable with past reports. The toxicity to the late third instar larvae of *Culex quinquefasciatus* by methanolic leaf extract of *Memordica charantia*, *Trichosanthus anguina*, *Luffa acutangula*, *Benincasa cerifera* and *Citrullus vulgaris* showed the LC50 values of 465.85, 567.81, 839.81, 1189.30 and 1636.04 ppm respectively [19]. The hexane, diethyl ether, dichloromethane, ethyl acetate and methanol extracts of shoot with leaves of *Leucas aspera* and *Vitex negundo* when tested against the larvae of *C. quinquefasciatus*, ethyl acetate and hexane extract provided maximum mortality respectively and also the ethyl acetate leaf extract of *Strychnos nuxvomica* against *C. Quinquefascaitus* [19]. The present study clearly proved the bioefficacy of *A. indica* extracts on *C. quinquefasciatus*.

A. indica extracts might lead to better application of botanical derivatives during the suitable developmental period and could also be helpful in usage of a natural mosquitocide which in future might be used directly as a larvicidal agent in small-volume aquatic habitats or breeding sites of limited size around human dwellings. Further studies such as mode of action, synergism with the biocides under field condition are essentially required among scientists. The mosquito larvicidal properties of the leaf extract of mosquito larvicidal properties of the leaf extract of a herbaceous plant, *Ocimum canum* against *Aedes aegypti*. The LC50 values for 2nd, 3rd and 4th instars larvae

were 177.82, 22.08 and 331.13ppm respectively [20]. The plant extracts of *Vinca rosea*, *Calatropis* sp.and *Adathoda sp*. posses larvicidal activity against *Ae.aegypti*. The Lc50 values of 34.06, 35.18 and 34.30ppm respectively [21]. All the concentrations of plant extracts used in the present study exhibited repellency activity against the starved female adults of *C. quinquefasciatus*. The repellent or antifeeding activity depends on the plant species, plant part, solvent used in extraction and the dose of the extract. The present study indicates that the ethanol extraction of the plants used was effective in exhibiting the repellent action against the mosquito tested. Many plant extracts and essential oils manifest repellency activity against different mosquito species. The present results are in accordance with previous study carried out in different leaf extracts viz., using neem oils against mosquito bites of *Anopheles* spp., *Culex* spp., and *Aedes* spp., [21], using methanolic extracts of *Pelargonium citrosa* against *Anopheles stephensi* [22], using methanol extracts from 23 aromatic medicinal plant species against female blood-starved *Aedes aegypti* [23], using extracts of the neem *Azadirachta indica* and methanolic extracts of leaves and seeds from the chinaberry tree, *Melia azedarach* against *Anopheles stephensi*, *Nepeta cataria* against *Aedes aegypti*, *A. vigilax*, *Culex annulirostris*, and *C. Quinquefasciatus* [24].

In general, it could be concluded that, plant extracts used in the present study act as larvicidal, adulticidal, and possess growth and emergence inhibiting, repellent and antifeeding activities against the mosquito vector, *C. pipiens*. Furthermore, the results of the present study may contribute to a reduction in the application of synthetic insecticides, which in turn increases the opportunity for natural control of various medically important pests by botanical pesticides. These botanical pesticides are often active against specific target insects, less expensive, easily biodegradable to nontoxic products and potentially suitable for use in mosquito control program [7]. The application of these plant extract along with silver nanoparticle on mosquito breeding places surely prevent environmental pollution and also protect the earth from toxic chemical pollutants. It is concluded from the present findings that, the biosynthesised silver nanoparticles of leaf aqueous extract of *A. indica* provided potential killing effect of mosquito larvae which could be used for prevention of several dreadful diseases.

Table 3: Insect growth regulator activity of Silver Nanoparticle synthezed by the leaf extracts of *Azadirachta indica* against *Culex quinquefasciatus*

Concentration (ppm)	Larval mortality (%)±SD	LC_{50} (ppm)	LC_{90} (ppm)	Regression equation	Chi square
125.0	90.0±2.2	515.11 (470.22-625.20)	954.25 (880.11-1252.15)	Y=1, 5+0.416X	0.04.35*
100.0	68.0±3.4				
75.0	48.0±3.0				
50.0	35.5±2.5				
25.0	22.0±1.8				
Control	00.0± 0.00				

*Significant at $P<0.05$ Values in parenthesis represent 95% confidence interval

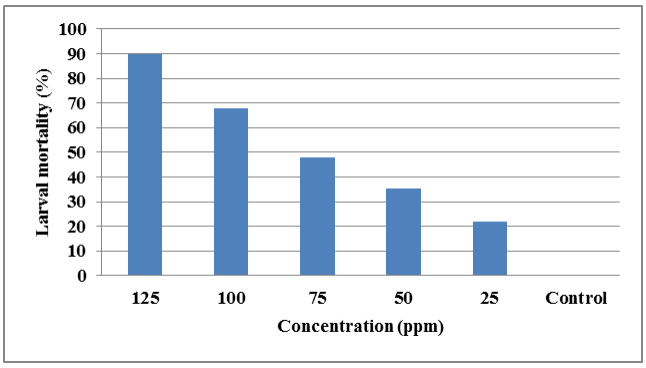

Fig 2: Insect growth regulator activity of Silver Nanoparticle synthezed by the leaf extracts of *Azadirachta indica* against *Culex quinquefasciatus*

4. Conclusion

If this method is applied in use, considerable decrease in the mosquito can be brought in a short period of time. With the above results obtained, we propose that these medically valuable plants, contains active compounds that are able to kill the mosquito larvae effectively. The plant extract (biological substances) are easily degraded and will not contaminate the environment, thus has greater advantage over the chemical methods. The disposed cups, coconut shells and other remains (cup shaped) should not through in the open area. This is the good breeding ground for mosquitoes.

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